Factors affecting the pathogenicity of *Yersinia ruckeri*

by

Maria Dolores Furones Nozal

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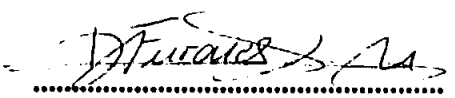
DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record has been performed by myself, and that all sources of information have been specifically acknowledged.



M.D. Furones Nozal

Colin B. Munn

(Supervisor)

Work in Chapter 6 was carried out in collaboration with the former Institute of Marine Biochemistry, Aberdeen, Scotland, where the diet preparation and the analytical testing of the samples was carried out.

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ABSTRACT

A group of 42 strains of *Yersinia ruckeri* were characterized and found to show homogenous biochemical profiles.

The optimal conditions for *in vitro* culture of a representative strain of *Y. ruckeri* were established. The virulence was increased by passage through fish. A standard method for infection of rainbow trout (*Oncorhynchus mykiss*) with *Y. ruckeri* was devised. As a result cultures of *Y. ruckeri* grown on full strength of BHIA for 5 h at 30°C in static conditions were found to be most suitable for infection by both intraperitoneal injection and immersion. Virulence of stored cultures was maintained for over 1 year at -20 and/or -70° C and virulence was not lost after up to six consecutive subcultures. The effect on mortality of susceptibility of various fish stocks, water temperature and stocking density is described. The size of fish (between 5 and 60 g) had no effect on virulence.

The injection and immersion protocols were used as *in vivo* models for the study of the effects of dietary vitamin E, with both laboratory prepared diets and commercially available diets. Comparative results of haematological and biochemical parameters, histology, mortality patterns, serum antibody levels and bacterial recovery from the four dietary groups of fish are presented.

A comparative *in vitro* and *in vivo* study of intra and inter strain variations among representatives of the serogroup I of *Y. ruckeri* was performed. Under the conditions used, some strains were virulent and other avirulent. Results of Western blotting and SDS-PAGE showed the presence of a heat sensitive factor (HSF), present only in the virulent strains of *Y. ruckeri*. To date this is the first putative virulence factor described for *Y. ruckeri*. Further work was undertaken in an attempt to characterize the HSF. It appears to be located in the periplasmic space and to have complex lipid and proteinaceous components. HSF is easily degraded, and is immunogenic and confers some protection when injected into rainbow trout. Selective media and detection methods for the HSF, and their potential use in laboratory and field studies are described.

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I want to dedicate this work to Manuel Furones Garzón and Rosalia Nozal Fernandez - my parents.

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ABBREVIATIONS

A	absorbance
ANOVA	analysis of variance
BSA	bovine serum albumin
BHI	brain heart infusion
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
BDH	British Drug Houses
cm	centimetre
c.f.u.	colony forming units
CR	congo red
CB	coomassie brilliant blue
CMC	critical micelle concentration
CV	crystal violet
CPE	cytopathic effect
°C	degree centigrade
DNA	deoxyribonucleic acid
ERM	enteric redmouth disease
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylene diamine-tetra acetic acid
EGTA	ethylene glycol-bis (ß-amino ethyl ether) - N,N,N^1,N^1 - tetra acetic acid
ECP	extracellular product
FDL	Fish Diseases Laboratory, Weymouth
FCA	Freunds complete adjuvant
FIA	Freunds incomplete adjuvant
FC	fusidic acid
g	gram
x g	acceleration due to gravity
HSF	heat sensitive factor
h	hour
IM	inner membrane
i.p.	intraperitoneal
kb	kilobase
kDa	kilodaltons
kg	kilogram
LD_{50}	lethal dose 50
LPS	lipopolysaccharide
l	litre
Mdal	megadalton
µg	microgram
µl	microlitre
µm	micrometre
mA	milliamp
mg	milligram
ml	millilitre
mM	millimolar
min	minute
M	molar
MS222	3-aminobenzoic acid ethyl ester
OM	outer membrane
OMP	outer membrane protein
%	percent
PBSa	phosphate buffered saline
PAGE	polyacrylamide gel electrophoresis
PUFA	polyunsaturated fatty acid

P/N	positive-negative ratio
PK	proteinase K
rpm	revolutions per minute
ROD	ribose ornithine deoxycholate
sv	score value
sec	seconds
SDS	sodium dodecyl sulphate
SB	sudan black
Σ	sum of
TLC	thin layer chromatography
TM	trimethoprim
Tris	tris (hydroxymethyl) amino methane
TSA	tryptone soya agar
TSB	tryptone soya broth
uv	ultra violet
v	volt
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

INTRODUCTION

Yersinia ruckeri is the bacterial causative agent of enteric redmouth disease (ERM), a condition affecting farmed salmonid fish. The disease was originally described in the USA (Ross *et al.*, 1966) and first recognised in the UK in the early 1980's (Roberts, 1983). Since then, it appears to have spread rapidly throughout Europe and Scandinavia, assuming major economic importance as a limiting factor which can affect the intensive aquaculture of trout and more recently, salmon. Vaccination against ERM has proved to have some efficacy in controlling the condition, but considerable problems remain, especially because of the intensive nature of modern fish culture and the consequent ease of disease transmission. An additional problem is the cyclic nature of the recurrence of ERM (Busch and Lingg, 1975) due to the presence of an asymptomatic carrier state.

Despite some success in the control of the disease, very little is known about the epidemiology and pathogenic mechanisms involved (reviews, Busch, 1978; Austin and Austin, 1987). The overall aim of this project was to obtain a better understanding of the underlying factors affecting the ability of the bacterium to infect fish and produce pathological effects, and to investigate the immunological response and defence mechanisms of the host fish.

The initial aim of the investigation was to establish experimental conditions for a reproducible laboratory method of infection, and to study various routes of administration of the pathogen. The effect of bacterial culture conditions and physiological factors affecting the development of the disease following artificial infection were also studied.

The next stage of the research project was to utilize the laboratory model of *Y. ruckeri* infection to investigate the influence of variations in nutritional factors (vitamin E) on the susceptibility of fish to ERM.

Finally, in an attempt to elucidate the factors affecting the pathogenicity of *Y. ruckeri* at a molecular level, a study of intra and inter strain variations of serotype I strains was made. During the course of this work a previously undescribed putative virulence factor was found. The summation of the research project was therefore the characterization of this factor and the use of the laboratory infection model to indicate its role in the pathogenesis of ERM.

CHAPTER 2

LITERATURE REVIEW

ENTERIC REDMOUTH DISEASE

2.1 PATHOLOGY

The name of the disease, enteric redmouth, (ERM) refers to one of its most common symptoms, i.e. the reddening in the mouth and throat, caused by subcutaneous haemorrhaging (Bullock, 1984). However, the classic "redmouth" haemorrhage cannot be reliably used in diagnosis, since the absence of symptoms has been described in fish suffering from *Y. ruckeri* infection during disease outbreaks in England (Bentley, 1982), Scotland (Frerichs *et al.*, 1985) and in West Germany (Furhmann *et al.*, 1983). A second disease, originating in Australia and known as salmonid blood spot (Llewellyn, 1980) does not present the typical haemorrhages in the mouth, although the disease is attributable to the same aetiological agent (Green & Austin, 1982). Subcutaneous haemorrhaging can also be seen along the base of the fins and anus (Busch, 1978). The often seen Exophthalmos, can be unilateral or bilateral, and is caused by tissue oedema and an increase in intraocular pressure, the eye can often rupture leading to lens opacity and blindness (Busch, 1978). Fish infected with ERM are characteristically sluggish and dark in colour (Rucker, 1966).

Internal gross pathology is distinguished by petechial haemorrhage of the viscera and tissues. The appearance of the lower intestine is probably the most significant clinical diagnostic sign of ERM, it can be inflamed and filled with a thick yellowish or whitish fluid (Busch, 1978; Bullock, 1984). The spleen appears swollen and congested (Wobeser, 1973), and the liver can also be enlarged, presenting a bright red colour (Bragg & Henton, 1986). The histopathology is typical of the haemorrhagic septicemic type of infection, with an inflammatory response in virtually all tissues and bacteria conspicuous in vascular tissue and areas of petechial haemorrhage (Rucker, 1966; Bullock, 1984). Extensive disruption of the structure of the renal haemopoietic tissue and areas of cell necrosis in the kidney, liver and spleen, are also principal histopathological changes (Frerichs *et al.*, 1985). The haemogram of fish in an advanced stage of infection shows a decrease in haematocrit, haemoglobulin, and in the number of red cells, lymphocytes and thrombocytes (Wobeser, 1973). Monocytes and neutrophil granulocytes are increased in numbers (Lehmann *et al.*, 1987). Destruction of α and ß-lipoproteins occurs in fish infected with *Y. ruckeri* (Gelev, *et al.*, 1984). In addition, glomerular nephritis and necrosis of the intestinal mucosa produces an increased clotting time as well as a plasma aproteinemia. As a result, there is a decrease of the plasma colloid osmotic pressure as well as disruption of the ionic balance (Busch, 1978). The specific causes of the pathological changes occurring during the disease are not known.

2.2 TRANSMISSION AND MORTALITY

Transmission is primarily of a horizontal nature from fish to fish (Bullock, 1984). Infection of healthy fish, by exposure to waterborne bacteria from infected animals has been demonstrated in trout by Rucker (1966). Bullock (1976) also infected Atlantic salmon (*Salmo salar*) by similar means. The pathogen can be readily recovered from infected broodstock at the time of spawning, but vertical transmission through the eggs has not been demonstrated, particularly when iodophor disinfection of the eggs is performed after fertilization (Dulin *et al.*, 1976). Asymptomatic carriers are probably the primary source and reservoir of *Y. ruckeri*. Busch and Lingg (1975) demonstrated the establishment of a clinical asymptomatic carrier state, in the lower intestine of 25% of surviving, experimentally infected rainbow trout (*Oncorhynchus mykiss*), 45 days post infection. Thereafter, regular intestinal shedding of the pathogen, causing recurrent infection and mortality within the population, occurred on a cyclical basis of 30-40 day intervals. These cycles depend upon other factors such as seasonal variations in water temperature, farming conditions, natural resistance of the fish and their immune status (Busch, 1983). Non clinical infections of *Y. ruckeri* have also been found in other aquatic organisms such as crayfish (Post, 1983).

It has been thought that *Y. ruckeri* could not survive well outside its host, and therefore, the aquatic environment could not act as a reservoir for ERM, (Post, 1983). However, Romalde *et al.* (1990), recently demonstrated

persistence and dormancy phenomena of the pathogen (with three different serotypes) under a range of environmental conditions, without a dramatic reduction of bacterial numbers over a 2 month period.

The incubation time for the development of ERM varies inversely with the water temperature (Busch, 1983). Experimental evidence suggests that the incubation time to the onset of disease signs is 5 to 10 days at 13-15°C (Bullock, 1984).

Enteric redmouth disease is an acute to chronic stress-related disease. Most epizootics of ERM occur at water temperatures between 11 and 18°C. Epizootics of subacute or chronic ERM disease may be noted between 8 and 11°C (Post, 1983). Busch (1978), reported that peracute to acute infections, occur usually in the spring and early summer, during the periods of rising water temperatures. Mortalities may be between 50-70 percent, corresponding mainly with young-of-the-year fish. Acute to subacute infections occur in yearling fish in the autumn and early winter, with the declining water temperatures and mortalities are usually in the 10 to 50 percent range. Chronic infections, although only producing low levels of mortality, can cause serious economic losses since valuable market size fish are involved.

Stress factors, such as overcrowding or poor water quality, can precipitate an ERM disease outbreak (Bentley, 1982; Meier, 1986). Hunter *et al.* (1980),

demonstrated that heat-stressed steelhead trout carriers, could transmit *Y. ruckeri* to recipient fish, whereas unstressed carrier fish did not transmit the bacterium. Other stress factors, such as exposure to heavy metals like cadmium and copper (Knittel 1978, 1981) or gamma irradiation of the lymphoid organs (Chilmonczyk and Oui, 1988) have been proved to increase the susceptibility of fish to *Y. ruckeri*. Feeding levels and diet composition are also factors which can condition the response of fish to the disease (Blazer & Wolke, 1984; Henken *et al.*, 1987).

2.3 GEOGRAPHIC RANGE

Enteric redmouth disease was first observed in rainbow trout in Idaho by Rucker in the 1950's (Cipriano *et al.*, 1986). Since then, isolations of the pathogen have been reported throughout the United States and Canada (Ross *et al.*, 1966; Rucker, 1966; McDaniel, 1971; Wobeser, 1973; Bullock & Snieszko, 1975; Bullock *et al.*, 1978; Busch, 1978; Stevenson & Daly, 1982). Shipment of carrier salmonids was thought to be the main cause of such spread (Bullock *et al.*, 1978). *Y. ruckeri* was isolated for the first time in Australia in the early 1960's from rainbow trout, although the isolates were not identified as *Y. ruckeri* until 1977 (Bullock *et al.*, 1977, 1978). Llewellyn (1980), described the "salmonid blood spot bacterium", isolated from brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar* L.) in Australia, and which showed similarities to *Y. ruckeri*. Stevenson & Airdrie (1984b)

have since confirmed that this bacterium was in fact *Y. ruckeri*. *Y. ruckeri* has now been isolated in many countries far away from North America such as Chile, where the pathogen was isolated from asymptomatic carrier carp (*Cyprinus carpio*) (Enriquez & Zamora, 1987) and Bragg & Henton (1986) reported the isolation of *Y. ruckeri* from rainbow trout in South Africa. Outbreaks of ERM were detected in Europe for the first time in the early 1980's (Roberts, 1983). Michel *et al.* (1986), blamed the entrance of the disease to the continent, on the importation of baitfish from the United States. Up to the present date, isolation of *Y. ruckeri* has been reported in Great Britain (Austin, 1982; Roberts, 1983). France (Lesel *et al.*, 1983), West Germany (Fuhrmann *et al.*, 1983), Finland (Rintamäki *et al.*, 1986), Italy (Giorgetti *et al.*, 1985), Denmark (Dalsgaard *et al.*, 1984), Bulgaria (Gelev *et al.*, 1984), Scotland (Frerichs *et al.*, 1985), Norway (Sparboe *et al.*, 1986), Switzerland (Meier, 1986), Spain (De la Cruz *et al.*, 1986), Ireland (McArdle & Dooley-Martin, 1985) and Yugoslavia (Ocvirk *et al.*, 1988).

2.4 HOST RANGE

The natural host of *Y. ruckeri* was initially thought to be rainbow trout (*O. mykiss*) by Ross *et al.* (1966) and Rucker (1966). Since then other salmonid species have been recognised as hosts of *Y. ruckeri*, namely sockeye salmon (*O. nerka*), Atlantic salmon (*Salmo salar*), chinook salmon (*O. tschawytscha*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), cut-throat trout

(*Salmo clarkii*) and coho salmon (*O. kisutch*) (Bullock *et al.*, 1978; Busch, 1983). *Y. ruckeri* has also been isolated from non-salmonid fish. Published reports include infection of emerald shiners (*Notemigonus atherinoides*) (Mitchum, 1981), cisco (*Coregonus artedi*) (Stevenson & Daly, 1982), minnow (*Pimephales promelas*) (Michel *et al.*, 1986), farmed whitefish (*Coregonus peled* and *C. muksun*) (Rintamaki *et al.*, 1986), farmed sturgeon (*Acipenser baeri*) (Vuillaume *et al.*, 1987), turbot (*Scophthalmus maximus*) (Michel *et al.*, 1986), carp (*C. carpio*) and eel (*Anguilla rostrata*) (Fuhrmann *et al.*, 1984), goldfish (*Carassius auratus*) (McArdle & Dooley-Martin, 1985), and burbot (*Lota lota*) (Dwilow *et al.*, 1987). *Y. ruckeri* has been transmitted experimentally to channel catfish (*Ictalurus punctatus*) (Lewis, 1981), sole and gilthead bream (Michel *et al.*, 1986).

Y. ruckeri has been isolated from sources other than fish, namely, from the intestine of a muskrat (Stevenson & Daly, 1982), from the faeces of a kestrel and a turkey vulture (Bangert *et al.*, 1988) and from a human specimen (Farmer *et al.*, 1985). Crayfish have been reported to act as a reservoir of the bacterium (Sullivan, 1981), and seagulls (*Larus* spp.) also may act as a vector of the disease (Willumsen, 1989).

2.5 CHARACTERISTICS OF THE PATHOGEN'S CLASSIFICATION

Y. ruckeri comprises a homogeneous group of gram-negative rods with a

controversial taxonomic location. Ross *et al.*, 1966, describing the bacterium for the first time, realized that heated O antigens prepared from 14 Redmouth (RM) organisms agglutinated strongly with O antiserum for *Arizona* (= *Salmonella arizonae*) O group 26, and weakly with O group 29. Negative results were obtained when the H antigens of *Salmonella* and *Arizona* were tested with H antiserum produced from a RM bacterium. The authors concluded that the RM bacteria may be considered a member of the family *Enterobacteriaceae*, but its position within this family was not clear, since despite the antigenic resemblance to *A. arizonae*, there were many differences in biochemical reaction. In later work, carried out by Ewing *et al.* (1978) cultures of the RM bacterium were characterized by means of their biochemical reactions, deoxyribonucleic acid (DNA) hybridization, and by determination of the guanine-plus-cytosine (G+C) ratio in DNA. The DNA relatedness studies confirmed that the RM bacteria was a member of the family *Enterobacteriaceae*, but that it should be considered as a single species. Using DNA hybridization, the organism was shown to be related (about 30%) to species of both *Serratia* and *Yersinia* (Table 2.1), but it was biochemically closer to *Yersinia*. The G+C ratios of RM bacteria (47.5-48.5%) were not only markedly different from those of *Serratia* (58%) but also closer to those of *Yersinia* (46-48%). On the basis of the biochemical reactions and the G+C ratios, the authors concluded that the RM bacteria should be considered a new species of *Yersinia*, and proposed the name *Yersinia ruckeri*, in honour of Robert R. Rucker. O'Leary *et al.* (1979), in a study of the characterization of the RM bacterium, stated that the organism did not

correlate well by DNA homologies or biochemical reactions to existing species of *Yersinia* but, on the basis of the G+C percentage content, they reached the same conclusions as Ewing *et al.* (1978), and supported *Yersinia ruckeri* as the genus and species designation for the RM bacterium. Later, Bercovier and Mollaret (1984), expressed their doubts about the location of *Y. ruckeri*. These authors considered that the bacterium might constitute a new genus by itself, since the DNA relatedness among *Yersinia* species is 40% or higher except for *Y. ruckeri* which is at most 38% related to other *Yersinia* species, and because the phenotypic characteristics of these are very different from those of *Y. ruckeri*.

After a numerical phenetic study of representative strains of *Y. ruckeri* and other taxa of *Enterobacteriaceae*, Green and Austin (1983) concluded that *Y. ruckeri* matched the description of *Salmonella arizonae*, rather than *Yersinia*. This conclusion has been contradicted by results obtained by De Grandis *et al.* (1988), studying DNA relatedness of serovars of *Y. ruckeri* and other *Enterobacteriaceae*. These authors found that, despite phenotypic similarities, *Y.ruckeri* does not show close genetic relationship with *Hafnia alvei* or with *S. cholerae-suis* subsp. *arizonae*. De Grandis *et al.* (1988), also reported very little hybridization between DNA from *Y. ruckeri* and *Y. enterocolitica* supporting the proposal of Bercovier and Mollaret (1984), of re-evaluation of the position of the ERM bacterium in the genus *Yersinia*.

2.6 MORPHOLOGY

Y. ruckeri is a short (1.5-2.0 μm x 0.5 μm) Gram negative, slightly curved rod, which is usually motile by means of peritrichous flagella. Colonies of the bacterium are smooth, circular, and slightly raised with entire edges. They are not fluorescent under ultraviolet light, but slightly iridescent when examined by reflected light (Ross *et al.*, 1966). Some variations in colonial morphology have been noticed, but while Ross *et al.* (1966), attributed them to an age effect of the cultures, other authors also found differences in cell size and shape, which could not be correlated with media composition, age of the culture (Austin *et al.*, 1982) or any biochemical characteristics of the strains (Davies and Frerichs, 1989). Stave *et al.* (1987), suggested that particular colony morphologies could be plasmid-mediated characteristics, since serotype I strains of *Y. ruckeri* have a different colony morphology than serotype II strains. Variations in colony morphology observed by Bullock *et al.* (1978), where some strains formed colonies with irregular edges, could not be correlated with any other factor at the time. Davies (1990), studying the lipopolysaccharide (LPS) patterns of *Y. ruckeri* pointed to the possibility of such differences in colony morphology being due to the possession of a "rough" LPS pattern.

Most strains of *Y. ruckeri* are motile (Busch, 1983), but motility depends upon temperature of incubation. Cells were reported to be not motile at 9 and 37°C, but were motile at 18, 22 and 27°C (O'Leary *et al.*, 1979). Davies and

Frerichs (1989) found that, while 95% of the North American isolates examined were motile, only 79% of the European strains were motile and, furthermore, all 24 non-motile isolates examined originated from the U.K. and Norway. These authors also found that all non-motile strains lacked lipolytic activity as determined by Tween 20 and Tween 80 hydrolysis tests, and they suggested that such strains be identified as *Y. ruckeri* biotype 2.

2.7 BIOCHEMICAL CHARACTERISTICS

The biochemical characteristics of strains of *Y. ruckeri* have been shown to be extremely uniform (Ross *et al.*, 1966; Busch, 1973; Wobeser, 1973; Bullock *et al.*, 1978; Ewing *et al.*, 1978; O'Leary *et al.*, 1979; Stevenson and Daly, 1982 and Pyle *et al.*, 1987).

Some variations between strains of *Y. ruckeri* occur in the tests for methyl red, Voges-Proskauer, lysine decarboxylase, arginine dihydrolase, gelatin hydrolysis, Tween 80 hydrolysis and sorbitol fermentation (Ewing *et al.*, 1978; Stevenson & Daly, 1982; Busch, 1983; Pyle *et al.*, 1987). These differences, excluding sorbitol fermentation, have been considered to be not significant, and therefore, indicate the existence of a single biotype (Busch, 1983).

Homogenicity among the taxon has been reported by Schill *et al.* (1984), by means of multilocus electrophoretic studies on a large number of *Y. ruckeri*

strains. Their work indicated that the genetic structure of *Y. ruckeri* is clonal, and they described four clonal groups one of which is predominant, but they do not seem to be related with the described serotypes.

2.8 SEROLOGICAL PROPERTIES OF *Yersinia ruckeri*

The terms serovar, serotype and serogroup have been used interchangeably by various authors; in addition, both roman and arabic numerals have been used as designations. In this review, the designations used by the original authors are given.

Initially, it was thought that *Y. ruckeri* was a very homogeneous taxon, not only biochemically, but also serologically with only one serotype recognized, namely the original Hagerman serotype (Ross *et al.*, 1966). O'Leary (1979) described a second serotype, isolated from Pacific salmon, which was designated as the "Big Creek" strain. Bullock *et al.*, (1978), comparing *Y. ruckeri* isolates from different geographic areas, described an Australian isolate which conformed to all the characteristics of *Y. ruckeri*, but failed to react with the antisera against types I and II and these authors therefore proposed this strain as a new serotype. These serotypes have been designated as type I (Hagerman), Type II (O'Leary) and Type III (Australian) (Bullock and Anderson, 1984). McCarthy and Johnson (1982) concluded after a serological study of North American strains of *Y. ruckeri*, that all but one of

the isolates tested comprised the classical "Hagerman" serotype. Stevenson and Daly (1982), however, characterising isolates from Ontario, found three isolates which biochemically resembled the sorbitol fermenting serovar II strain, but gave high titres when tested with antiserum against serotype I strain. The authors suggested the role of a heat-denatured surface antigen in this phenomenon, since agglutination titres of those strains dropped dramatically after the cells had been heated. A fourth isolate from brown trout appeared to be different from the three previously mentioned serovars. These findings, suggested that the diversity in the serological characteristics of *Y. ruckeri* was probably greater than originally thought. Stevenson and Airdrie (1984b) studied the serological variation among *Y. ruckeri* strains from North America and Australia. These authors designated two new serovars (IV and V) on the basis of their serological and biochemical profiles. However, it was subsequently shown, using DNA-DNA hybridization studies, that serotype IV was not in fact *Y. ruckeri* (De Grandis *et al.*, 1988). Stevenson and Airdrie (1984b) also considered the salmonid blood spot bacterium (Llewellyn, 1980) to be an anomalous serovar I strain of *Y. ruckeri*, although no relationship was established, between this bacterium and the "Australian" serotype (serovar III) mentioned by Bullock *et al.* (1978). A new serological variety (serovar VI) was found by Daly *et al.* (1986). These authors also reported a sorbitol-fermenting serovar III isolate from British Columbia, which was the first report of this serological group outside Australia. Therefore, on the basis of whole cell serology, Stevenson and Airdrie (1984b) and Daly *et al.* (1986), designated six serovars of *Y. ruckeri*:

serovar I (Hagerman), serovar I' (SBS), serovar II (Oregon and O'Leary), serovar III (Australian), serovar V (Colorado), and serovar VI (Ontario).

Pyle and Schill (1985) studied the antigenic variations among seventeen sorbitol fermenting isolates, which had been previously designated as type II. The technique used by these authors consisted of the screening of proteinase K-treated bacterial lysates by electroblot and immunoenzymatic detection of the somatic antigen (LPS). On the basis of homologous LPS banding patterns, four O-serotypes were identified among sorbitol fermenting isolates, which were designated serotypes 2, 4, 5 and 6.

Pyle *et al.*, (1987), using an indirect fluorescent antibody test (IFAT) for the detection of whole cell antigens, distinguished at least six serotypes designated serotypes 1-6 among 79 *Y. ruckeri* isolates from North America and Australia.

Flett (1989) also established an O-antigen serogrouping based, as Pyle and Schill (1985), on western blotting of LPS. Flett (1989) found that *Y. ruckeri* could be placed into six O-serogroups based on LPS banding patterns and immunoblotting reactions. Flett's O-serogroups correlated with the designations used by Stevenson and Airdrie (1984b) and Daly *et al.* (1986) as follows: Serovars I and III were placed together in serogroup 0:1. Serovar II, comprised strains with three different LPS banding patterns, which were placed into the serogroups 0:2, 0:3 and 0:4. The LPS banding patterns of

serovars V and VI were unique and consequently were placed in serogroups 0:5 and 0:6 respectively.

Another O-serotyping scheme has been published recently. Davies (1990) studied the serological characteristics of 131 isolates of *Y. ruckeri*, from a wide geographic range, using a microplate agglutination assay to test whole-cell heat-stable O-antigens. The numerical designations used in this study, (serotypes 01, 02, 05, 06 and 07), were based on those of Stevenson and Airdrie (1984b) and Daly *et al.* (1986), although in Davies' case the scheme was based on heat-stable O-antigens as opposed to unheated whole cells. The method used by Davies did not differentiate between the serotypes I and III of the Stevenson and Daly (1982) or Daly *et al.* (1986) schemes, and therefore, the relationship between both serotyping schemes was as follows: Davies' serotype 01 corresponds with serotypes I and III, serotypes 02, 05, 06 and 07, correspond with serotypes II, V, VI and "not typed" respectively.

It was further suggested by Davies (1990), that the serotype III isolate described by Bullock *et al.* (1978), is a rough-type mutant, and that serotype III (or 03) does not exist. The work carried out by Davies included representative isolates of the various serotypes described by Stevenson and Airdrie (1984b), Pyle and Schill (1985), Daly *et al.* (1986) and Pyle *et al.* (1987), and therefore, a direct comparison between all schemes was possible. With the exception of serotype 2, the designations used by Pyle and Schill (1985) did not correspond with any other scheme. However, serotypes 1, 2

and 5 of the scheme proposed by Pyle *et al.* (1987) correspond with those of Stevenson and Airdrie (1984b), Daly *et al.* (1986), Flett (1989) and Davies (1990), whereas only serotype 2 corresponds with the scheme of Pyle and Schill (1985). Briefly, it is clear that the serology of *Y. ruckeri* is more complex than was initially thought. Consequently standard methodology leads to commonly accepted designations, it will remain confusing with limited use in the identification of *Y. ruckeri* and in the criteria for the implementation of measures for disease control and movements of fish.

2.9 OTHER CHARACTERISTICS OF SEROVARS OF *Y. ruckeri*

O'Leary (1977) initially indicated that *Y. ruckeri* could be divided into two distinct serotypes. In addition to antigenic differences which accounted for this serotypic differentiation, the Hagerman serotype did not ferment sorbitol, whereas the other serotype did. It was later established that sorbitol non-fermenters only comprised serotype I isolates whereas sorbitol fermenters comprised a variety of serotypes (Stevenson and Airdrie, 1984b; Daly *et al.*, 1986; Pyle *et al.*, 1987). Nevertheless, serotype I sorbitol fermenting isolates have been identified on some occasions, Stevenson and Airdrie (1984b), Michel *et al.* (1986), Rintämaki *et al.* (1986) and Pyle *et al.* (1987). Therefore, when sorbitol is used as an indicator of serotype, it can only indicate serotype I strains that are sorbitol negative.

Stevenson and Airdrie (1984a) isolated eight bacteriophages effective against *Y. ruckeri*. One particular phage, Yer A41, was of interest because it lysed all strains except one autoagglutinating strain which had been assigned to serovar I. Phage Yer A41 did not lyse strains of serovars II and V or representatives of the salmonid blood spot bacterium (serovar I'). The specificity of Yer A41 for serovar I strains, makes this phage potentially valuable as a diagnostic tool (Stevenson and Airdrie, 1984a). However, since Yer A41 has also been shown to lyse a range of bacterial species, the host range of the phage should be studied, since it could lyse other bacteria commonly present in the gut flora of fish. Another phage of interest found by Stevenson and Airdrie (1984a) was Yer L62, which only lysed strains of *Y. ruckeri* serovar V. Phage lysis patterns could be useful also to rule out taxonomically misplaced isolates, as was shown with two strains designated serovar IV which were not recognised by any of the *Y. ruckeri* phages. Subsequently, DNA-DNA hybridization results showed that those strains were not members of the *Y. ruckeri* species (Stevenson and Airdrie, 1984a).

Most of the serovar I strains of *Y. ruckeri* (10/12), tested by De Grandis and Stevenson (1982) carried a large plasmid (40-50 Mdal). A large plasmid has also been reported in serovar I *Y. ruckeri* by other workers (Cook and Gemski, 1982; Toranzo *et al.*, 1983; Stave *et al.*, 1987 and Guilvout *et al.*, 1988). Although the reported size of this plasmid seems to vary with each author (40 to 78 Mdal), the apparent discrepancies in size can possibly be explained by the different DNA isolation and purification methods employed.

The mobility of plasmid DNA in agarose gels can be affected by both the conformational state of the plasmid molecule (i.e. supercoiled, closed-circular and linearised) and also by the purity of the DNA applied to the gels, which will depend on the chosen DNA isolation method (Maniatis *et al.*, 1982).

Other plasmids of smaller sizes have been reported in other serovars (II and V) of *Y. ruckeri* (De Grandis, 1987). The large plasmid of the serovar I strains was of interest because it was thought that it could be related to the plasmid (40-48 Mdal) of other species of *Yersinia* (Portnoy and Falkow, 1981), which confers a temperature-dependent calcium requirement. No calcium dependence was observed in *Y. ruckeri* serovar I strains bearing the 40-50 Mdal plasmid (De Grandis and Stevenson, 1982). Later work (Guilvout *et al.*, 1988) in which the plasmid profiles and Bam HI restriction endonuclease patterns of *Y. ruckeri* were studied in comparison to those of other members of genus *Yersinia*, showed that they had different restriction patterns. Furthermore, hybridization of restriction-digested *Y. ruckeri* plasmids with the *Y. enterocolitica* plasmid probe IP 383, showed a complete absence of recognition of the sequences in the *Y. ruckeri* plasmid by the probe. These results suggested that the *Y. ruckeri* serovar I plasmid has a different origin to the common *Yersinia* plasmid. This gives further evidence for the questionable taxonomic position of *Y. ruckeri*. De Grandis and Stevenson (1982) found that the serovar I strains carrying the 40-50 Mdal plasmid were unable to grow at 37°C, leading to the suggestion that the plasmid could be related to temperature sensitivity. De Grandis and Stevenson (1985), studied

the susceptibility patterns of 50 strains of *Y. ruckeri* to 23 antimicrobial agents. They found that the serological varieties of *Y. ruckeri* did not differ significantly in their *in vitro* response to most antimicrobial agents, with the exception of polymyxin B to which strains II, III and V were highly resistant, whereas most serovars I were susceptible. However, six serovar I strains were highly resistant to polymyxin B, and these strains were also able to grow at 37°C. These authors found no correlation between the susceptibility to polymyxin B and the presence of the 50-Mdal plasmid of *Y. ruckeri*, and they suggested that these characteristics may reflect variations in the cell surface of serovar I strains. The possible role of the plasmid in virulence and its relation with susceptibility to polymyxin B and growth at 37°C awaits further research.

2.10 CONTROL OF ERM

2.10.1 Prevention

Wherever ERM is geographically restricted, prevention of further dissemination should be achieved by implementation of fish health inspections and certification procedures to regulate fish movements (Busch, 1983). In areas where ERM is already established, the severity of the disease could be reduced by optimising husbandry conditions, including reducing stress, control of water quality, provision of adequate diets, and maintenance of good sanitary practices, since ERM is a stress-related disease (Busch, 1983;

Bullock, 1984; Horne *et al.*, 1984; Schlotfeldt *et al.*, 1986; Horne and Robertson, 1987). However, such improved husbandry conditions may be difficult to achieve in intensive aquaculture systems.

2.10.2 Vaccination

Enteric redmouth disease was the first fish disease for which a practical, commercially available bacterin was developed (Bullock, 1984). Despite this fact, the nature of the protective effect and the mechanism by which the vaccine is taken up by the fish, remains unknown (Austin and Austin, 1987). The first reported vaccine against ERM, was an oral bacterin developed by Klontz (1963). This was later improved by Ross and Klontz (1965), working with a phenol-killed cell preparation incorporated in the feed, which achieved a 90% survival in immunized fish, compared with only 29% in non-immunized controls. Anderson and Ross (1972), compared the effectiveness of four different oral bacterins by inactivating cells either with 0.5% phenol or 3% chloroform. These authors also prepared bacterins with sonicated cells followed by adding either formalin to 1% or phenol to 3%. The fish immunized with the chloroform-prepared vaccine showed greater protection after challenge, although all preparations protected the fish to some degree. Amend *et al.* (1983) found that the potency of bacterins grown on tryptic soya broth at room temperature was not affected by pH (6.5 to 7.7) or age of the culture (9 to 96 h). Chloroform and formalin-inactivated bacterins were equally potent, and the potency was not increased when cells were extracted with either butanol or phenol. However, bacterins prepared from alkaline-

lysed cells (pH 9.8 for 60-120 min) followed by inactivation in 0.3% formalin, resulted in a significant increase in protection (Amend *et al.*, 1983). Bacterins prepared in this way could be diluted up to 1:2000 without significant loss in protection, when delivered by immersion (20 sec).

Anderson and Nelson (1974) showed that fish vaccinated by subcutaneous inoculation had higher levels of protection, after virulent challenge, than fish orally vaccinated and protection in the former group was retained longer than in the fish vaccinated orally. Antibody levels were only detectable in fish vaccinated by injection (Anderson and Nelson, 1974). Although injection vaccination was more efficient than oral administration, the economic cost of injection procedures is much higher since it involves individual handling of the fish, therefore, other delivery systems which allow mass immunization are desirable.

The early commercial enteric redmouth bacterins were usually crude formalin killed broth cultures of serotype I (Hagerman strain) *Y. ruckeri* administered to the fish (0.45 g) by an "hyperosmotic immersion" procedure (30 sec in 5% solution of salt followed by 30-60 sec in the bacterin; Busch, 1983). Anderson *et al.* (1979) questioned the value of the hyperosmotic step, by showing that preceding antigen immersion with a 2 min immersion in either a 5.2 or a 2.6% NaCl solution, did not affect the numbers of plaque-forming cells (PFC) or antibodies in immunized rainbow trout. Johnson and Amend (1983a), compared the efficacy of several methods of vaccinating rainbow trout. The

best protective immunity was achieved by intraperitoneal (i.p.) injection, followed by direct immersion, shower and spray respectively.

Johnson and Amend (1983b) showed that anally vaccinated fish were significantly better protected than fish vaccinated either orally or by immersion. The author pointed to alternatives to improve the performance of oral vaccines such as micro-encapsulation, enteric coating or polymer entrapment.

The optimum immersion time for effective vaccination of salmonid fry was reported to be 5 sec, and protection against either *Y. ruckeri* or *Vibrio anguillarum* occurred within 5 days at 18°C and within 10 days at 10°C (Johnson *et al.*, 1982a). The same authors showed that 1 g was the minimum size at which salmonids could be effectively immunized by immersion, with immunity being a function of size rather than age. Johnson *et al.* (1982b) reported that the duration of immunity in salmonids, vaccinated by immersion with *Y. ruckeri* and *V. anguillarum* bacterins varied not only as a function of size but also with species of fish and bacterin concentration. Immunity lasted longer in bigger fish, but duration could be prolonged by increasing the concentration of bacterin.

The relationship between vaccine dilution and length of immersion is important to achieve effective vaccination. It has been found that the higher the dilution of the vaccine the longer the immersion time required to achieve

an equivalent protection of brown trout compared to a more concentrated bacterin (Tatner and Horne, 1985). Field trials to evaluate the efficacy of vaccines in fish farms where natural challenges occur, have been well documented (Amend and Eshenour, 1980; Tebbit *et al.*, 1981; Johnson *et al.*, 1982b; Bullock and Anderson, 1984; Schlotfeldt *et al.*, 1985; Schlotfeldt *et al.*, 1986; Horne and Robertson, 1987). Field trials produced encouraging results and showed the effectiveness of vaccination for the control of ERM. Even though vaccines are being extensively used to control *Y. ruckeri* infection in fish, they do not completely eliminate the pathogen (Busch, 1983). *Y. ruckeri* was isolated from kidney and rectum samples of Atlantic salmon after being vaccinated with the ERM bacterin, suggesting that vaccination does not prevent the establishment of asymptomatic carrier populations (Bruno and Munro, 1989). This may mean that vaccination could be, in fact, a means of spreading ERM. Therefore, new formulations of ERM vaccines able to eliminate the establishment of a carrier state are most desirable. Although some work has been done in the study of a secondary response in salmonids after a booster vaccination (Tatner and Horne, 1985; Cossarini-Dunier, 1986), more work is needed to determine the optimal conditions to perform booster vaccination in an effective and economic way. Despite the considerable amount of work carried out using *Y. ruckeri* as a "model antigen", the nature of the protection acquired by the fish after vaccination remains largely unknown. Protection against *Y. ruckeri* does not seem to be due to antibodies (Cossarini-Dunier, 1986; Cipriano and Ruppenthal, 1987).

Vaccination can enhance the bactericidal activity of trout serum, although non-specific factors could also play an important defensive function (Grayson *et al.*, 1987). ERM bacterins were originally produced using the serotype I strain (Hagerman strain) since it was thought to be the only one responsible for ERM disease outbreaks. O'Leary *et al.* (1982), prepared formalin-killed bacterins of *Y. ruckeri* serotypes I and II and studied the cross-protection among them. Bacterin made with serotype II bacterium and bivalent bacterin (I and II) conferred protection against infection with either serotype, whereas serotype I bacterin only conferred protection against its homologous serotype. By contrast, Cipriano and Ruppenthal (1987) immunized brook trout with bacterins containing either serotype 1 or 2 of *Y. ruckeri* to determine the degree of cross-protection against challenge with the homologous and heterologous serotype. They found that all fish were protected, regardless of the bacterin used. Despite the apparent contradiction in the results of these two teams, its seems obvious that further work, in the formulation of bacterin preparation to include more than one serotype of *Y. ruckeri*, should be considered.

2.10.3 Chemotherapy

Antimicrobial compounds are routinely used on fish farms to combat diseases. These compounds are used as food additives, baths (dips or flushes) or introduced by injection (Austin, 1985). Injection delivery poses hardly any risk of environmental contamination, and medicated diets, if used sensibly, should also lead to release of minimal amounts of the compounds into the

water. Bath systems, by contrast, lead to the discharge of the chemotherapeutic agent into the water surrounding the fish farm facility, which may serve to induce the development of a resistant microflora in the aquatic environment (Austin, 1985). Unexpected resistance to antibiotics can appear on a farm, thereby reducing the spectrum of antibiotics available for chemotherapy (Schlotfeldt *et al.*, 1986). It is therefore important to perform differential diagnosis and antibiotic sensitivity testing before proceeding with treatment. The wrong dosage or choice of antibiotic can lead to the development of drug resistant strains (Busch, 1983), this has been particularly problematic in countries like Italy, where vaccine prophylaxis was previously not allowed, and fish disease control relied on chemotherapy (Ceschia *et al.*, 1987).

In vitro sensitivity tests of *Y. ruckeri* to specific antibiotics, have been performed either with therapeutic aims or for descriptive purposes (Bosse and Post, 1983; Ghittino *et al.*, 1983; Lesel *et al.*, 1983; Schlotfeldt *et al.*, 1985; De Grandis and Stevenson, 1985; Baath, 1986; De la Cruz *et al.*, 1986; Michel *et al.*, 1986; Ceschia *et al.*, 1987; Bowser and House, 1990). De Grandis and Stevenson (1985) studied the susceptibility of 50 strains of *Y. ruckeri* to 23 antimicrobial agents and showed that the susceptibility patterns were very similar for all strains independent of their serological type. Two of the strains tested carried resistance plasmids for tetracyclines and sulfonamides, which were transferable to *Escherichia coli* and *Y. ruckeri* recipients. Tetracycline resistance plasmids in *Y. ruckeri* had been reported before by

Cook and Gemski (1982). The linkage of resistance to antibiotics with a transferable plasmid is a matter of concern, especially as the two antibiotics are commonly used in aquaculture (De Grandis and Stevenson, 1985).

The use of antimicrobial compounds in fish farming is limited by the current legislations operating in different countries. Austin (1985) presented the list of substances and dosages used in Great Britain for the control of fish diseases.

Several antimicrobial agents have been used effectively to treat ERM. Rucker (1966), recommended a combination of sulfamerazine (20 g/100 kg of fish per day for 5 days), followed by oxytetracycline or chloramphenicol (5.0 g/100 kg of fish per day for 3 days). Bosse and Post (1983) found that Tribissen, a combination of 80 parts of trimethoprim and 400 parts of sulphadiazine (1 mg per kg fish per day) and tiamulin (5 mg per kg fish per day), when administered orally for 14 days were effective against *Y. ruckeri*, and were also found to be non-toxic for the fish.

Bullock *et al.* (1983), effectively controlled ERM with a potentiated sulfonamide,Ro5-0037 (50 mg per kg of fish for 5 days). Despite the success of those treatments, none of the above drugs was registered for use in the United States for treatment of ERM in fish intended for human consumption (Bullock, 1984).

Apurone (80 g per 100 g of food at 1.5% of live weight of fish for 5 to 6 days), stopped mortalities occurring in rainbow trout during an outbreak of ERM, but failed to completely eliminate *Y. ruckeri* from the fish, which remained asymptomatic carriers (Lesel *et al.*, 1983). Rodgers and Austin (1983) reported that oxolinic acid can act both as a prophylactic and a chemotherapeutic agent in rainbow trout. Tetracycline was fed to rainbow trout (2 g per kg of food) for 7 days, during an ERM outbreak (Rebsämen and Weis, 1985). Mortalities declined but did not cease completely until the stocking density was reduced. The same authors used chloramphenicol (1 g per kg of food during 7 days) in a second ERM case. In this instance, mortalities ceased completely.

Recent work carried out by Siwicki *et al.* (1989) showed that oxolinic acid, used at the recommended dosage, did not cause immunosuppression in either the non-specific or in the specific immune defence system of young rainbow trout, whereas tetracycline (10 mg per kg) caused reduced activity in both. Therefore, care should be taken when a particular antimicrobial compound is chosen and used for subsequent therapy.

2.11 *Yersinia ruckeri* AS A MODEL ANTIGEN

Y. ruckeri has been used as a model antigen in a wide variety of studies, such as the development of techniques for diagnostic work, either for antibody

detection (Hansen and Lingg, 1976; Toranzo *et al.*, 1987), for antigen detection (Johnson *et al.*, 1974; Lewis, 1981; Austin *et al.*, 1986) or for detection of changes in blood serum proteins by immunoelectrophoresis (Gelev *et al.*, 1984). In several studies of the primary and secondary immune response in fish, *Y. ruckeri* has been the antigen of choice (Busch, 1978; Anderson *et al.*, 1979a, b, c, d; Anderson and Dixon, 1980; Lamers and Pilarczyk, 1982; Anderson *et al.*, 1983; Neumann and Tripp, 1986; Cossarini-Dunier, 1986).

Y. ruckeri has also been used in studies of immunosuppression caused by manganese (Cossarini-Dunier *et al.*, 1988); corticosteroids (Anderson *et al.*, 1982); gamma irradiation of lymphoid organs (Chilmonczyk and Oui, 1988); exposure to copper (Knittel, 1981) and tetracycline treatment (Siwicki *et al.*, 1989). Griffin (1983) and Stave *et al.* (1987), used *Y. ruckeri* to study the responses of fish phagocytes. The effect of diets on the immunoperformance of fish which had been immunized with *Y. ruckeri* has also been reported (Blazer and Wolke, 1984a-b; Henken *et al.*, 1987). The localization and trapping of *Y. ruckeri* cells in fish tissues was studied by Lamers and Pilarczyk (1982); Herraez and Zapata (1987); Zapata *et al.* (1987).

2.12 MECHANISMS OF PATHOGENICITY

The terms pathogenicity and virulence are synonymous, meaning the capacity

to produce disease by a micro-organism (Smith, 1984) although virulence is often retained for use in a quantitative sense. While the term infection refers to successful persistence or multiplication of a pathogen on or within the host, the term disease implies an infection which causes overt damage to the host (Finlay and Falkow, 1989).

Microbial pathogenesis is usually complex and multifactorial. The pathogenic micro-organism has to be able to succeed in penetrating the mucous surfaces of the host, which act as a physical barrier; it also has to be able to multiply in the environment of the host, resist or interfere with the host defence mechanisms and finally cause damage to the tissues of the host (Smith, 1984). Microbial pathogens need an array of biochemical mechanisms to succeed in these different steps and removal of any of those components will result in a loss of virulence (Finlay and Falkow, 1989).

An increased interest in the study of microbial pathogenicity happened during the 1960's and 1970's, due in part to the failure of antibiotics to eliminate bacterial diseases, but, mainly, to the improvement of techniques in cell biology, genetics and molecular biology (Smith, 1989). Such work has produced a voluminous literature which is too large to be covered in the present work.

Excellent general revisions have been compiled by Smith (1984, 1989); Brubaker (1985); Mims (1987); Roth (1988); Williams *et al.* (1988) and Finlay

and Falkow (1989). The vast majority of the published work on microbial pathogenicity refers to diseases of homeotherm hosts, mainly mammals, and their responses to infection and disease are not necessarily comparable to those of poikilotherm hosts, such as fish. In order to be able to understand how fish pathogens cause disease, it is of paramount importance to study the virulence processes within the relevant host context, for this reason, the reader is referred to the following reviews of the immune system in fish: Ingram (1980); Ellis (1981); Fletcher (1982); Dorson (1984); Ingram (1986) and Ellis (1989).

The first step in studies of microbial pathogenicity is the establishment of methods for measuring and comparing the virulence of different strains of the chosen pathogenic species (Smith, 1984). The degree of virulence can be modulated by variables such as route of inoculation, bacterial strain and the culture medium used, the growth stage of the inoculated culture together with species of test animal used and their physiological state (age, nutrition, immunological status) (Davis *et al.*, 1981). The virulence of an organism (or of a toxin) is usually expressed as the LD_{50}: the dose that will kill 50% of the inoculated animals within a given time (Davis *et al.*, 1981). The quantification of the ability of a bacterium to cause a demonstrable infection is also important in diseases like ERM which can be of both acute and chronic nature. The ID_{50} refers to the dose required to cause infection in 50% of the animals (Davis *et al.*, 1981).

As mentioned previously, the various stages in colonization of the host, avoidance of host defence mechanisms and production of damage can be identified. The first stages, such as adherence and entry of a bacterium to the host are key steps in the pathogenic process. The pathogen overcomes the problems of removal by fluids and the host defence mechanisms found in the mucous surfaces by means of highly specialized adherence factors, (i.e. fimbriae or pili). A receptor and an adhesin are the minimal requirements for the microbial attachment process (Finlay and Falkow, 1989). Adhesion phenomena have also been reported for fish pathogens. Parker and Munn (1985) working with *Aeromonas salmonicida*, the causal agent of furunculosis, correlated the presence of the external layer, A-protein, with an increased adhesive ability *in vitro*, when A^+ strains of *A. salmonicida* were compared with A^- strains.

It has been reported that another fish pathogen, *Vibrio anguillarum*, can adhere and colonize the intestine of rainbow trout within 100 min of exposure (Horne and Baxendale, 1983).

In the case of intracellular pathogens invasion is particularly important. Many different strategies have evolved in various bacterial taxons to enable successful invasion. Host cell invasion is often mediated by the presence of a receptor (integrin) on the surface of the eukaryotic cell (Finlay and Falkow, 1989). Of the known fish pathogens *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease (BKD) in salmonid fish, is probably the best

example of a bacterial intracellular pathogen. Virulent strains of *R. salmoninarum* have increased cell surface hydrophobicity, which has been suggested could contribute to serum resistance and intracellular survival by avoidance of the action of lysosomal enzymes within the phagosome (Bruno, 1988; Munro and Bruno, 1989). The hydrophobic nature of the cell surface of *R. salmoninarum* could be due to the presence of a haemagglutinin molecule associated non-covalently with the cell surface (Daly and Stevenson, 1987). Progress in the investigations on the virulence mechanisms of *R. salmoninarum* has been handicapped by the difficulty in growing this pathogen in the laboratory, due to the lengthy culture periods required. This problem has been overcome in recent work carried out by Evenden *et al.* (1990) using molecular biology techniques. These authors reported the cloning of *R. salmoninarum* haemolysin into *E. coli*, and suggested that the molecule may be a virulence factor operating as a membrane active cytotoxin.

Establishment of the bacterial pathogen within the host will usually require destruction or damage of host tissues by means of bacterial toxins which can be both intracellular and extracellular (Smith, 1984). Among fish pathogens, *A. salmonicida* is the one on which most work on the study of extracellular products (ECP) has been concentrated. The ECP of *A. salmonicida* is lethal when injected (i.p.) in rainbow trout (Munro *et al.*, 1980). The toxic activity of the ECP of *A. salmonicida* has been attributed to enzymes such as proteases and phospholipases and to haemo- or leukocytolytic substances (Munro, 1984). Work carried out by Cipriano (1981, 1982 a, b, c)

fractionating ECP of *A. salmonicida* has shown that different fractions display different activities both in tissue culture and in *in vivo* experiments. Three of the isolated fractions (I, II and III) were toxic when injected into fish. Substantial progress has been made in the isolation and characterization of the components of the *A. salmonicida* ECP. Various activities have been detected (haemolysins, proteases and leucocidins), but the interrelationships remain unclear (Austin and Austin, 1987). It has been reported that there is a lack of relationship between the proteolytic and haemolytic activities of *A. salmonicida*. However, ECP is associated with lethality (Ellis *et al.*, 1988) and recent work (Lee and Ellis, 1989) indicates that the high molecular weight haemolysin of *A. salmonicida*, formed by a complex of glycerophospholipid: cholesterol acyltransferase (GCAT) and lipopolysaccharide (LPS), can combine with the protease in an additive way to kill fish. The lethal dose of the GCAT/LPS complex is 55 times higher than the lethal dose of the protease. However, the lethal dose of the *A. salmonicida* ECP will depend not only on the absolute concentrations of protease and haemolysin but also on their relative amounts in the ECP.

V. anguillarum can also display toxins of haemolytic (Munn 1978, 1980) and proteolytic nature (Inamura *et al.*, 1984; Kodama *et al.*, 1984). These may be virulence factors which facilitate the establishment of the pathogen in the fish host. However, in order to be able to grow and multiply *in vivo*, organisms like *V. anguillarum*, which use the blood stream for dissemination often have developed mechanisms to acquire iron which can successfully compete with

the iron binding system of the host (Finlay and Falkow, 1989). The ability of the host to withold iron is often referred to as "nutritional immunity" and is achieved by the production of iron ligands as ferritin, transferrin and lactoferrin which are both within the host cells and in extracellular body fluids (Williams *et al.*, 1988). Micro-organisms synthesize low molecular weight iron-specific ligands called siderophores capable of competing with host iron-binding proteins for the iron which they require for growth (Hider, 1984). Virulent *V. anguillarum* contain a 47 Mdal plasmid (pJM1) which codes for a siderophore and an outer membrane protein (OM2) which, together with a chromosomal coded outer membrane protein (OM3), constitute a rapid iron-sequestering system of *V. anguillarum*, only present in virulent strains (Crosa *et al.*, 1977; Crosa, 1980; Crosa *et al.*, 1980; Crosa and Hodges, 1981; and Crosa *et al.*, 1983).

Toranzo *et al.* (1983) reported the existence of plasmid-less virulent *V. anguillarum*, which were able to grow in iron-limiting conditions with production of siderophore and outer membrane proteins, which were coded by a sequence of chromosomal DNA homologous with the pJM1 plasmid. Recent work carried out by Conchas *et al.* (1990), reported that all serotype 02 strains of *V. anguillarum* seem to have the same iron uptake system, which is distinct from the one present in strains of the 01 serotype.

In order to survive and proliferate after invasion, the pathogen has to be able to avoid the host immune system. This can be achieved in various ways such

as resistance to the bactericidal effect of serum, antigenic variation and resistance to phagocytosis (Williams *et al.*, 1988). The A-layer of *A. salmonicida* seems to be responsible for conferring the pathogen with the capacity of resistance to the complement bacteriocidal activity of rainbow trout serum either in the presence or absence of specific antibody to *A. salmonicida* (Munn *et al.*, 1982). Recent work carried out by Sharp and Secombes (1990), indicates that virulent *A. salmonicida* are more resistant to the killing action of rainbow trout macrophages than avirulent strains of the pathogen. In fact, A^+ strains of *A. salmonicida* were able to multiply in the spleen of fish challenged with the pathogen (Munn and Trust, 1984).

2.12.1 Virulence Mechanisms of *Y. ruckeri*

A large number of experiments have been carried out using *Y. ruckeri*, and some information about virulence can be deduced from these. A summary of the methods and the results obtained in virulence work carried out by various teams using *Y. ruckeri* as a pathogen is given in Table 2.2. Both parenteral and waterborne infection routes have been used with various fish species. Various serotypes of *Y. ruckeri* have also been considered, because although it was thought originally that only serovar I was virulent (Bullock *et al.*, 1978), later observations indicated that serotype II strains could also be responsible for epizootics, as reported by Cipriano *et al.* (1986), who found a *Y. ruckeri* serovar 2 responsible for an ERM outbreak in a chinook salmon hatchery. More evidence of the virulence of serotype II strains of *Y. ruckeri* has been produced by means of laboratory infections (Table 2.2).

Other serotypes of *Y. ruckeri* have been tested for virulence in the laboratory, both by i.p. and bath infections (Flett, 1989), but they are apparently of lower virulence than serotypes I and II (Table 2.2).

Despite the information available on virulence of *Y. ruckeri*, a comparison of results obtained in different laboratories proves very difficult due to the lack of uniformity in the experimental protocols (inoculum preparation, infection dose, water temperature), variation in fish species and, in some cases, non-reported details such as bacterial serotype used and the weight of experimental fish.

Despite the fact that *Y. ruckeri* has been recognised as a fish pathogen for over three decades, since Rucker isolated the bacterium in the early 1950's, and despite the amount of work involving *Y. ruckeri*; very little is still known about its virulence mechanisms. Immunological studies carried out mainly in salmonid fish, indicated that although the pathogen induces a humoral immune response (primary and secondary) in fish, and specific antibodies against it can be detected in immunized fish, the role of such antibodies is not clear, since they do not seem to be correlated with level of protection. A possible role of specific antibodies in the ERM disease process has been outlined by Griffin (1983) who studied the influence of specific antibody on phagocytic uptake and intracellular killing of *Y. ruckeri* by partially purified rainbow trout peripheral blood leucocytes. This work indicated that specific antibodies against *Y. ruckeri* exerted a significant opsonic effect on

phagocytosis of homologous bacteria by normal trout phagocytes. However, intracellular killing of bacteria by normal trout leucocytes was not affected by opsonization with antibody. The possible reason for the high survival rates of *Y. ruckeri* within the trout leucocytes were, however, not studied in Griffin's work. The presence of a 70 Mdal plasmid in some *Y. ruckeri* strains, was correlated directly with virulence by Cook and Gemski (1982). These authors referred to virulent and non-virulent serotypes of *Y. ruckeri*, although no indication of how virulence had been assessed is provided. Stave *et al.* (1987), studied the chemiluminescent responses of phagocytes of striped bass (*Morone saxatilis*) to serotype I and serotype II strains of *Y. ruckeri*. They found that, in general, serotype I strains, carrying the 70 Mdal plasmid, elicited weak chemiluminescent responses from phagocytes, while plasmidless-serotype II strains, generated up to 14 times stronger responses. In addition the work also showed the capacity of some strains of *Y. ruckeri* to circumvent the phagocytic defence mechanisms of the fish. Initially it was thought that the 70 Mdal plasmid carried by the serotype I strains, was responsible for the ability of such strains to avoid phagocytosis, but the low chemiluminescent responses registered when a *Y. ruckeri* plasmid-less serotype II was tested, indicated that factors unrelated to the plasmid could be involved. Stave *et al.* (1987), therefore hypothesized that an antiphagocytic antigen may be expressed by *Y. ruckeri* resulting in the avoidance of phagocytic cells, as is the case in other members of *Yersinia*, such as *Y. pestis*, which is able to produce antiphagocytic factors (Charnetzky and Shuford, 1985) to reduce phagocytic microbiocidal activity. It is known now, however, that the plasmid of *Y.*

ruckeri is completely different from the plasmid of the virulent members of the genus *Yersinia*. O'Leary *et al.* (1979), showed that although *Y. ruckeri* was non-pathogenic for mice, the bacterium produced gamma haemolysis in mammalian blood (5% sheep blood agar). Romalde *et al.* (1988), tested 24 strains of three different serotypes of *Y. ruckeri*, on trout and human blood but none of the strains tested produced haemolysis of either blood type. The same *Y. ruckeri* strains were used in a series of tests comprising autoagglutination, hydrophobicity, haemagglutination, cytotoxic activities and siderophore production. All but one of the strains tested, were negative for all tests conducted, indicating that different tests other than those mentioned above, should be tried to study further the pathogenicity mechanisms of *Y. ruckeri*.

Gelev *et al.* (1984) pointed out the strong lipolytic activity exhibited by the Bulgarian isolates of *Y. ruckeri* that they were using. In addition, during immunoelectrophoretic diagnostic testing of blood sera from trout suffering ERM, precipitation arcs, due to the molecular destruction of α and ß-lipoproteins appeared. These "breakdowns" of lipoproteins did not occur in fish suffering diseases of very similar pathology to ERM such as viral haemorrhagic septicaemia or in fish with non-infectious degeneration of the liver. The implications of this fact in the pathogenesis of *Y. ruckeri* were not explained. Miller (1983) suggested an endotoxin activity of the LPS of *Y. ruckeri*, which would cause the severe intravascular coagulation syndrome found in ERM.

Further work is needed on the study of the virulence of *Y. ruckeri*, which takes into account that, although it is a phenotypically homogenous taxon, its antigenic make-up can be quite complex, as has been suggested by Davies (1990). This could indicate that different strategies are followed by different serotypes in their interaction with the host and subsequent establishment of disease.

TABLE 2.1: PERCENT RELATIVE BINDING RATIOS AND PERCENT DIVERGENCE IN RELATED SEQUENCES IN THE GENUS *Yersinia*[a]

Source of Unlabelled DNA	Source of Labelled DNA					
	Y. ruckeri	*Y. pseudotuberculosis*	*Y. enterocolitica*	*Y. intermedia*	*Y. frederiksenii*	*Y. kristensenii*
Y. ruckeri	95(0.1)	33(13)	30(15)	38		
Y. pseudotuberculosis	30(15)	92(1)	59(12)	64(12)		50
Y. enterocolitica	30(15)	48(11.5)	96(2.5)	58(11)	60(12.5)	69(9)
Y. intermedia		44(12)	59(12)	95(1.5)	61(11)	62(12)
Y. frederiksenii		44(11)	67(11)	58(12.5)	81(5)	59(10)
Y. kristensenii		44(11)	70(9.5)	62(12.5)	55(12)	84(4)

[a]Data are from Bercovier *et al.* (1980), Brenner *et al.* (1980), Ewing *et al.* (1978) and Ursing *et al.* (1980). Table is adapted from Bercovier and Mollaret (1984). Hybridizations were carried out at 60°C. The first number is the percent relative binding ratio of all unlabelled DNA with the specific labelled DNA. The second number, in parenthesesis the percentage of divergence calculated on the basis of 1% unpaired bases per 1°C decrease in duplex stability.

TABLE 2.2 VIRULENCE OF *Y.ruckeri* AS INDICATED IN PUBLISHED STUDIES.

INFECTION METHODS.

1. INTRAPERITONEAL INJECTION.

SEROVAR	INOCULUM PREP. (a).	DOSE+WATER T°C	FISH (b).	RESULTS (c).	REFERENCE
I	TSB. 24-48 h.	$1.3x10^3$ cfu/fish 17-18 °C.	RT (> 1.3g)	68% in 10 days.	Amend *et al* (1983)
	TSA. 18 h.	18 bacteria/fish 14.5°C	RT (185g)	LD_{26} = 18 bacteria	Busch & Lingg (1975)
	NR	$3x10^3$ cfu/fish	RT (20g)	0-60%	Chilmonczyk & Oui (1988)
	TSB, 48 h, 18°C (3 strains)	$2.2x10^6$-$2.7x10^6$ cfu/0.5ml 10±2°C	RT (48.7-61.2g)	10-80% in 10-14 days	Flett (1989)
	NR	$4x10^6$ cfu/fish 15°C	RT (> 28g)	86%	O'Leary (1982)
	NR	$2.5x10^8$ cfu/fish	RT (NR)	92% in 24 h	McArdle and Dooley-Martyn (1985)
	NR	$1x10^{10}$ cfu/fish	RT (12-18 cm)	100% in 62 h	Wobeser (1973)
II	TSB, 48 h, 18°C (2 strains)	$2.5x10^6$ cfu 0.5 ml 10°C±2°C	RT (56.5-60.3 g)	10-100% in 10-14 days	Flett (1989)
	TSB, 20 h	$4.2x10^8$-$2x10^8$ cfu x ml^{-1} 15°C	Coho salmon (> 5 g)	70-83% in 14 days	Newman & Majnarich (1982)
	TSB, 20 h	$2x10^6$-$3x10^6$ cfu 15°C	RT (> 5 g)	72.1-78.3% in 14 days	Newman & Majnarich (1982
	NR	$5.95x10^6$ cfu/fish 15°C	RT (> 28 g)	76%	O'Leary (1982)
III	TSB, 48 h, 18°C (3 strains)	$2.5x10^6$-$4.4x10^6$ cfu/0.5ml 10°C±2°C	RT (39.5-57.2 g)	0-18% in 10-14 days	Flett (1989)

Continued 2

TABLE 2.2 VIRULENCE OF *Y.ruckeri* AS INDICATED IN PUBLISHED STUDIES.

SEROVAR	INOCULUM PREP. (a).	DOSE+WATER T°C	FISH (b).	RESULTS (c).	REFERENCE
V	TSB, 48 h, 18°C (2 strains)	$1.7x10^8$-$2.8x10^8$ cfu/0.5 ml 10°C±2°C	RT (39.5-40.8g)	0-18% in 10-14 days	Flett (1989)
Unknown	TSB	0.1ml of dose equivalent to 0.0001mg dry weight of bacteria per ml	RT	LD_{75} in 14 days	Bosse and Post (1983)
	TSA, 48 h, 20-30°C	0.05ml of $6.5x10^7$-$6.5x10^5$ cfu 12.5°C	Atlantic salmon 2.15 g	100% in 21 days	Bullock *et al.* (1976)
	TSB, 18 h, 22°C	$1.2x10^7$ cfu/fish 14°C	RT (> 100 g)	23/26	Cossarini-Dunier (1986)
	NR	$1x10^8$ cfu/fish	RT (200 g)	100% in 3 days	Dalsgaard *et al.* (1984)
	NR	$1x10^8$ cfu/fish 18°C	RT (6 g).	76-84% in 28 days	Rodgers and Austin (1983)
	NR	$1x10^8$ cfu/fish	RT (> 180 g)	48.3%	Schlotfeldt (1986)
	TSB, 24 h, 20°C	$2x10^5$ cfu/fish 8-10°C	Brown Trout (> 0.3 g)	96%	Tatner and Horne (1985)
2. INTRAMUSCULAR INJECTION					
Unknown	NR	10^3 cfu/fish	Channel Catfish (@ 25 cm)	100% in 96 h	Lewis (1981)
3. SUBCUTANEOUS INJECTION					
Unknown	NR	$4x10^7$ cfu/fish. 0.1ml of 10-fold dilutions of a $4x10^9$ cfu/ml 17-18°C	RT (> 2 g)	$LD_{50} < 4x10^5$ cfu/fish	Anderson and Nelson (1974)
		0.1ml of 10-fold dilutions of a $4x10^9$ cfu/ml ($4x10^7$ cfu/fish) 17-18°c	RT (9 months old)	$LD_{50} = 8.5x10^5$ cfu/fish	Anderson and Ross (1972)

Continued 3

TABLE 2.2 VIRULENCE OF *Y.ruckeri* AS INDICATED IN PUBLISHED STUDIES.

4. IMMERSION CHALLENGE

SEROVAR	INOCULUM PREP. (a).	DOSE+WATER T°C	FISH (b).	RESULTS (c).	REFERENCE
I	TSB, 24-48 h	1h. $1x10^8$ cfu x ml^{-1} 17-18°C	RT (>3.2g-10.6g)	85-54% in 10 days	Amend *et al.* (1983)
	NR	90s. 10^8-10^9 cfu x ml^{-1}	RT (10 g)	LD^{50} = $3x10^5$ cfuxml^{-1} in 15 days	Bullock and Anderson (1984)
	TSA. 18 h	1 h. $2.75x10^8$ cfu x ml^{-1} 20°C	RT (185 g)	LD_2 = 2.75×10^8 cfu x ml^{-1}	Busch and Lingg (1975)
	NR	60s. $1.4x10^9$ cfu x ml^{-1}	Brook Trout (52g) Atlantic salmon (36g)	70-80% in 14 days 100% in 14 days	Cipriano *et al.* (1986)
	TSB, 48 h, 25°C	(1min). $1x10^8$ cfu x ml^{-1} 12.5°C	Brook Trout (62.8 g)	6/20 and 5/20 in 14 days	Cipriano and Ruppental (1987)
	TSB, 48 h, 25°C (17 strains)	(1min). 10^9 cfu x ml^{-1} 12.5°C	Brook Trout (42.6 g)	65% (15-100%) in 14 days	Cipriano *et al.* (1987)
	TSB, 24-48 h	1 h. $2x10^8$ cfu x ml^{-1} 17-18°C	RT (>3 g)	76%	McCarthy and Johnson (1982)
	TSB, 48 h, 18°C (4 strains)	1 h. $2x10^8$-$3.8x10^8$ cfuxml^{-1} 10±2°C	RT 15.6-42.6 g	0-8% in 7-14 days	Flett (1989)
	TSB, 48 h, 18°C	1 h. $2.8x10^8$ cfu x ml^{-1}	Chinook Salmon (37 g)	0% in 14 days	Flett (1989)
	TSB, 24-48 h	1 h. $4.6x10^8$ cfu x ml^{-1} 17°C	RT (> 9.5 g)	65% in 7 days	Johnson and Amend (1983)
II	NR	(90s) 10^8-10^9 cfu x ml^{-1}	RT (10 g)	LD_{50} = $1x10^7$ cfu x ml^{-1} in 15 days	Bullock and Anderson (1984)
	NR	(60s) $3.5x10^9$ cfu x ml^{-1}	Brook Trout (52 g) Atlantic salmon (36 g)	90% in 14 days 70% in 14 days	Cipriano *et al.* (1986)

Continued 4

TABLE 2.2 VIRULENCE OF *Y.ruckeri* AS INDICATED IN PUBLISHED STUDIES.					
SEROVAR	INOCULUM PREP. (a).	DOSE+WATER T°C	FISH (b).	RESULTS (c).	REFERENCE
II	TSB, 48 h, 25°C	(1 min), $1x10^8$ cfu x ml^{-1} 12.5°C	Brook Trout	70% in 14 days	Cipriano and Ruppental (1987)
	TSB, 48 h, 25°C (16 strains)	(1 min) 10^9 cfu x ml^{-1} 12.5°C	Brook Trout (42.8 g)	59% (12-90%) in 14 days	Cipriano *et al.* (1987)
	TSB, 48 h, 18°C (2 strains)	(1h)$1x10^8$-$1.5x10^6$ cfuxml^{-1} 10±2°C	RT 56.6-61.8 g	0-90% in 14 days	Flett (1989)
	TSB, 48 h, 18°C (3 strains)	(1h)$3.4x10^8$-$3.8x10^8$cfuxml^{-1} 10±2°C	Chinook salmon 38.5-41.9 g	0-50%	Flett (1989)
III	NR	(90s) 10^8-10^9 cfu x ml^{-1}	RT (10 g)	0% in 15 days	Bullock and Anderson (1984)
	TSB, 48 h, 18°C (3 strains)	(1h)$1.6x10^8$-$2.4x10^8$cfuxml^{-1} 10±2°C	RT (30.9-33.2 g)	0% in 7 days	Flett (1989)
V	TSB, 48 h, 18°C (2 strains)	(1 h) $2.6x10^8$ cfu x ml^{-1} 10±2°C	RT (23.8-38.2 g)	0% in 7 days	Flett (1989)
Unknown	NR	(30 min) $2x10^5$ cfu x ml^{-1}	Atlantic salmon (> 4 g)	37-42% in 12-14 days 78% (when strain was passaged through fish)	Bruno and Munro (1989)
	NR. TSA, 48 h 20-30°C	(30 min) 10^7 cfu/1500ml 12.5°C	Atlantic salmon(2.15g)	30-50% in 21 days	Bullock *et al.* (1976)
	BHI, 24 h	(90s) $1x10^9$ cfu x ml^{-1}	RT (79 g)	75% in 2 weeks	Bullock *et al.* (1983)
	TSB, 18-24 h	200 ml of broth culture in 10 l water for 1 h.	Steelhead Trout (27 g)	0%	Hunter *et al.* (1980)
	TSB, 24 h, 20°C	(1 h)$1x10^6$-$2x10^6$ cfu x ml^{-1} 12°C	Steelhead Trout (8 g)	30-35%	Knittel (1980)
	TSB, 24 h, 25°C	(1 h) $1x10^3$ cfu x ml^{-1}	Steelhead Trout (8.7 g)	LD_{50} = $1.1x10^6$ cfu/ml	Knittel (1981)

Continued 5

TABLE 2.2 VIRULENCE OF *Y.ruckeri* AS INDICATED IN PUBLISHED STUDIES.					
SEROVAR	INOCULUM PREP. (a).	DOSE+WATER T°C	FISH (b).	RESULTS (c).	REFERENCE
Unknown	NR	6 h, @ 10^5 cfu x ml^{-1}	Channel Catfish (@25cm)	0% in 30 days	Lewis (1981)
	NR	500 ml broth per day for 4 days. 15°C	RT (10 cm)	27% in 18 days	Rucker (1966)

N.R. - Not Reported

(a) - Incubation conditions of the inoculum and serovar used.

(b) - Fish species used and average weight. **b-1:** if weight is expressed as two figures - indicates that there was more than one group of fish, and each figure is the average weight of each group.

b-2: ">" indicates weight at immunization with no recorded weight at challenge.

b-3: RT = Rainbow Trout

(c) - LD_{50} or % cummulative mortality

CHAPTER 3

MATERIALS AND METHODS

3.1 CHEMICALS, REAGENTS AND MEDIA

Unless otherwise stated all chemicals in this work were obtained from Sigma Chemical Company Ltd. (Poole, U.K.) or BDH (Poole, UK), Oxoid Ltd. (Basingstoke, U.K.) or LabM (Toddington, U.K.).

3.2 BACTERIOLOGY

3.2.1 Bacterial strains

The strains of *Y. ruckeri* available for this study are listed in Table 3.1.

3.2.2 Storage of bacteria

Working cultures (25°C, 48 h) were prepared by using colonies of *Y. ruckeri* grown on tryptone-soya agar (TSA) to inoculate nutrient agar (NA) or TSA slopes. After 24 h incubation at 25°C, slopes were stored at 4°C. New slants were prepared every four months. For long-term preservation, overnight broth cultures grown in brain heart infusion broth (BHIB) were distributed in 1 ml aliquots to which 0.2 ml of sterile glycerol were added as a cryoprotectant. After thorough mixing, bacteria were stored at both -20°C and -70°C.

3.2.3 Bacterial cultivation

3.2.3.1 Routine cultivation

For standard culture purposes and phenotypic characterization, bacteria were grown on TSA or brain heart infusion agar (BHIA) plates for 24 or 48 h at 28°C. Broth cultures were prepared from isolated colonies and grown (18 h, 25°C) in tryptone-soy broth (TSB) or BHIB. Cells in broth were grown with shaking on an orbital platform shaker (60 strokes/min) unless otherwise stated. BHIA and BHIB were routinely used to obtain bacterial inocula for use in *in vivo* experiments. Where BHIB was used it was inoculated with bacteria first grown on BHIA (24 h, 25°C). Cultures were normally incubated, unshaken, for 5 h at 30°C.

3.2.3.2 Bacterial growth curves

Conical flasks containing 100 ml of BHIB were inoculated with 1 ml of standard overnight cultures. Samples (5.5 ml) were taken at regular intervals to evaluate growth. A 0.5 ml aliquot was used to determine viable counts and 5.0 ml were centrifuged (2000 x g, 20 min, 4°C), the supernatant removed and cells resuspended in 5.0 ml of phosphate buffer saline (PBSa). Absorbance was read at 625 nm on a double beam spectrophotometer (SP 1800 Pye Unicam, Cambridge) against a PBSa control.

In experiments to determine the effect of medium composition on growth, inocula were prepared by pelleting and washing with PBSa in order to eliminate the medium from the inoculum. A standard growth curve of

absorbance of *Y. ruckeri* strain 5 against viable count was prepared in order to predict the number of viable bacteria present in the inoculum, by measuring only the absorbance at 625 nm for further experiments.

3.2.3.3 Viable Counts

Viable counts were performed by plating 0.1 ml amounts of appropriate serial dilutions (in 0.85% w/v saline) on TSA, using the spread plate method. After incubation (25°C, 48 h) colonies were recorded from plates with between 30 and 300 colonies. Results are expressed as colony forming units (c.f.u.) ml^{-1}.

3.2.3.4 Growth in kidney extract agars

The kidney of a rainbow trout (15 g), dying after artificial infection (i.p.) with *Y. ruckeri* was extracted aseptically, immersed in saline (1 ml) and homogenised. After cell debris was separated by centrifugation (200 x g, 10 min), the supernatant (kidney extract) was mixed with an equal volume of saline containing molten agar (3% w/v) at 50°C. The mixture was poured into petri dishes (50 x 13 mm). The same procedure was followed with kidney material from a non-infected rainbow trout. At least two plates were prepared from each kidney in order to use one as a contamination control.

3.2.4 Biochemical characterization of strains

Strains were subcultured two or three times before being tested. Gram staining, catalase, oxidase, Hugh and Leifson oxidation - fermentation and motility tests were performed according to Collins and Taylor (1967). The

API 20E and API 50CH kits (API Laboratory Products Ltd., Basingstoke) were used to provide an initial characterization of the strains. Test strips were inoculated following the manufacturer's instructions, incubated at 25°C and read at 24 h (API 20E) and 3, 6, 24 and 48 h (API 50CH).

3.2.5 Differential and selective media

3.2.5.1 Ribose ornithine deoxycholate (ROD)

ROD agar media was prepared according to Rodgers (1990), inoculated and incubated at 25°C for up to 10 days. Changes in colour (from red to yellow), due to acid production and formation of a yellow opaque precipitate were noted. Various modifications of the original ROD formulation were used. Their compositions are listed in Table 3.2.

3.2.5.2 Sodium Dodecyl Sulphate (SDS) - TSA media (TSA-SDS)

Filter-sterilized (0.22 μm, Millipore filter) solutions of SDS (20% w/v) were prepared in distilled water and added to molten TSA to achieve final concentrations of 1 to 5% SDS. Plates were incubated at 25°C, and growth recorded daily for up to 7 days.

3.2.5.3 Dye-supplemented media

Filter-sterilized solutions (10 mg ml^{-1}) of Sudan Black (SB) (Aldrich Chemical Co. Ltd. U.K.), Coomassie Brilliant Blue (CB) (Koch-Light Ltd. U.K.), Congo Red (CR), Crystal Violet (CV) and Alcian Blue (AB) were prepared.

Solutions were added to molten TSA to provide final concentrations of 5-100 μg ml^{-1}, inoculated and incubated at 10, 15, 20, 25 and 30°C.

For Alcian Blue and Sudan Black, 5% absolute ethanol was used to aid dissolution since they presented solubility problems. Consequently they were used only at the concentration of 100 μg ml^{-1}, and incubated at 25°C. Results were recorded after 48 h by examining plates under a double incident light beam, illuminating the colonies from opposite directions with an incident angle of approximately 45°. Intensity of the colour in the centre of the colonies was recorded on the basis of an arbitrary scale of 1 (no colouration) to 5 (maximum colouration).

3.2.5.4 TSA-SDS Dye Supplemented Media

Combined media were prepared as above, with final concentration of 1% (w/v) of SDS and 100 μg ml^{-1} of the dyes. Plates were incubated at 25 and 30°C and results were recorded after 48 h.

3.2.6 Antibiotic sensitivity tests

3.2.6.1 Antibiograms

Aliquots (2.5 ml) of broth cultures of *Y. ruckeri* (18 h, BHIB, 25°C) were mixed with 300 ml of melted TSA (45°C) and immediately poured into petri-dishes (20 ml per plate). After the agar had solidified, antibiotic multidisks, were placed on the centre of the plates. Plates were incubated at 25°C and inhibition zones recorded after 48 h. The following multidisks were tested:-

- MASTRING-S (Mast Laboratories Ltd., U.K.): M12, M26, M27, M31, M41 and M43.
- OXOID: 30-12L and U3.
- SENS TEST 8 (Labm. U.K.).

3.2.6.2 Susceptibility to Polymyxin B Phosphate

Cultures grown on BHIA (24 h) were used to inoculate tubes containing penassay medium (Difco Ltd.) which were incubated at 25°C for 24 h, and 0.1 ml was then mixed with 20 ml of diagnostic sensitivity test agar (DSTA) at 45°C and quickly poured into a petri dish. Following solidification of the agar, 7 wells (7 mm diameter) were cut in each plate. Aliquots (20 μl) of different polymyxin B solutions (2, 4, 16, 64, 128 and 270 μg/ml) were added to 6 wells and the remaining well was filled with 20·μl of sterile distilled water. Inhibition zones were recorded after 24 and 48 h incubation (25°C). Strains were considered to be sensitive to polymyxin B with a minimum inhibitory concentration (MIC) of less than 16 μg/ml and resistant at concentrations higher than or equal to 64 μg/ml.

3.2.7 Differential staining of whole bacteria

A series of stains were used on fixed-whole cells of *Y. ruckeri* to examine if there were interstrain variations. Cell smears were prepared on glass microscope slides from bacterial colonies grown on TSA (24 h, 25°C). Smears were air-dried and then fixed (30 sec) with buffered formalin-acetone (pH 6.6) (0.02% w/v of Na_2HPO_4, 0.1% w/v of KH_2PO_4, 45% v/v of acetone

and 25% v/v of 37% formaldehyde made up in distilled water). Fixative was washed out with distilled water (three 3 min washes). The following stains were used:

a) Crystal Violet (1% w/v), aqueous solution for 10 min, and destained with ethanol (70% v/v).

b) Coomassie Brilliant Blue (0.5% w/v in 40% methanol:10% acetic acid) for 10 min and destained with 40% methanol:10% acetic acid.

c) Alcian blue prepared as in Section 3.2.5.3, was applied for 20 min (30°C) and then decanted and washed with distilled water.

d) Sudan black B, (1% w/v in 70% v/v ethanol) was applied for 20 min and destained with 70% ethanol.

e) Periodic acid - Schiff's stain. 1% (w/v) aqueous periodic acid was applied on the cell smears (5 min) and then washed with distilled water (5 min), followed by Schiff's reagent (20 min). After washing with distilled water (10 min), smears were counterstained with Harris haematoxylin.

f) Congo Red (1% w/v aqueous solution) for 20 min, decanted and washed with distilled water.

g) Iodine vapour (solid iodine crystals heated to 60°C). Smears were treated with vapour for 10 min.

3.2.8 Induction of mutants

Four procedures were used in an attempt to obtain mutants with altered colonial characteristics.

3.2.8.1 Acridine Orange (AO) treatment

AO solution (205 mg ml^{-1}, filter sterilized with a 0.22 μm Millipore filter), were added to 20 ml of BHIB, to give a final concentration of 0.02 mg ml^{-1}, inoculated with 0.2 ml of an overnight culture (18 h, BHIB) and incubated on a shaker (15 hours, 25°C) (Salisbury *et al.*, 1972). Serial 10-fold dilutions of this culture were made in 0.85% saline, and 0.1 ml aliquots of the 10^{-5} to 10^{-9} dilution range were plated, in duplicate, onto BHIA plates. After incubation, (48 h, 25°C) growth on plates with less than 100 colonies were transferred to differential media plates (ROD, TSA-CR, TSA-SDS) by a replica plating technique using sterile velvet (12-15 cm square) held taut over a 8 cm diameter cylinder (Lederberg and Lederberg, 1952). Resultant colonies which were not opaque and surrounded by a precipitate ring on ROD and TSA-SDS or colonies with a very dark colour on TSA-CR plates, were to be isolated as potential mutants.

3.2.8.2 Ultraviolet Irradiation

For ultraviolet light (UVL) treatment (Miller, 1972), cell suspensions were placed in glass petri dishes, 75 cm below a warmed UVL lamp, in a dark room and irradiated (in duplicate) for 5-150 sec, stirring constantly. Subsequently one of the plates was left in the dark, while the other was

exposed to daylight for 30 min. Two millilitres of each set of suspensions were then added to 10 ml of BHIB, protected from any light by covering the bottles with aluminium foil and incubated overnight with vigorous shaking. Aliquots (0.1 ml) from ten-fold serial dilutions of the UVL irradiated cultures were spread on TSA-CR, TSA-CB and ROD plates, to screen for mutants showing altered colonial characteristics.

3.2.8.3 Bacteriophage induction

Two approaches were followed. Firstly, a possible induction of lysogenic phages by UVL irradiation was used. Cell lawns were prepared by mixing 0.2 ml of cultures (BHIB, 25°C, shaking) in the logarithmic phase (4 h) and stationary phase (18 h) with 3 ml of melted soft agar (45°C) and after quick, vigorous mixing, poured onto BHIA plates. Once the overlays had set, 10 μl drops of supernatant from a serial dilution of an UVL-irradiated culture were pipetted onto the surface. Plates were incubated at room temperature and checked every 12 h for 48 h. Secondly, an attempt to isolate *Y. ruckeri* phages was performed following the method of Dhillon and Dhillon (1972), using river water taken at the outflow point of a rainbow trout farm. Sixty ml of river water were treated with 4 ml of chloroform, to eliminate any bacteria present. After thorough mixing, the aqueous layer was decanted. Enrichment was conducted by mixing 5 ml of the aqueous layer with 5 ml of double-strength BHIB and 1 ml of a culture of the host strain in logarithmic phase. The mixtures were incubated for 6-24 hours at 15-37°C. The cultures were then centrifuged (2000 x g, 20 min, 4°C). The supernatants (10 ml) were

collected very carefully, mixed with 0.75 ml of chloroform, and the aqueous layer decanted. Undiluted samples of supernatants were applied to agar cultures of various strains and the plates were incubated at 25°C. Lytic phage release was denoted by observation of plaques in the bacterial overlay.

3.2.8.4 Transformation

Transformation was attempted following the method of Mandel and Higa (1970), in which bacteria are made competent, i.e. able to take up chromosomal or plasmid DNA (Cohen *et al.*, 1973) by treatment with DNA. A mixture of chromosomal and plasmid DNA of *Y. ruckeri* strain 48 was obtained by the method of Marmur (1961) (see Section 3.4.8) and DNA preparations (0.25 μg DNA/μl) were diluted as follows: 129 μl of the DNA preparation were mixed with 20 μl of Sau medium (Maniatis, *et al.*, 1982) and 60 μl of sterile Analar water. The diluted DNA (200 μl) was distributed in 9 microfuge tubes (40 μl in the first tube and 20 μl in the others). Digestion was carried out using the restriction enzyme Sau 3A (Boehringer, Mannheim). Enzyme (1 μl of 3 units per μl concentration) was added to the first tube and mixed thoroughly, and serial doubling dilutions (20 μl + 20 μl) were then performed quickly in the next 7 tubes. The last sample was left uncut. Digestion took place for 20 min (37°C) and was stopped by transferring the tubes to 70°C for 10 min. Aliquots (5 μl) of each dilution and the uncut DNA, were resolved on 0.5% agarose gel (see Section 3.4.8). Phage λ DNA treated with restriction enzyme Hind III and 1Kb DNA ladder, were run on the same gel as controls to determine the size of the DNA fragments

obtained. Competent bacteria were obtained by growing *Y. ruckeri* (18 h, 10 ml TSB, 25°C, shaking), centrifuging (6000 x g, 20 min) and resuspending in 1 ml of ice-cold 75 mM $CaCl_2$, incubated on ice for 20 min. The competent bacteria were pelleted and resuspended in 0.5 ml of ice-cold 75 mM $CaCl_2$. Cells were kept on ice, until transformation, for approximately 2 h. Competent bacterial preparations of *Y. ruckeri* were transformed with restriction fragments of *Y. ruckeri* DNA (chromosomal and plasmid) of various sizes and the uncut DNA. DNA from plasmid pUC18, which confers ampicillin resistance, was also used in a separater experiment in order to examine the capacity of *Y. ruckeri* to become competent under these experimental conditions. Bacterial suspension (0.2 ml) was added to approximately 0.5 μg of DNA and mixed (0°C, 10 min) before incubating (25°C, 15 min), followed by a further 45 min on ice. The bacteria were heat-shocked at 37°C for 2 min and allowed to recover by adding 1 ml of TSB and incubating at room temperature for 90 min, without shaking. Selection of transformants was carried out by plating on the appropriate medium. When plasmid pUC18 had been used, selection was carried out on plates containing 50 μg ml^{-1} of ampicillin. When *Y. ruckeri* DNA had been used, TSA-SDS plates were used to select transformants.

3.3 EXPERIMENTAL ANIMALS

3.3.1 Rainbow trout

Fish used in this study were rainbow trout (*Oncorhynchus mykiss*), obtained from ERM-free sites. All fish used for the standardization of a challenge

protocol (Chapter 5) were obtained from Upwey Trout Farm (Dorset, U.K.). Fish were supplied in 13 shipments, throughout the experimental period, in order to keep fish size as homogeneous as possible. These fish were the offspring of a broodstock, set up in 1983, formed of 31 hens and 10 cocks. Fish used for the dietary study of Vitamin E (Chapter 6), were also supplied by Upwey Trout Farm, but in this case they originated from a Danish brood stock different from the 1983 stock. For experiments presented in Chapter 7, fish were obtained from Hook Springs Farm (Beaminster, Dorset). No data about the broodstocks' female:male ratio of these last two groups were available, but fish were purchased as single shipments, and they came from the same lot of eggs. Fish were always unvaccinated females, weighing between 2.5 g or 5 g average when received. On entrance to the Fish Diseases Laboratory aquarium facilities, fish were held at 10°C for at least 2 weeks until needed in circular 720 litre, fibre-glass tanks, supplied with flow-through dechlorinated tap water. Aeration was supplied with airstones.

Infection experiments were carried out in 30 litre rectangular tanks with the same water supply as the holding tanks but with controlled flow rates (unless otherwise stated, 0.5 l min^{-1}). Before and during challenge experiments, chlorine levels in the water were monitored twice daily to ensure it's total absence. Fish were distributed randomly for infectivity experiments, although wherever possible negative control fish were separated from infected fish by empty tanks. Except when temperature was the variable under study, water

was supplied at 13°C for experimental infections. The number of fish used per tank and their sizes will be indicated for each particular experiment.

3.3.2 Dietary regimes of fish

Fish were fed twice daily with commercial dry pellets (EWOS, U.K.), except when the effect of dietary Vitamin E was under study, in which case six diets were used. Three were commercially available and contained 81, 123 and 158 mg vitamin E kg^{-1}. The other three diets were prepared in the laboratory (Cowey *et al.*, 1975) with the composition given in Table 6.1. Essentially the basal diet contained 7 mg vitamin E kg^{-1} and was supplemented to give diets with 86 and 806 mg vitamin E kg^{-1}. Diets were prepared every three weeks and stored at

-20°C until required. Food pellets varied in diameter from 1.4 mm to 4 mm, depending on the size of the fish. Experimental groups of fish in this dietary study consisted of 500 trout (2.5 g average weight at the beginning of the experiment), which were fed at the rate of 20 g kg^{-1} biomass per day, given in four daily feeds. The feeding rates were adjusted every two weeks after weighing 20 fish from each group. Fish were maintained on the different diets for 12 weeks prior to the start of the experiment, to allow the establishment of the different vitamin E levels.

3.3.3 Infection experiments

Modifications to the conditions described below are given in the appropriate Results section.

3.3.3.1 Bacteria preparation for challenge

Bacterial inocula, grown as described above (3.2.3.1) were prepared as 100 ml cultures in 250 ml conical flasks for intraperitoneal (i.p.) infection or 500 ml cultures in 1.5 litre conical flasks for immersion infection challenge. After growth (5 h, 30°C, unshaken), bacteria were collected by centrifugation (2000 x g, 20 min, 4°C) and resuspended in PBSa. Bacterial numbers were estimated spectrophotometrically (3.2.8.2). Viable counts (c.f.u. ml^{-1}), were always assessed on TSA to confirm the inoculum dose used. Two strains of *Y. ruckeri* (5 and 19) were passaged through fish in an attempt to increase their virulence. Bacteria were reisolated from kidneys of infected fish, onto BHIA (24 h, 25°C), and used either to infect more fish, in which case they were grown as usual (5 h, 30°C, BHIB), or they were stored over glycerol as aliquots. When these stored isolates had to be used for further experiments, they were cultured straight from glycerol on BHIA and then the standard procedure was followed. Whenever possible, aliquots containing the passaged isolates were used only once for infectivity experiments. Purity checks were always carried out on TSA.

3.3.3.2 Field challenge

A field experiment was conducted in order to provide a natural challenge, to test the effect of dietary vitamin E on the response of rainbow trout to ERM, with fish previously adapted at the laboratory to different levels of vitamin E in their diets (Chapter 6). Three hundred fish from each dietary group were transferred to a field site on the River Test (Hampshire), known to have

suffered an autumn outbreak of ERM for a number of years. Fish were fed by automatic feeders, which were adjusted weekly after the increase in fish weight had been assessed, by measuring the weight of 20 fish in each group.

3.3.3.3 Anaesthesis

Fish were anaesthetized with MS222 (Sandoz, Switzerland) diluted 1:10000 (w/v) in aquaria water. Where necessary, anaesthetised fish were killed by severing the chordal nerve.

3.3.3.4 Standard laboratory infection

Intraperitoneal (i.p.) and immersion procedures were used to perform laboratory infections with *Y. ruckeri* in order to assess the effect of different variables determined as described in Results. Inocula were prepared in a similar way for both i.p. or immersion infections (Section 3.3.2.1). Cells were pelleted and washed before parenteral (i.p.) delivery (except when the influence of presence of the media was being studied). Immersion was performed either with bacteria suspended in the growth medium (BHIB) or pelleted and washed before delivery. Fish were injected under the effect of anaesthetic with 0.1 ml of bacterial suspension. When fish were infected by immersion, the bacterial dose was adjusted by the combination of a known inoculum and regulation of the water volume.

Control tanks were set up for the different experiments treating the fish in an identical manner to the experimental groups but in absence of the bacterial

pathogen. After challenge, mortalities were recorded twice daily, and dead or moribund fish collected and sampled for the presence of the pathogen. Unless otherwise stated, the duration of the experiments was 2 weeks for i.p. and 4 weeks for immersion infection.

3.3.3.5 Bacteriological sampling

Kidneys from each group of fish were sampled aseptically and plated onto TSA plates. Faecal swabs were plated onto ROD medium, to test for the presence of *Y. ruckeri* in the lower intestine. Sampling was carried out always in the same way, in order to allow for comparison of results. Growth at 25°C was recorded after 72 h for TSA plates and at 10 days for ROD medium. The presence of *Y. ruckeri* was checked for each plate using a bacterial slide agglutination test with a rabbit antiserum to the pathogen. The API 20E biochemical test system was used on random samples to confirm the slide agglutination results. A system of scoring was employed to record *Y. ruckeri* plate growth as follows: 0, no colonies; 1, 1-300 colonies; 2, 300 or more colonies but countable; 3, confluent growth. Growth of *Y. ruckeri* was given a score value (s.v.) as follows:

$$\text{score value (s.v.)} = \frac{\Sigma \text{ score of the plates (a) x 100}}{\Sigma \text{ maximum possible score of the sample (b)}}$$

(a) calculated adding the values (0→3) obtained from each plate.

(b) calculated as if all samples (i.e. number of fish tested) would have had the maximum score (3).

3.3.3.6 Blood samples

Blood samples were obtained either by aspiration from the caudal vein using a hypodermic syringe or by severing the caudal peduncle.

3.3.3.7 *In vivo* passage of *Y. ruckeri*

Fish were injected i.p. in the usual way and after the development of first symptoms, fish were killed and kidney swabs plated onto BHIA (25°C, 24 h). These plates were used to prepare a standard inoculum for i.p. injection of further fish. The process was repeated several times, and isolates collected at the various stages were stored over glycerol at -20°C and -70°C (after checking for purity).

3.3.4 Immunological procedures

3.3.4.1 Production of trout antisera against *Y. ruckeri*

Bacterial strains 1, 5, 7, 19, and 26, were grown on BHIA (24 h, 25°C), then colonies were transferred to 10 ml of BHIB and incubated with shaking (60 strokes/min, 18 h, 25°C). Bacteria were killed either by treatment with formalin (0.6% v/v, 12 h) or by heat treatment (120°C, 20 min), washed 3 times in PBSa and finally resuspended in 0.85% saline to an approximate density of 10^9 bacteria ml^{-1}. Suspensions were injected (0.1 ml) i.p. into groups of 5 rainbow trout (65 g average weight). The fish were kept at 13°C for 4 weeks, after which time they were bled by suction from the caudal vein (approximately 1 ml blood per fish). Pooled blood was allowed to coagulate for 1 h at 20°C and treated as in section 3.3.4.3.

3.3.4.2 Production of rabbit antisera against *Y. ruckeri*

Bacterial suspensions (10^9 c.f.u. ml^{-1}) of strains 1, 7, 19 and 26 were prepared as above, and 1 ml was emulsified with an equal volume of Freund's complete adjuvant (FCA). One ml was injected intramuscularly (0.5 ml in each of two sites) in Dutch rabbits. Three or four subcutaneous injections were given at weekly intervals thereafter, using equivalent bacterial suspensions emulsified (v/v) in Freund's incomplete adjuvant (FIA). The last two inoculations were performed with 0.1 ml of a bacterial suspension (approximately 10^7 c.f.u. ml^{-1}) in saline, injected in the marginal ear vein. Rabbits were bled from the marginal ear vein 4 or 5 days after the last injection. Blood was incubated at 37°C for 1 h and treated as in section 3.3.4.3.

3.3.4.3 Separation and storage of serum

One hour after extraction, trout blood samples (at 20°C) or rabbit blood samples (at 37°C) were transferred to 4°C overnight, to allow coagulation. Serum was separated by centrifugation (1000 g, 20 min, 4°C), aliquoted and stored at either -20°C, -70°C or 4°C, in which case sodium azide was added (0.2 mg ml^{-1} serum).

3.3.4.4 Titration of antibodies in serum

Serum titration was performed by the doubling dilution method in U-shaped 96 well microtitre plates (Flow Laboratories, Irvine, U.K.). Whole cells and unabsorbed anti-whole-cell antisera were used in all assays. Wells were filled

with 50 μl of PBSa, with undiluted antisera (50 μl) added to the first well and a series of two-fold dilutions were made. The last well of each row was used as a negative control and contained only PBSa. *Y. ruckeri* grown on BHIA (48 h, 25°C), was resuspended in PBSa to an approximate density of 10^7 c.f.u. ml^{-1} and 50 μl of this suspension was added to all wells. Plates were incubated for 4 h at 37°C followed by 18 h at 4°C. Results were read against a black background, and were recorded as the reciprocal of the highest dilution showing a macroscopic agglutination. Positive reactions were recorded as those in which cells formed a uniform plaque on the bottom of the well and negative reactions were recorded when cells formed a compact round sediment on the bottom of the well.

3.3.4.5 Slide agglutination test

Ten microlitres of immune antisera (diluted 1:10 in distilled water) were mixed, on a glass slide, with bacteria (BHIA, 48 h, 25°C) taken directly from plate culture. Negative controls were performed using sterile PBSa instead of antisera. Reactions were considered positive when rapid agglutination (1-2 sec) occurred only in the suspensions mixed with antisera. Negative reactions occurred as a homogeneous suspension of the bacteria in the liquid. Reactions slower than 1-2 sec were recorded as "weak" and repeated using undiluted antisera.

3.3.4.6 Enzyme-linked immunosorbent assay (ELISA)

The indirect ELISA method used in this work was developed by H. de Groot and P.F. Dixon (Fish Diseases Laboratory, Weymouth, manuscript in preparation). It was used to monitor the appearance of serum antibodies to *Y. ruckeri* in the experimental trout. Flat-bottomed polystyrene cuvette racks (Gilford) were coated with *Y. ruckeri* heat stable antigen, diluted 1:200 in Panacoat buffer (Biogenesis, Bournemouth, UK) according to the manufacturer's instructions. Buffers and solutions in this method are listed in Table 3.3. Solutions of the antigen (200 μl) in the coating buffer were added to each well and incubated for 24 h at 20°C in a humid box. After coating, racks were washed (3 x 3 min) with PBSa + sodium azide (PBN) and excess solution was removed onto absorbent paper. Racks were treated with 250 μl of 1% bovine serum albumin (BSA) in carbonate buffer (1.5 h at 37°C in humid box) to block non-specific binding, washed quickly, followed by three 5-minute rinses with PBSa and tween (PBST). Excess PBST was blotted with absorbent paper, and racks were dried overnight at room temperature (20°C), sealed and stored at -20°C. Racks were left to warm to room temperature for 1-2 h before being used. Rainbow trout serum samples, stored at 4°C, were diluted 1:10 in PBST and 200 μl were added to duplicate coated racks. Outside wells were not used to avoid gradient temperature effects. Serum samples were incubated for 1 h at 20°C in a humid chamber. They were then removed and the cuvettes were washed with PBST (one quick rinse followed by three 3-min rinses). Excess PBST was blotted with absorbent paper.

Conjugate (rabbit anti-trout immunoglobulin (Ig) - alkaline phosphatase) kindly donated by H. de Groot, was diluted 1:600 in PBST with 1% BSA. 200 μl of the conjugate dilution were added to each well, and incubated for 0.5 h at 20°C in a humid box. After incubation, the cuvettes were washed and dried as before (three 3-min with PBST). Bound conjugate was detected by adding alkaline phosphatase substrate as 1 mg/ml of p-nitrophenyl-phosphate made up in substrate buffer. Substrate was prepared immediately before use, 250 μl were added to each cuvette, and incubated for 1.5 h at 37°C in a humid chamber. The reaction was stopped by adding 50 μl of 3M NaOH to each cuvette. Results were read immediately on an EIA reader (Gilford instruments) at 405 nm. Negative serum samples were used in all assays, including both FDL normal negative sera and sera from control fish fed the vitamin E experimental diets. Blank cuvettes were also included in all tests, to evaluate the background absorbance produced when sera was not present in the system.

Sample values were calculated as a positive-negative ratio (P/N). The mean of the absorbance value of the sample was divided by the mean of the absorbance value of a known negative sample. A ratio value equal to or higher than two was considered positive.

3.3.5 Haematological and biochemical parameters

Fish fed on diets with different levels of vitamin E were anaesthetized and their livers removed and immediately placed in liquid nitrogen. Samples

were stored at -70°C and, at various time intervals, sent (in liquid nitrogen) to the Institute of Marine Biochemistry (IMB, Aberdeen, Scotland) for analysis. Vitamin E was extracted from the livers by the method of Cowey *et al.* (1981), and measured by high performance liquid chromatography (HPLC). Levels of vitamin C in the livers, were determined by colorimetry as described by McGown *et al.* (1981).

Haematological parameters were examined *in situ* immediately after fish sampling. Erythrocyte fragility measurements and plasma pyruvate kinase (EC 2.7.1.40) activity, were determined as described by Bell *et al.* (1985). Haematocrit values were obtained by collecting blood samples into a heparinised capillary tube and calculating the percentage of the cell-packed volume in the sample after centrifugation.

3.3.6 Histology

Histological examination of tissues obtained from fish fed on different levels of vitamin E was performed. Five fish from each group were randomly selected for examination, 28 weeks after the start of the feeding experiment. From each fish, samples of skeletal muscle, liver and spleen were processed to 5 μm paraffin wax sections using standard techniques. All sections were stained with haematoxylin and eosin for morphological changes, and to identify pigmented materials in spleens, the sections were subjected to the Perl's Prussian blue reaction for haemosiderin and Schmorl's reaction for

lipofuscin. Sections were examined under daylight filter using a Polyvar Reichert-Jung photomicroscope.

3.4 STUDY OF CELL COMPONENTS OF *Y. ruckeri*

3.4.1 Effect of cell components of *Y. ruckeri* on cell monolayers

For this study the following cell lines were used: Atlantic salmon (AS), baby hamster kidney (BHK), chinese hamster ovary (CHO), Madin Darby canine kidney (MDCK) and rainbow trout gonad (RTG). Cell lines were obtained from the Polytechnic South West collection, and maintained as confluent monolayers, in polystyrene, sterile, 24 well microtitre plates (Flow Laboratories). Minimal essential modified medium (MEM, Flow Laboratories) was used to maintain the cell lines during the assays. The strains of *Y. ruckeri* used were 35 and 26. Bacteria grown overnight (BHIB, 18 h, 25°C) were centrifuged for 1 h at 10,000 rpm on a MSE High Speed 18 centrifuge. Cell pellets were resuspended in Ringers and adjusted to the desired concentration (approximately 10^8 bacteria ml^{-1}). Increasing amounts of both supernatants and cell suspensions (0.1 - 0.5 ml) were added in duplicate to the cell monolayers. The pH of supernatants was corrected to 7 when necessary. Events were followed over a 24 h period, under an inverted light microscope (Olympus) to monitor any possible cytopathic effects.

3.4.2 Preparation of cell-components and extracellular products for electrophoretic studies

3.4.2.1 Soluble proteins from whole-cells

Bacterial cultures were centrifuged (2000 x g, 4°C, 20 min), resuspended in PBSa to an absorbance (625 nm) of 2. Suspensions (0.5 ml) were centrifuged in microtubes and resuspended in 50 μl of electrophoresis loading buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v SDS, 0.01% w/v bromophenol blue in 0.05 M Tris-HCl pH 6.8). Samples were boiled (5 min), centrifuged (6000 x g, 3 min) and 20 μl of the supernatant was used for electrophoresis (Section 3.4.4).

3.4.2.2 Sonicated cell extracts

Bacterial suspensions were disrupted by sonication (W-385 Ultrasonic Processor, Heat Systems Ultrasonics) on ice (5s cycle, 50% duty cycle for 3 min). Unbroken cells and debris were removed by centrifugation (6000 x g, 10 min), and supernatants were mixed (2:1) with double strength loading buffer. Samples were electrophoresed before and after heating (100°C, 5 min). For preparation of native gels (Section 3.4.4), loading buffer did not contain SDS.

3.4.2.3 Envelope protein preparation

The supernatant from sonicated cells was centrifuged to pellet the total cell envelope fractions (40000 x g, 1 h, 4°C), which was then resuspended in 10

ml of 0.5% (w/v) sodium N-lauryl sarcosinate (sarcosyl) for 2 min at room temperature (Filip *et al.* 1973). The insoluble fraction (outer membrane, OM) was obtained by centrifugation (40000 x g, 1 h, 4°C), and resuspended in 5 ml 20 mM Tris HCl. The supernatant (lauryl sarcosyl soluble fraction, inner membrane, IM) and OM fractions were mixed with loading buffer, in duplicate. One sample was boiled (5 min) and the other left at room temperature. All fractions were resolved by electrophoresis (Section 3.4.4).

3.4.2.4 Extraction of periplasmic proteins

Periplasmic proteins of *Y. ruckeri* were released from the cell by chloroform treatment following the method of F.L. Ames *et al.* (1984). Pellets (6000 g, 1 min) of 3 ml bacterial suspension were treated with 20 μl of chloroform (15 min, 25°C) and followed by 0.2 ml of 0.01 M Tris HCl (pH 8) (20 min, 25°C). Supernatants (6000 x g, 20 min) of these suspensions were mixed with loading buffer and electrophoresed (Section 3.4.4).

3.4.2.5 Detection of lipopolysaccharide

Cell lysates were subjected to proteinase K (PK) digestion following the method of Hitchcock and Brown (1983). Cell pellets (1 ml) were diluted in 50 μl loading buffer and heated at 100°C for 5 min. Duplicate samples were prepared and after cooling, half of the samples were treated with 25 μg of proteinase K (60°C, 1 h). After further cooling, cell debris was removed (6000 x g, 3 min) and supernatants electrophoresed.

3.4.2.6 Separation of cell components by chromatography

Sonicated cell extracts (Section 3.4.2.2) (4 ml) were centrifuged (40000 x g, 15 min, 4°C). Clear supernatants were loaded into a column (90 x 1.6 cm) composed of polyacrylamide and agarose (AcA 22, LKB, Bromma, Sweden) with a fractionation range for proteins of 1.2×10^5 - 1.2×10^6 daltons. Flow rate was set at 4 ml h^{-1}, and fraction volumes of approximately 2-5 ml were collected automatically using a Pharmacia Frac 100 fraction collector. The column was eluted with PBSa, using a constant head reservoir. The optical density of the column eluent was continuously monitored (280 nm) using a LKB Uvicord S, linked to a chopper bar recorder (LKB). The resultant fractions were aliquoted, and stored at 4°C, -20°C and -70°C. Fractions were resolved by SDS-PAGE and PAGE electrophoresis.

3.4.2.7 Extracellular products

For production of extracellular products using the agar overlay technique molten TSA (250 ml) was poured into sterile 30 x 30 cm glass assay plates. When set, sterile 325 PV cellophane (British Cellophane Company) was placed on top of the agar. Fifty ml of inoculum (Section 3.2.3.1) were spread over the surface of the cellophane and incubated for 48 h at 25°C. Growth was then carefully scraped off the cellophane and mixed with 5 ml of PBSa which had also been used to wash the cellophane. After centrifugation (2000 x g, 20 min, 4°C), cells were washed (x3) with PBSa. Samples of supernatants and cells were then electrophoresed.

Extracellular products of *Y. ruckeri* grown in calcium deficient medium were prepared following the method of Heesemann *et al.* (1986). Stock solutions of ethylene glycol-bis (B-aminoethyl ether) - N,N,N´,N´-tetracetic acid (EGTA) (0.25 mM), $CaCl_2$ (1M) and $MgCl_2$ (1M) were prepared. Different volumes of these stock solutions were then added to 50 ml of *Y. ruckeri* cultures in logarithmic phase (A_{625}=1.1), in order to achieve the following final concentrations: 10 - 0.1 mM of EGTA, 20 mM of $MgCl_2$ with 10 mM of EGTA, and 20 - 1 mM of $CaCl_2$ with 10 mM of EGTA.

After 90 min incubation at 30°C (A_{625} = 1.5), the supernatants were precipitated with ammonium sulphate (40 x g/100 ml) for 24 h at 4°C, then centrifuged to concentrate the protein. The precipitates were resuspended in 20 mM Tris buffer (pH 7.2) and loaded onto a 12% SDS-PAGE (Section 3.4.4) gel.

3.4.3 Treatment of cell extracts prior to electrophoresis

3.4.3.1 Enzymatic digestions

Proteinase K (25 μg) was added to 55 μl of cell extracts from either whole cells or sonicates (3:1) in loading buffer (x2). Digestions were conducted at 30°C for 0-180 min with both digested samples and non-digested controls being loaded for electrophoresis. Similar 60 min digestions, were performed with fractions obtained by chromatography (Section 3.4.2.6).

Cell extracts (60 μl) of *Y. ruckeri* in Tris buffer (pH 7.5), were separately digested (30°C) for 18 h or 2 h, with 20 μg of Lipase I (animal origin, No. L-3001), Lipase II (plant origin, No. L-3126) or Lipase VII (microbial origin, No. L-1754) (Sigma), made up as stock solutions (10 mg/ml) in Tris buffer (pH 7.5). Digests and their non-digested control, were mixed (v/v) with loading buffer (x2) and electrophoresed.

3.4.3.2 Treatment of cell extracts with detergents and organic solvents

Cell extracts in electrophoresis sampling buffer (3:1) without SDS were treated prior to PAGE (Section 3.4.4) with a number of detergents: SDS, Tween 20, Tween 80, Triton X-100 and 10% (w/v) Zwittergent (Calbiochem, CA, U.S.A.) (in citric acid pH 4) and 1.0% (w/v) saponin. Chloroform, methanol and 1-butanol were also used for treating the extracts. All these mixtures were made up in a 3:1 ratio. After mixing, samples were loaded on a native acrylamide gel. Lipid solvents: chloroform/methanol (2:1) and diethyl ether were also mixed with cell extracts (3:1), and mixed on an orbital shaker (60 strokes min^{-1}) for 3 h. Mixtures were centrifuged (2000 x g, 3 min), the three phases were separated (aqueous, organic and interphase), mixed (v/v) with loading buffer and resolved on a 12% SDS-PAGE.

3.4.4 Electrophoresis

Preparations were separated using the mini-Protein II dual slab cell (Bio-Rad, Richmond, Ca, U.S.A.) apparatus, according to the method of Laemmli

(1970). A discontinuous electrophoretic system was employed using polyacrylamide gels with an acrylamide:bisacrylamide ratio of 37.5:1. All gel mixtures and buffers were prepared following the Bio-Rad instruction manual. For general purposes, preparations in sampling buffer were applied (20 μl) to stacking gels (4.5% acrylamide) and electrophoresed under a constant voltage setting (200V, 45 min). The stacked samples were resolved in 12% acrylamide separation gels (SDS-PAGE), until the tracking dye front was within 1 cm of the end of the gel.

To resolve LPS samples, the system was as above except that neither the 4.5% stacking gel nor the 12% separating gel contained SDS.

Native gels (PAGE), containing no SDS were also used (12% and 7.5% separating gels). In this case, neither the sampling buffer nor the running buffer contained SDS. Gels were run pre-cooled and in a refrigerator at 4°C to avoid overheating. Standard molecular weight markers, covering the range 29-205 kilo Daltons (MW-SDS200, Sigma), were run simultaneously with the preparations. Prestained molecular weight standards (MW-SDS Blue kit, Sigma), covering the range 26.6 - 180 kiloDaltons, were used in gels for western blotting (Section 3.4.6).

3.4.5 Staining of polyacrylamide gels

3.4.5.1 Silver nitrate for proteins

Staining was performed using the Sigma kit (AG-5), following the manufacturer's instructions.

3.4.5.2 Coomassie brilliant blue

CBB, 0.1% (w/v) dissolved in 40% methanol-10% acetic acid was used for routine protein staining (overnight at room temperature, constant shaking). Gels were destained with a 40% methanol: 10% acetic acid solution. Protein bands appear blue.

3.4.5.3 Copper chloride stain (Lee *et al.*, 1987)

Gels were stained in a 0.3M $CuCl_2$ solution for 5 min and bands visualized against a black background. As the $CuCl_2$ enters the gel, a white precipitate is formed in the regions of the gel that do not contain protein.

3.4.5.4 Silver nitrate stain for LPS (Hitchcock and Brown, 1983)

After overnight fixation in 25% (v/v) of isopropanol in 7% (v/v) acetic acid, gels were oxidised (5 min) in 0.68% (w/v) periodic acid in a 2.597% (v/v) solution of the fixative in distilled water. After thorough washing with distilled water, gels were stained for 10 minutes in a fresh solution consisting of 0.1 N NaOH (28 ml), 29.4% (v/v) ammonium hydroxide (1 ml), 20% (w/v) silver nitrate (5 ml); all in 115 ml of distilled water. After staining, gels were intensively washed (4 x 10 min) in distilled water and then developed (10-20

min, 25°C) in a solution containing citric acid (50 mg), 37% formaldehyde (0.5 ml) in 1 l of distilled water. The stop bath consisted of 200 ml of distilled water with 10 ml of 7% (v/v) acetic acid. The colour of the silver stained bands differed from ochre to black.

3.4.5.5 Periodic acid Schiff stain for glycoproteins

After gel fixation (28 h, 25°C) in 7% (v/v) acetic acid, gels were oxidized in 0.2% periodic acid (45 min, 4°C) and then transferred to Schiff's reagent (BDH) (45 min, 4°C). Gels were destained in 10% acetic acid. Positive bands are shown by a red-purple colour.

3.4.5.6 Alcian blue stain for sialomucins and sulphated sialomucins

Gels were stained in 100 ml of distilled water containing 0.5 g of Alcian blue and 3 ml of glacial acetic acid (20 min, 55°C). Gels were destained in distilled water (18 h, 25°C). Staining for 48 h was also performed. Positively stained components appear blue.

3.4.5.7 Congo red for amyloid

Gels were stained in a 1% (w/v) aqueous solution of Congo red (BDH) (25°C, 48 h). Gels were destained with distilled water. Positive staining appears as a red colour.

3.4.5.8 Sudan black B - general staining for lipids (1)

Gels were stained with 1% (w/v) of filtered (Whatman No.1) Sudan black B in 70% (v/v) ethanol (25°C, 30 min). Ethanol (70%) was used to destain the gels. Extended staining times (48 hours) were also used. Stained bands are black.

3.4.5.9 Bromine - Sudan black B for lipids (Bayliss-High, 1984) (2)

Gels were immersed in 2.5% aqueous bromine (30 min, 25°C), washed with distilled water and treated with 0.5% (w/v) sodium metabisulphite to remove excess bromine. Gels were washed in 70% ethanol; stained in Sudan black B in 70% ethanol (15 min) and differentiated in 70% ethanol (1-2 min).

3.4.5.10 Bromine - acetone - Sudan black B - method for phospholipids (Bayliss-High, 1984) (3)

Gels were treated with bromine as indicated in method (2) but before staining fats were extracted with acetone (20 min, 4°C). Gels were then stained with saturated Sudan black in 70% ethanol (15 min) and differentiated with 70% ethanol. Sudan black B alone will stain unsaturated cholesterol and glycerol esters dark blue whereas some phospholipids appear grey. Bromination enhances the reaction and, in addition, lecithins, free fatty acids and free cholesterol will also be stained. However, after bromination and acetone extraction, only phospholipids will stain.

3.4.5.11 Nile blue sulphate methods for acidic lipids and neutral fats (Bayliss-High, 1984)

The staining solution consisted of 10 ml of 1% (v/v) H_2SO_4, added to 200 ml of 1% (w/v) of nile blue SO_4. The mixture (pH 2) was boiled under reflux for 4 h and was filtered after cooling. Gels were stained in the nile blue SO_4 solution (37°C) for 30 minutes, and then differentiated in 1% (v/v) acetic acid (2 mins). After nile blue SO_4 staining, unsaturated hydrophobic lipids appear pink, whereas free fatty acids and phospholipids are blue.

3.4.5.12 Acetone - nile blue sulphate method for phospholipids (Bayliss-High, 1984)

Gels were treated with 1N HCl (1 h), to desaponify any calcium soaps and then extracted with acetone (20 min, 4°C). After extraction, gels were stained as in the previous method. Phospholipids are stained blue.

3.4.5.13 Phosphine 3R method for neutral fats (Bayliss-High, 1984)

Gels were stained in 0.1% (w/v) aqueous phosphine 3R (3 min, 25°C) and after brief rinsing in distilled water, they were examined in ultraviolet (u.v.) light (360 μm). Cholesterol and glycerol esters fluoresce silvery-white.

3.4.5.14 Neutral red

Gels were stained in 0.1% (w/v) aqueous neutral red, rinsed with distilled water and examined under u.v. light (360 μm).

3.4.6 Western Blotting

After separation by SDS-PAGE or PAGE, electrophoretic transfer from the slab gel to nitrocellulose was carried out according to the Tris-glycine-methanol method described by Towin *et al.* (1979). A Bio-Rad Trans-Blot Cell was used, following the manufacturer's instructions. Transfer was carried out overnight (30 mA, room temperature), in buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3. The nitrocellulose membrane was washed in PBSa (2 x 5 min). Non-specific binding was blocked by incubation for 1 h at 37°C, in 5% (w/v) skimmed milk (Marvel, UK) (blocking solution). Excess blocking agent was removed by two 5-minute washes in PBSa and then the membrane was incubated (2 h, 25°C) in primary rabbit antiserum, diluted 1:200 in blocking solution with constant shaking (10 strokes min^{-1}), washed four times (5 min) in PBSa and then incubated for 90 min in peroxidase-conjugate porcine anti-rabbit immunoglobulin (Dako P217, Denmark), diluted 1:500 in blocking solution. The membrane was washed in PBSa (three 5-minute washes) and in Tris-saline (pH 7.4) for 5 min and then developed in a substrate solution (15 mg of 1, 4-chloronaphthol, 5 ml Analar ethanol, 45 ml Tris-saline pH 7.4, and 50 μl H_2O_2). Development was stopped by immersing the membrane in distilled water for 10 min, the nitrocellulose was then dried and photographed.

3.4.7 Thin-layer chromatography (TLC)

Cell supernatants were extracted either with chloroform/methanol (2:1) or diethyl ether, by adding 3 ml of the solvent mixture to 1 ml of the cell

supernatant. After 1 h mixing (orbital shaker, 60 strokes min^{-1}) at room temperature, samples were centrifuged (6000 x g, 5 min) and phases carefully separated by means of a pasteur pipette. The organic phase was evaporated under a nitrogen atmosphere, on ice, and, shortly after complete evaporation, lipid extracts were resuspended in 20 μl of chloroform. Extracted lipids (1-2 μl) were applied on thin layer chromatography, using a 0.25 mm silica gel layer (Polygram SILG/UV254), containing fluorescent indicator UV254. Samples were run with chloroform in a chromatographic chamber, pre-equilibrated before use (1-2 h), with the same solvent. Chromatography was stopped when the solvent front was 5 cm below the end of the gel. The filter was allowed to dry slightly and then examined under u.v. light (360 μm).

3.4.8 Isolation and electrophoresis of bacterial DNA

For transformation experiments, bacterial DNA, kindly provided by M.L. Gilpin (Polytechnic South West), had been extracted following the procedure of Marmur (1961) for both plasmid and chromosomal DNA. For comparison of plasmid profiles between *Y. ruckeri* strains, DNA was extracted following an alkaline lysis method (Birnboim and Doly, 1979), purified by centrifugation to equilibrium in caesium chloride-ethidium bromide density gradients (Maniatis *et al.*, 1982). Ethidium bromide was removed from the purified DNA by isopropanol extraction (x3), and dialysed against Tris-EDTA (TE) buffer (10 mM Tris pH 8.0, 1 mM EDTA) to remove caesium chloride. After this, plasmid DNA was resolved in agarose gels.

Agarose gel-electrophoresis for DNA was carried out using a Tris/boric acid/EDTA buffer (0.089 mM Tris, 0.089 mM boric-acid and 2.5 mM Na_2 EDTA, pH 8.5, TBE). The buffer was made up at 10 x strength, autoclaved, and stored at 4°C, to be diluted when required. The loading buffer contained 40% sucrose and 0.25% bromophenol blue. Agarose gels (0.3% w/v agarose in 1 x TBE buffer, and 0.5 $\mu g\ ml^{-1}$ ethidium bromide) were run in a horizontal electrophoresis tank (Pharmacia) at 100V for 3 to 4 hours and then examined under u.v. light (300 nm, Trans-illuminator, UV Products, Cambridge).

3.5 STATISTICS

The following statistical analyses were used: (a) Reed and Muench (1938) and Probit method, for estimation of LD_{50}; (b) chi-square and logrank (Reto *et al.* 1977) to determine significant differences amongst mortalities and recovery of bacteria in infection experiments; (c) one way analysis of variance (ANOVA), for the data treatment of the immunological test (ELISA); (d) student T test for the analysis of the physiological data.

A probability value of 5% (0.05) was considered as the cut off point for all analysis.

TABLE 3.1: LIST OF STRAINS OF *Y. ruckeri* USED IN THIS STUDY SOURCE AND ORIGIN				
LABORATORY STRAIN NUMBER	OTHER STRAIN DESIGNATIONS	GEOGRAPHIC ORIGIN	FISH SOURCE	DONORS
1	Yr.V187/09/700 (RS745)	Norway	*Salmo salar* 1987	C.J.R
2	138/76	Canada	*Oncorhynchus mykiss*	C.J.R
3	2/85	U.S.A.	Unknown	C.J.R
4	165/76	U.S.A.	Unknown	C.J.R
5	13/86	England	*O. mykiss* 1986	C.J.R
7	NCMB.1316, RM12	U.S.A.	*O. mykiss* 1965	C.J.R
9	35/85	Denmark	*O. mykiss*	C.J.R
10	29/78	England	*Salmo trutta* 1978	C.J.R
11	8R	Spain	*O. mykiss*	A.T
12	AG52	Spain	*O. mykiss*	A.T
13	18/83	Italy	*O. mykiss*	C.J.R
14	39/81	Australia	*O. mykiss*	C.J.R
15	MR21	Ireland	*Carassius auratus*	C.J.R
16	MR1	France	*O. mykiss*	C.J.R
17	RS6	Canada	*Salvelinus fontinalis*	C.B.M
18	RS24	U.S.A.	*O. mykiss* 1978	C.B.M
19	RS11 ATCC 29473	U.S.A.	*O. mykiss*	C.B.M
20	MR64	England	*Rutilus*	C.J.R
21	MR19	West Germany	*O. mykiss*	C.J.R
22	Yr.V187/09/115 (RS744)	Norway	*S. salar* 1987	C.J.R
23	31/86	England	Fish farm in-flow water 1986	C.J.R
24	6/85	England	*O. mykiss*	C.J.R
25	p.p.31	Spain	*O. mykiss* 1986	A.T.
26	13/86-P	England	*O. mykiss* 1988	C.J.R.
27	FDL21	England	*O. mykiss* 1988	C.J.R
28	FDL20	England	*O. mykiss* 1988	C.J.R

TABLE 3.1: LIST OF STRAINS OF *Y. ruckeri* USED IN THIS STUDY SOURCE AND ORIGIN

LABORATORY STRAIN NUMBER	OTHER STRAIN DESIGNATIONS	GEOGRAPHIC ORIGIN	FISH SOURCE	DONORS
29	RD36	U.S.A.	*O. mykiss* 1978	R.D
30	RD38	U.S.A.	*Micropterus salmonoides* 1978	R.D
31	RD50	U.S.A.	*O. mykiss* 1978	R.D
32	RD54	Canada	*Coregonus artedii* 1981	R.D
33	139/76	U.S.A.	*O. tschawytscha* 1976	C.J.R
34	140/76	U.S.A.	*O. mykiss* 1976	C.J.R
35	137/76	U.S.A.	*O. mykiss* 1970	C.J.R
36	167/76	U.S.A.	Unknown 1976	C.J.R
37	136/76	U.S.A.	Unknown 1976	C.J.R
38	NCTC 10478 RMI	U.S.A.	*O. mykiss* 1967	C.J.R
39	NCMB 1315 NCTC 10476 RM3	U.S.A.	*O. mykiss* 1967	C.J.R
40	NCTC 10477 RM12	U.S.A.	*O. mykiss* 1967	C.J.R
41	141/76	U.S.A.	*O. mykiss* 1976	C.J.R
42	166/76	U.S.A.	Unknown	C.J.R
43	BA3	England	Unknown	C.J.R
44	12/6*5	England	Unknown	C.J.R
45	30/75 RMR NCMB 1316	U.S.A.	*O. mykiss* 1965	C.J.R
46	29/75 RMR NCMB 1316	U.S.A.	*O. mykiss* 1965	C.J.R
47	RD194 RS80	Canada	*O. mykiss*	R.D
48	12/6	England	*O. mykiss*	C.J.R
49	NCIMB 1316	U.S.A.	*O. mykiss* 1965	NCIMB
50	NCIMB 1315 RM3	U.S.A.	*O. mykiss* 1965	NCIMB
51	NCIMB 2194*	U.S.A.	*O. mykiss*	NCIMB
52	NCTC 10478	U.S.A.	*O. mykiss* 1967	NCTC

TABLE 3.1: LIST OF STRAINS OF *Y. ruckeri* USED IN THIS STUDY SOURCE AND ORIGIN				
LABORATORY STRAIN NUMBER	OTHER STRAIN DESIGNATIONS	GEOGRAPHIC ORIGIN	FISH SOURCE	DONORS
53	11.31	U.S.A.	*O. mykiss*	A.T
54	AL 3000	Norway	Unknown 1987	R.N
55	AL 3007	Norway	Unknown 1987	R.N
56	AL 3010	Norway	Unknown 1987	R.N
57	AL 3017	Norway	Unknown 1987	R.N
58	3129	Scotland	Unknown 1989	C.J.R
59	3206	Scotland	Unknown 1989	C.J.R
60	3424	Scotland	Unknown 1989	C.J.R
61	3520	Scotland	Unknown 1989	C.J.R
62	MR19	West Germany	*O. mykiss* 1982	C.J.R
63	MR20	Unknown	Unknown	C.J.R
64	724.771	U.S.A.	Human bile	C.J.R
65	724.772	U.S.A.	Human bile	C.J.R
66	SVA 254/89/2	Sweden	*O. mykiss* 1989	C.J.R
67	SVA 254/89/1	Sweden	*O. mykiss* 1989	C.J.R
68	RD20	Finland	*Corregenus peled*	R.D
69	RD22	Finland	*Salmo salar*	R.D
70	RD62	West Germany	*O. mykiss*	R.D
71	RD126	Unknown, U.S.A?	Unknown	R.D
72	RD138	France	Unknown	R.D
73	RD140	France	Unknown	R.D
74	RD156	Australia	*Salvelinus fortinalis*	R.D
75	1p71	England	*O. mykiss*	
76	RD150, 38/85	Denmark	*Anguilla anguilla*	C.J.R

Donor Key:-

AT: Dr. A. Toranzo - Universidad Santiago de Compostela, Spain.
CBM: Dr. C.B. Munn - Polytechnic South West, Plymouth, England.
CJR: Mr. C.J. Rodgers - Fish Diseases Laboratory (M.A.F.F.), Weymouth, England.
NCIMB:National Collections of Industrial and Marine Bacteria.
NCTC:National Collection of Type Cultures.
RD: Dr. R.L. Davies - Institute of Aquaculture, Stirling, Scotland.
RN: Dr. Ragnhild. Nilsen - Apothekenes Laboratorium, Trosϕ, Norway.

TABLE 3.2: COMPOSITION OF THE MEDIUM RIBOSE ORNITHINE DESOXYCHOLATE (ROD).	
BASAL MEDIA (BM)	
Yeast extract	3g
Sodium desoxycholate	1g
Sodium chloride	5g
Sodium thiosulphate	6.8g
Ferric ammonium citrate	0.8g
Agar No, 1	12.5g
Distilled water	1l
pH 7.4	
SUPPLEMENTS	
Ribose	3.75g
Maltose	7.5g
Ornithine hydrochloride	3.75
Sodium dodecyl sulphate	1%
Phenol red	0.08g
ROD- MODIFICATIONS	
1: BM + 10g tryptone/l + 1% SDS	
2: BM + 3.75g ornithine/l + 1% SDS	
3: BM + 10g tryptone/l + 3.75g ornithine/l + 1% SDS	
4: BM + 10g tryptone/l + 3.75g ornithine/l	
5: BM + 0.75g ornithine/l + 1.5g maltose/l + phenol red + 1% SDS	

TABLE 3.3 COMPOSITION OF THE BASAL DIET (g/kg DRY DIET) FOR DIETARY VITAMIN E STUDIES	
Casein, vitamin free	345
White fish meal	200
Starch, precooked	150
α-Cellulose	117
Corn oil, α-tocopherol stripped[1]	60
Lard[1]	50
Vitamin premix[2]	28
Mineral premix[3]	20
Canning oil[4]	20
Cystine	10
Vitamin A, D_3[5]	0.07

1 ICN Biomedicals, Inc., High Wycombe, U.K.

2 Supplied (/kg diet): choline bitartrate, 9 g; myo-inositol, 2 g; ascorbic acid, 1 g; nictinic acid, 0.75 g; calcium pantothenate, 0.5 g; riboflavin, 0.2 g; thiamin hydrochloride, 50 mg; pyridoxine, 50 mg; menaphthone, 40 mg; folic acid, 15 mg; biotin, 5 mg; cyanocobalamin, 0.09 mg.

3 Supplied (/kg diet): $Ca(H_2PO_4)_2.H_2O$, 8.3 g; K_2HPO_4, 4.1 g; $NaH_2PO_4.2H_2O$, 2.6g; $MgCO_3$, 1.8 g; NaCl, 1.3 g; KCl, 1.0 g; $FeSO_4.7H_2O$, 0.6 g; $CaCO_3$, 0.4 g; $ZnSO_4.7H_2O$, 80 mg; $MnSO_4.4H_2O$, 72 mg; $CuSO_4.5H_2O$, 20 mg; $CoSO_4.7H_2O$, 20 mg; KI, 4 mg.

4 Marfleet Refining Co. Ltd., Hull, U.K.

5 Danochemo A/S, Ballerup, Denmark. Supplied: Vitamin A, 35,000 I.U. and Vitamin D_3, 11,690 I.U./kg diet.

TABLE 3.4 BUFFERS AND SOLUTIONS FOR ELISA

Buffers

1. (a) PBS (phosphate buffered saline):

8.0 g/1 NaCl	: sodium chloride
0.2 g/1 KH_2PO_4	: potassium dihydrogen orthophosphate
1.4 g/1 Na_2HPO_4-$2H_2O$	: disodium hydrogen orthophosphate
0.2 g/1 KCl	: potassium chloride
0.2 g/1 NaN_3	: sodium azide

(b) PBST (0.05%):

PBS containing 0.5 ml Tween 20*/litre

*(Polyoxyethylene sorbitan monolaurate)

2. (a) Substrate buffer:

97 ml diethanolamine

0.2 g NaN_3	: sodium azide
0.1 g $MgCl_2$-$6H_2O$	: magnesium chloride

Make up to 800 ml with distilled water, adjust the pH to 9.8 with 1 M HCl and make up

to 1 litre with distilled water. Store in brown bottle at room temperature.

(b) Substrate:

Use 1 mg/ml p-nitrophenyl phosphate in substrate buffer (40°C), dissolve immediately

before use.

3. Carbonate buffer (pH 9.6):

1.5 g/1 Na_2CO_3	: sodium carbonate
2.93 g/1 $NaHCO_3$	: sodium bicarbonate
0.2 g/1 NaN_3	: sodium azide

Store at room temperature up to 2 weeks.

CHAPTER 4

CHARACTERIZATION OF *Y. ruckeri*

4.1 INTRODUCTION

Members of the species *Yersinia ruckeri* comprise a homogeneous group of Gram-negative rods, usually motile. Its taxonomic position as a member of the genus *Yersinia* has been questioned by Green and Austin (1983) and De Grandis *et al.* (1988), and it has been suggested that *Y. ruckeri* could belong in a new genus of the *Enterobacteriaceae* (Bercovier and Mollaret, 1984). While the taxon is phenotypically very homogeneous, its serology has proved complex and controversial (Davies, 1990). The objective of this part of the study was to examine and compare a large collection of *Y. ruckeri* isolates in order to select those members of the serotype 01 which were to be used in further *in vivo* and pathogenicity work.

4.2 RESULTS

A group of 42 strains of *Y. ruckeri* from the Polytechnic South West collection were characterized. Details of the geographical origin of the strains and their donors are given in Materials and Methods, Table 3.1.

Primary tests consisted of Gram stain (negative), catalase (positive), oxidase

(negative) and fermentation of glucose (positive). Biochemical characterization was carried out using API 20E (for 25 strains) and API 50CH (for 17 strains) kits. Biochemical profiles were, as expected, very homogeneous for both sets of tests.

In the API 20E system, the 25 strains used gave positive reactions in the following tests: lysine decarboxylase, ornithine decarboxylase, utilization of glucose and mannitol. All strains, except number 17, were also positive for ortho-nitro-phenyl-galactosidase. Negative reaction always occurred with the following tests: arginine dihydrolase, hydrogen sulphide, urease, tryptophan deaminase, indole, inositol, rhamnose, sucrose, melibiose, amygdaline and arabinose. Variable results were obtained with the following tests: citrate utilization (strains 17 and 18 positive), acetoin production (strains 1, 16, 22, 30, 39, 40 and 44 were negative; strains 2, 4, 7, 11, 13, 18 and 19 gave a weak reaction, and the rest of the strains, 3, 5, 9, 10, 12, 14, 15, 17, 35, 45 and 46, were positive); gelatin hydrolysis (strains 2, 4, 7, 10,11, 13, 14, 16, 17, 18, 19, 39 and 44 were negative) and sorbitol (strains 1, 3, 10, 17 and 18 negative). These variations fitted within the usual API 20E profiles for *Y. ruckeri* (Rodgers pers. com.).

Seventeen of the strains (1 to 19) tested with the API 20E system, were selected for further characterisation using the API 50CH gallery tests. All strains tested were able to utilize the following substrates: glycerol, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetyl glucosamine,

maltose and trehalose. Only strains 1, 3, 10, 13, 17 and 18 were able to utilize sorbitol, confirming the result obtained with the API 20E system. Results were read at different times (3, 6, 24 and 48 h) after inoculation. Reaction times for the substrates were very similar for the strains tested, although strain 7 presented a general delay in most substrates. This strain was generally observed to grow slowly.

Additional tests to further characterize the strains were carried out and the results are listed in Table 4.1. Polymyxin B produced a zone of inhibition around the *Y. ruckeri* colonies which varied in diameter according to the antibiotic concentration. Such inhibition occurred for all strains tested when plates were read after 24 h. At 48 h some strains presented growth inside the rings and they were recorded as resistant in Table 4.1.

All strains which agglutinated with antisera against serotype O1 were unable to utilize sorbitol. Most of them grew weakly or not at all at 37°C and were also sensitive to polymyxin B. There were, nevertheless, exceptions in these two last tests. Sorbitol positive strains were lower in number, and did not agglutinate with serotype O1 antisera.

4.3 DISCUSSION

The biochemical tests performed using the API 20E and API 50CH strips

showed that strain variations occurred only in reactions for citrate, gelatin liquefaction, Voges-Proskauer (acetoin production) and fermentation of sorbitol. These results indicate that *Y. ruckeri* is a very homogeneous species and they basically agree with reports by other authors (Pyle *et al.*, 1987; De Grandis *et al.*, 1988). Citrate, gelatin liquefaction and Voges-Proskauer are variable reactions which have previously been shown to bear no correlation with serological groups (De Grandis *et al.*, 1988). Sorbitol fermentation has been reported as one of the phenotypic characteristics of *Y. ruckeri* which could be used to help serological grouping (O'Leary *et al.*, 1982; Stevenson and Daly, 1982). Initially, sorbitol fermentation was suggested as being a characteristic of a second serological variety of *Y. ruckeri*, but more recent work, reporting the existence of more than two serotypes of *Y. ruckeri* has shown that sorbitol fermenting isolates are not restricted to a unique group of the species (Daly *et al.*, 1986; De Grandis *et al.*, 1988). In the biochemical study of *Y. ruckeri* performed by De Grandis (1987), sorbitol non-fermenting isolates were found in serotypes I and III. Davies (1989) studied the biochemical and serological characteristics of 148 strains of *Y. ruckeri* and concluded that sorbitol non-fermenting strains were restricted to the serogroup O1 and that all sorbitol-fermenting isolates, with the exception of four strains, were members of the other serotypes. This apparent contradiction between both authors can be explained, since Davies' serotyping scheme considers in the same serotype O1, the groups designated I and III by the schemes of Stevenson and Airdrie (1984b), Daly *et al.* (1986) which De Grandis (1987) also used in a subsequent work. Therefore, it could be

concluded that sorbitol fermentation is a useful initial indication of serotype if, as in the present work, the interest of the study is centred on strains of the serotype O1 group.

Tests for the ability to grow at 37°C and resistance to polymyxin B were also included in this study since they have been reported to be concomitant characteristics in *Y. ruckeri* and related with serotype in the same way as the sorbitol fermentation reaction (De Grandis, 1987). Most serotype I strains are unable to grow at 37°C and they are also sensitive to polymyxin B. Except for strains 14 and 25, which grew at 37°C but were susceptible to polymyxin B, the remaining non-fermenting sorbitol strains used in this work, were either unable to grow at 37°C or presented very poor growth, correlating with their susceptibility to polymyxin B. It is important to bear in mind that ability to grow at 37°C was tested here in a non-quantitative way whereas De Grandis (1987) used a dilution method to assess the results.

The motility test showed that most strains were positive (36/41) with only five isolates being negative. Of the seven UK isolates tested only two were non-motile, although two of the motile ones showed a slow reaction (positive at 48 h). This result contrasts with that of Davies and Frerichs (1989), who found all their non-motile isolates (24) originated from the UK and Norway, and that none of their UK isolates were motile.

Davies and Frerichs (1989) used the "hanging drop" technique to assess

motility, but in this study, growth in soft agar was used which was thought to be a more reliable method. Apart from use of a different technique, the strains used were different, and selection processes suffered by the isolates when growing in laboratory media could account for some of the differences. It would be interesting to test the capacity of the UK motile strains, used in this study, to hydrolyse Tween 80, since Davies and Frerichs (1989) found that all non-motile strains lacked lipolytic activity. With these criteria they established a biotype designation for *Y. ruckeri*.

Y. ruckeri strains were selected for this study on the basis of their reactivity with antiserum against strain 5 (serotype O1). Although, initially there appeared to be only two serological varieties of the ERM bacterium (Bullock, 1984) work carried out in the last few years by various workers have shown the serology of *Y. ruckeri* to be complex and confusing. Stevenson and Airdrie (1984b) and Daly *et al.* (1986) described six serotypes (designated as I-VI) using whole-cell antigens with both unadsorbed and cross-adsorbed antisera. Pyle and Schill (1985) described four O-serotypes (2-6) but they worked only with sorbitol fermenting isolates from North America. Pyle *et al.* (1987) working with a wider range of strains and based on whole-cell antigens, differentiated six serotypes (1-6). However, such schemes only agreed on the designations for serotypes 1 and 2. Both Flett (1989) and Davies (1989), based their schemes on the heat-stable O-antigens, but while Flett (1989) proposed six O serogroups, Davies (1989) proposed only five. With the exception of serotype O1 of *Y. ruckeri*, the location of an isolate in

a particular serotype can prove to be difficult.

Serotype O1 isolates account for the majority of the existing *Y. ruckeri* strains, and are responsible for most of the disease outbreaks, at least in Europe (Davies, 1990). Since the aim of this study was only to select typical representative isolates of the serotype O1 strains, only a presumptive slide agglutination test was carried out to screen the strain collection, and strains able to react with antiserum against serotype O1 but not O2 were considered for further work. Additional phenotypic characteristics, like sorbitol fermentation, were studied, as already mentioned, to help differentiate serotype O1 isolates from non-serotype O1. This system could miss the small number of non-typical serotype O1 strains which are able to ferment sorbitol.

TABLE 4.1: CHARACTERIZATION OF *Y. ruckeri* STRAINS						
TRAIN NUMBER	WHOLE CELL AGGLUTINATION		SORBITOL	GROWTH AT 37°C	SENSITIVITY TO POLYMYXIN B	MOTILITY
	Serotype I antisera	Serotype II antisera				
1	-	+	+	+	R	+
2	+	-	-	-	S	+
3	-	NT	+	+	R	+
4	+	-	-	-	S	+
5	+	-	-	-	S	+s
7	+	-	-	-	S	_
9	+	-	-	-	S	+s
10	-	NT	+	+	R	+
11	+	-	-	-	S	+
12	+	-	-	-	NT	+
13	+	-	-	-	S	+
14	+	-	-	+	S	+s
15	+	-	-	+w	S	+
16	+	NT	-	-	S	+
17	-	NT	+	+	R	+s
18	-	NT	+	+	R	+
19	+	-	-	-	S	-
20	+	-	-	+w	S	-
21	-	NT	+	NT	R	+
22	+	-	-	+	R	+s
23	+	NT	-	NT	S	+
24	-	NT	+	NT	R	+
25	+	-	-	+	S	+
26	+	-	-	NT	S	NT
27	+	-	-	-	NT	-
28	+	-	-	-	S	+s
29	-	NT	+	NT	NT	+
30	+	-	-	+w	R	+s
31	-	+	+	+w	S	+
32	-	+	+	+vw	S	+
33	-	NT	NT	NT	NT	NT
34	+	-	-	+w	R	NT
35	+	-	-	+w	NT	+
36	+	-	-	+w	S	+

TABLE 4.1: CHARACTERIZATION OF *Y.ruckeri* STRAINS (CONTINUED).						
STRAIN NUMBER	WHOLE CELL AGGLUTINATION		SORBITOL	GROWTH AT 37°	SENSITIVITY TO POLYMYXIN B	MOTILITY
	Serotype I antisera	Serotype II antisera				
37	NT	NT	+	+vw	NT	NT
38	+	-	-	-	S	+
39	?	-	-	-	S	+
40	+	-	-	+w	S	+
41	+	-	-	+	R	+
42	+	-	-	+w	S	+s
43	NT	NT	NT	-	NT	NT
44	+	-	-	+w	S	NT

Strains 1-19 API 120E and API 50CH.
Strains 22, 30, 35, 39 and 40 API 120E only.
NT Not Tested.
s Motility Slow.
w Weak Growth at 37°c.
vw Very Weak Growth at 37°c.

CHAPTER 5

DEVELOPMENT OF A LABORATORY MODEL FOR INFECTION OF RAINBOW TROUT WITH *Y. ruckeri*

5.1 INTRODUCTION

The necessity of developing standard challenge protocols has been emphasised by Amend (1981), since the lack of such information is one of the more important limiting factors in the evaluation and comparison of commercial fish vaccines. Amend proposed the criteria required to perform potency testing of fish vaccines. Some protocols for infection of fish by *A. salmonicida* (Michel, 1980; McCarthy *et al.*, 1983; Adams *et al.*, 1987) and *V. anguillarum* (Croy and Amend, 1977) under standardised laboratory conditions have been published. More detailed work in this field has been carried out with the infectious pancreatic necrosis virus (IPNV) (McAllister and Owens, 1986; Okamoto *et al.* 1987).

Although there are several reports in the literature about investigations using *Y. ruckeri* as a model for infectivity work, there is a lack of uniformity in the experimental conditions under which such research has been conducted. Conditions of growth of the pathogen for use as an inoculum in infection trials change almost with each author (see Table 2.2). More systematic

studies attempting to elucidate the optimal growth conditions of *Y. ruckeri* for its use in bacterin production have been defined by Amend *et al.* (1983), but detail referring to the relationship between such conditions and virulence of the pathogen is lacking. This is possibly due to the early success of vaccines against ERM. A standard laboratory model for infectivity tests using *Y. ruckeri* could allow a comparison of work from different laboratories enabling a more profitable discussion and give more value to work in vaccine testing, chemotherapy, prophylaxis and virulence studies.

The aim of this section therefore, was to develop such a protocol by performing a series of preliminary studies on the optimal conditions for *in vitro* culture of *Y. ruckeri* under laboratory conditions. Further investigations were then undertaken *in vivo*, to monitor any potential variations in the pathogen's virulence related to these laboratory conditions. In addition the route of infection, host and environmental conditions were studied in order to standardise a laboratory model for infection of rainbow trout (*O. mykiss*) with *Y. ruckeri*.

5.2 RESULTS

5.2.1 Growth conditions for *Y. ruckeri in vitro*

5.2.1.1 Effect of shaking on bacterial growth

When *Y. ruckeri* was grown in both static and shaken cultures the absorbance values were higher in cultures under shaking conditions, although the shape

of the growth curve was similar for the two conditions studied (Fig. 5.1). Since static growth proved to be reproducible, it was preferred for future experiments because it allowed a higher number of culture flasks to be used simultaneously.

5.2.1.2 Effect of temperature on growth

The absorbance values of *Y. ruckeri* cultures grown at a range of temperatures between 15 and 37°C over a 12 h period are presented in Fig. 5.2. Growth was lower at 15°C. The higher absorbance readings during the exponential phase of growth were at 30°C, although after approximately 9 h there was an inflexion between the growth at 26°C and at 30°C, giving the cultures at 26°C slightly higher readings in the stationary phase.

5.2.1.3 Growth of *Y. ruckeri* at a range of pH between 4.5 and 10.5

There was a slight decrease in the pH of the medium after autoclaving. pH was modified by adding either HCl or NaOH (1M)
Bacterial growth also modified these values as is shown in table 5.1. There was only markedly affected growth at the lowest (4.5) and highest (10.5) pH, with both showing a reduced capacity for supporting bacterial growth. *Y. ruckeri* grown within the 6.5 to 8.5 range produced similar curves and close absorbance values throughout the 28 h that the experiment was followed. However, BHIB with an initial pH of 7.5 supported the highest growth during the exponential phase (Fig. 5.3).

5.2.1.4 Effect of medium composition on growth

Standard growth curves were also obtained for static *Y. ruckeri* cultures grown in BHIB of different concentrations (37, 7.4, 3.7 g/l of BHIB). Fig. 5.4 shows the absorbance values and viable count values for growth at three concentrations of BHIB over an 11 h period. Growth increased with the concentration of media.

5.2.1.5 Relationship between absorbance and viable count

A linear regression curve was calculated from the absorbance values and colony counts obtained during the exponential phase of growth (30°C, static) of *Y. ruckeri* strain 5 (Fig. 5.5). This calibration curve was used for an estimation of the viable count in samples taken from cultures in the exponential growth phase.

5.2.2 Factors affecting the virulence of *Y. ruckeri in vivo*

5.2.2.1 Selection for increased virulence of *Y. ruckeri*

In vivo passage of *Y. ruckeri* strain 5 through fish was used to increase virulence. Two groups of five fish weighing 5 g and 160 g were utilized. Figure 5.6 shows the effect of sequential *in vivo* passage on the mortalities of fish receiving 10^7 and 10^5 bacteria by i.p. injection. When fish were injected with 10^7 viable bacteria, mortalities reached 100% after the first passage in the group of 5 g fish, and after the third in the group of 160 g fish. A reduction in the time to death, 7 to 2 days in the small fish and 14 to 4 days

in the bigger fish, occurred progressively during the first six passages. Aliquots of the seventh *in vivo* passaged strain (designated strain number 26) were kept at -20°C in glycerol for use in further experiments.

5.2.2.2 Comparison of mortalities and recovery of bacteria from fish challenged with the non-passaged and the passaged isolate

Groups of 30 fish (10 g average weight) were injected i.p. with equivalent doses of either *Y. ruckeri* strain 5 or strain 26. It was found that when fish were injected with 10^6 c.f.u., the passaged strain caused a 100% mortality after 14 days, whereas the non-passaged strain caused only a 57% mortality over the same time period. A lower dose of the passaged strain (10^3 c.f.u. per fish) produced a 43% mortality, but no mortalities were observed in fish given the same dose of the original strain 5 (Fig 5.7). The number of fish from which *Y. ruckeri* could be recovered 2 weeks after challenge was determined, and results are shown in Table 5.2. Essentially, kidneys from all sampled fish irrespective of the group were positive for the pathogen, and although the degree of bacterial growth recovered (sv) was dependent on the size of the inoculum used, the difference was not significant ($p > 0.05$). The recovery of bacteria from faeces was significantly different ($p < 0.001$), depending on the challenge dose used. Serum samples were tested for the presence of antibodies to *Y. ruckeri* using an indirect ELISA. Clear positive results were only obtained with samples from fish injected with the passaged strain (Table 5.2).

5.2.2.3 Effect of *in vitro* culture on virulence

A portion of kidney from a moribund fish infected with the passaged strain 26 was sampled onto a BHIA plate, grown at 26°C and after a purity check, stored in glycerol for 3 days at -20°C. This isolate was designated 26-P. A larger portion of the kidney was removed aseptically, homogenised in saline and centrifuged (200 x g/10min). The supernatant was diluted (1:10) in saline and 0.1 ml were injected (i.p.) into 20 fish of 15 g average weight. A viable count was carried out to assess the inoculum and this was found to be 5×10^4 c.f.u. per fish. A standard inoculum was then produced from the culture kept in glycerol and 20 fish were injected (i.p.) with 1.3×10^4 c.f.u. per fish. Results (Fig. 5.8) show that kidney material injected directly into fish, caused the highest mortality of 90% after 10 days compared with a single *in vitro* culture which produced a lower mortality of 35% after 21 days. In addition, the first mortality, following injection with infected kidney material occurred after 3 days, whereas for the plate culture this was 13 days. Since both mortalities were of the same order of magnitude, and due to the difficulties of standardising and quantifying the inocula prepared from infected kidney material, it was decided for future experiments to subculture the bacteria once to allow accurate determination of bacterial cell age and number.

5.2.2.4 Effect of repeated subculturing on virulence

The passaged strain 26 was subcultured onto BHIA 1,2,4 or 6 times and grown for 24 h at each step. A standard static growth in BHIB, incubated for

5 h at 30°C, was carried out with all these cultures prior to challenge. An overnight culture was also grown directly on BHIB, as well as a direct injection of strain 26 which had been kept in glycerol (in which the concentration of bacteria had been previously determined). Inocula were adjusted for all cultures to 10^4 and 10^5 c.f.u. per fish. Higher inocula than normal were required in this experiment since the stock of fish available had proved to be quite resistant to infection. Groups of 30 fish of an average weight of 9 g were used. Mortalities occurred in all groups after inoculation with either 10^4 or 10^5 c.f.u. per fish, except in those injected with the lower dose (4×10^4 c.f.u. per fish) of bacteria cells obtained directly from glycerol storage without subculture (Fig. 5.9). With the exception of the fish injected with *Y. ruckeri* suspended in glycerol, differences in mortalities between the various groups were not significant ($p > 0.05$). Mortalities started to occur between 5 and 6 days post-injection for all groups of fish injected with the higher dose of pathogen (10^5 c.f.u. per fish), in contrast to those receiving the culture obtained directly from glycerol storage, where the first death was recorded after 8 days. In groups of fish injected with subcultured bacteria at the lower dose of pathogen (10^4 c.f.u. per fish), mortalities first occurred at 6 or 7 days post-injection. However, fish injected with the overnight culture of *Y. ruckeri* and with the glycerol preparation, had delayed mortalities at days 9 and 11 respectively. Table 5.3 shows the effect of subculture on the recovery of *Y. ruckeri* from kidney and faeces from 10 of the survivor fish from each group, 2 weeks after challenge with 10^4 c.f.u.. The number of fish positive for *Y. ruckeri* in kidney samples and the degree of bacterial growth

on plate culture (sv), showed no correlation with the number of times that the isolate was subcultured. On the contrary, when faecal samples were examined, a correlation was indicated. It was observed that a significantly higher number of fish ($p < 0.05$) were positive for *Y. ruckeri* in those groups inoculated with cultures grown overnight or taken directly from glycerol (40% and 50% respectively), in comparison with fish groups injected with bacteria cultured on solid media. To ensure that bacteria taken directly from glycerol did not merely require a longer incubation period within the fish to develop disease, fish were kept for 2 months rather than the usual 2 weeks, but no mortalities were recorded over this time. The ELISA test was performed with pooled serum samples (10 fish) from the survivors at 2 weeks post-challenge. None of the sera had an absorbance value at least twice as high as that of the negative sample, therefore all groups were considered negative.

5.2.2.5 Effect of storage time and temperature on virulence

Two experiments were performed to study whether the length of time and the temperature conditions under which the pathogen would be commonly kept could modify its virulence.

The effect of storage time was studied using two isolates of *Y. ruckeri*, strain 26 which had been kept over glycerol at -20°C for one year and strain 75 which had been isolated on BHIA from the kidney of a moribund fish injected with *Y. ruckeri* strain 26, incubated at 25°C for 24 h and then stored at -20°C in glycerol for one week before use. Inocula using both isolates

were prepared as detailed in section 3.2.3.1 and groups of 30 rainbow trout (15 g average weight) were injected i.p. with 8×10^3, 8×10^2, or 8×10^1 c.f.u. of strain 75 and 4×10^3, 4×10^2 or 4×10^1 c.f.u. of strain 26. The recorded mortalities for the higher dose injected were 27% for strain 75 and 43% for strain 5 (see Table 5.4). Mortalities started occuring in both groups at day 7 post-challenge. These results indicated that maintenance of the bacteria in glycerol for a year did not decrease virulence compared to an isolate stored just for one week under the same conditions. The levels of *Y. ruckeri* recovered from 10 surviving fish from each group, killed and examined at day 14 after challenge are presented in Table 5.4. The recovery of the pathogen from kidney samples was very homogeneous in all groups ($p > 0.05$) and markedly higher than the levels recovered from faecal swabs. Additionally, no significant differences ($p > 0.05$) were detected between groups, for the recovery of *Y. ruckeri* from faecal samples.

Bacteria were routinely maintained at either -20°C or -70°C over glycerol. Since it was occasionally necessary to move cultures from one freezer to the other, the effect of variations of temperature during storage was investigated. A culture of *Y. ruckeri* strain 26 kept at -20°C was transferred for 2 weeks to -70°C and then transferred back to the original -20°C. This strain was designated 26(T). Standard inocula were then prepared with *Y. ruckeri* strain 26 (kept at -20°C at all times) and strain 26(T) and used to inject groups of 5 fish (average size 125 g) i.p. with 1×10^6, 1×10^5, 1×10^4 c.f.u. per fish. There was no significant difference between mortalities produced by either

isolate ($p > 0.05$), and LD_{50} values of approximately 4×10^5 c.f.u. for each were obtained.

5.2.2.6 Susceptibility of different fish stocks to laboratory infection

Groups of fish used for experiments were as uniform as possible, in regard to their origin (see section 3.3.1). The LD_{50} of *Y. ruckeri* strain 26 was determined for 11 of the 13 lots of fish received prior to their use for any standardization experiment. The tests were performed by i.p. injection of 0.1 ml of serial bacterial dilutions within the 10^3 - 10^7 bacteria ml^{-1} range. The number of fish per group used in the different experiments varied from 15 to 30 depending on availability. The size of the fish was between 5 and 57 g. LD_{50} values calculated by both the Reed and Muench and the Probit method (Table 5.5), gave comparative results, for all but two lots of fish (lots I and K). Increased resistance to *Y. ruckeri* infection was observed in fish from late-winter or early-spring batches (I and K) during the two consecutive years that the experiments took place. An example of this resistance is presented in Fig. 5.10, showing that a winter batch (5 g average size), had much lower mortalities than a summer batch (57 g average size) for a given concentration of bacteria. Table 5.6 represents the results obtained from the bacteriological samples taken from the surviving fish 2 weeks after challenge. Recovery of *Y. ruckeri* was apparently lower in infected fish from the winter batch, unfortunately the difference in number of fish sampled in each group did not allow for statistical comparison. Antibody levels against *Y. ruckeri*, in serum samples measured by ELISA were negative for all groups of fish. Another

experiment comparing two stocks of fish, one of them a "winter" stock, gave similar results to the experiment described above (data not shown). Wherever possible, stocks of fish thought to have unusually high resistance were not used for experiments. However, stock K (Table 5.5) had to be used to perform a series of infectivity tests due to unavailability of other fish at the time. In this case, bacterial dose and number of fish per group were increased accordingly.

5.2.3 Factors affecting infection by i.p. injection

5.2.3.1 Effect of water temperature on infection

The effect of the water temperature on the development of disease following laboratory challenge was studied by comparing groups of 15 fish from three different stocks of rainbow trout (A: 8 g, B: 13 g and C: 35 g average weights) held at three different temperatures (10, 13 and 18°C). Before challenge all fish were kept at 13°C. Infection was carried out by i.p. injection of 6×10^2 c.f.u. of strain 26 per fish. Mortalities were monitored over 14 days, and the results, expressed as percentage cumulative mortality, are presented in Fig. 5.11. Significant differences due to temperature effect, occurred only for stock C, ($p < 0.01$) in which mortality levels varied concomitantly with temperature. In all groups, first mortalities appeared 4 to 5 days earlier in

fish maintained at the higher temperature of 18°C than those kept at 10°C. The recovery of *Y. ruckeri* from surviving fish held at different temperatures is shown in Table 5.7. The percentage of fish from which *Y. ruckeri* was recovered from kidney samples was similar in all groups, although the relative growth of bacteria was apparently higher from the organs of fish held at 10°C. Results obtained from faecal samples shown that both the percentage of fish positive for the pathogen and its relative growth were higher in fish maintained at 10°C ($p < 0.01$). Since low numbers of fish survived infection when kept at 18°C, those groups were not included for comparison.

5.2.3.2 Effect of stocking density on infection

Rainbow trout (7.3 g) were injected i.p. with 2×10^3 c.f.u. of *Y. ruckeri* strain 26 and divided into groups of 10, 20, 30, 40 and 50 fish per tank (30 l volume). Mortality results at 2 weeks post infection, are presented in Fig. 5.12. Percentage mortalities were significantly higher in fish held at a density of 10, 40 and 50 per tank compared with those at 20 to 30 per tank ($p < 0.001$).

5.2.3.3 Effect of fish size on infection

Groups of 15 fish of 4 different stocks of rainbow trout (A:6.4 g, B:12 g, C:28 g and D:53 g average size) were injected i.p. with 2.3×10^2, 2.3×10^4 and 2.3×10^6 c.f.u. of *Y. ruckeri* strain 26 grown under the standard conditions (see section 3.3.2.1). Fig. 5.13 shows the percentage cumulative mortalities for each stock over the 14 days of the experiment. In all groups of fish,

mortalities increased in correlation with the bacterial dose injected. However, no significant differences in mortalities were observed between fish of different sizes injected with equivalent bacterial inocula ($p > 0.05$). The approximate LD_{50} values obtained from this experiment by the Reed and Muench method and the Probit method are presented in Table 5.8. After two weeks post infection *Y. ruckeri* was isolated from the kidneys of all surviving fish sampled (Table 5.9). The relative bacterial growth (sv) varied between 39% and 62% and was independent of either the size of fish or number of bacteria injected. The percentage of positive isolations of *Y. ruckeri* from the faeces of surviving fish varied significantly ($p < 0.05$) for the different stocks (Table 5.9), although such variations were not correlated with fish stock size.

To provide further insight into the effect of size on response to *Y. ruckeri*, a further experiment was conducted using the same stock of rainbow trout but at different stages of its growth, covering a similar range of sizes (6-52 g) as that used in the previous experiment and using also 15 fish per tank. This overcame possible effects due to genetic variation between different fish stocks. The LD_{50} value was obtained for the three sizes (6 g, 28 g and 52 g) of fish, following injection of *Y. ruckeri* (strain 26) within the range of 10^5 to 10^2 c.f.u.. The LD_{50} value was between 2×10^3 and 1×10^3 bacteria for all groups independently of their size.

5.2.3.4 Effect of time and incubation temperature of the inoculum on virulence

A series of seven experiments involving different time periods and/or temperatures of incubation for *Y. ruckeri* prior to injection into fish was carried out. In five of these experiments mortalities were either very low or non-existent due to the high resistance of the stock of fish to the pathogen. Therefore, results are only presented for those from experiments to which statistical treatment was applicable. *Y. ruckeri* strain 26 was grown on BHIA plates and incubated at 22°C and 30°C for 24 h, or at 15°C for 36 h. Bacterial colonies from the different plates were used to inoculate BHIB, at the same temperatures in which they had grown and incubated for 5 h in static conditions. A modified experiment was also performed in which strain 26 was grown for 24 h at 25°C on BHIA, before static culture in BHIB for 5, 15 or 25 h. Preparation of bacteria for challenge was done as usual (see section 3.3.3.1), adjusting all suspensions to approximately 10^5 c.f.u. per ml. Groups of 50 fish (6.3 g average weight) were i.p. injected with 0.1 ml of the various challenge preparations. The recorded mortalities during the first 2 weeks were lower than expected, being between 10 and 20 percent for all groups (Fig. 5.14). Survivor fish were maintained for a further 2 weeks, during which time the water temperature was slowly raised (approximately 1°C per day) from 13°C to 18°C, and kept at this temperature for 9 days. No additional mortalities occurred despite the heat stress. It was observed that the highest mortality of 20% occurred in the group of fish injected with *Y. ruckeri* grown for 5 h (Fig. 5.14b) despite the dose of the inoculum (6.4×10^3

c.f.u. per fish) being at least one half logarithm lower than the doses corresponding to the inocula grown for 15 and 25 h (1.3×10^4 and 1.5×10^4 c.f.u. per fish respectively). Table 5.10 shows the summary of the mortalities and bacteriological results obtained from this experiment. The effects of different temperatures and times of incubation of the inoculum on mortalities were not significant ($p > 0.05$). All surviving fish sampled at two weeks post-infection gave positive isolation of *Y. ruckeri* from the kidney, with the percentage of the relative bacterial growth (sv) being within the 40-50 range. The number of fish containing the pathogen in their faeces varied from 0 to 50% being this difference significant ($p < 0.01$), while the extent of bacterial growth (sv) varied from 7 to 17%. Fish injected with *Y. ruckeri* cultured for 5 h gave the highest recovery of the pathogen in faeces. At four weeks post challenge kidney isolations from the groups inoculated with bacteria grown at various temperatures gave identical results. Those inoculated with bacteria grown for different lengths of time also gave similar results for kidney isolations but only fish in the 15 and 5 h groups were still carrying bacteria in their lower intestine.

A second experiment comparing the mortalities induced by injecting *Y. ruckeri* grown for two different times and at two incubation temperatures was performed. Cultures of *Y. ruckeri* (strain 26) were grown either for 5 h (exponential phase of growth) or for 15 h (late stationary phase of growth). Both 25°C and 30°C temperatures were used to incubate the static cultures. Inoculums were prepared (see section 3.3.3.4) to infect groups of 15 fish (5

g average weight) by i.p. injection of serial dilutions of the pathogen (approximately 1 x 10^7, 1 x 10^6 and 1 x 10^5 c.f.u. per fish). The challenge doses were high because the stock of fish used for this experiment had proved to be quite resistant to *Y. ruckeri*. The highest cummulative mortality after 2 weeks post challenge occurred in the groups of fish which had been infected with *Y. ruckeri* grown for 5 h at 30°C, however, such a difference was only significant ($p < 0.05$) when results obtained with the higher inoculum dose (10^6 c.f.u. per fish) were compared. Table 5.11 presents a summary of the results, expressed as LD_{50} values for the 4 protocols used.

5.2.3.5 Effect on virulence of concentration of the media for inoculum preparation

Y. ruckeri strain 26 was grown on BHIA of three different concentrations, 37 g/l, 7.4 g/l and 3.7 g/l for 24 h at 25°C, and then inoculated into BHIB at the same concentrations for 5 h at 30°C in static conditions. One additional full strength BHIB (37 g/l) was used but incubated with shaking. Inocula for challenge were calculated using the standard growth curves for each of the different strengths (see Section 5.2.1.5), and adjusted to a density of approximately 2 x 10^3 c.f.u.. Groups of 60 fish (15 g average weight) were used for this experiment. Mortalities over the first two weeks were recorded and results are shown in Fig. 5.15. No significant differences in mortality were observed ($p > 0.05$). The results of the bacteriological sampling of faecal and kidney samples carried out with 10 survivor fish from each tank are presented in Table 5.12. While the recovery of *Y. ruckeri* from the kidney was

similar for all groups of fish ($p>0.5$), the permanence of the bacteria in the lower intestine of the fish seemed to be correlated with the strength of the media. Both shaken and unshaken cultures grown in BHI (37 g/l) produced a higher number of faecal carrier fish than the other cultures ($p<0.01$).

5.2.3.6 Effect on virulence of other conditions of culture of the inoculum

In order to determine whether the way in which the "pre-inoculum" was prepared had any effect on virulence, *Y. ruckeri* strain 26 was grown in liquid (BHIB, both shaken and unshaken) and solid (BHIA) media for 24 h at 25°C. Growth was then transferred to BHIB for 5 h at 30°C to prepare the inoculum for the infectivity test. Groups of 30 fish (67 g average weight) were injected i.p. with 2×10^3 c.f.u.. Mortalities occurred in the three groups of injected trout (Fig. 5.16), but the difference among total final mortalities was not significant ($p>0.05$) after 2 weeks. Isolation from samples taken from survivors was not performed, since virulence did not seem to be affected by the conditions studied, in this case. Growth of the "pre-inoculum" on plates was chosen for future work, since it allowed better control of potential contamination.

5.2.3.7 Effect on virulence of the presence of culture supernatant in the inoculum

Inocula of *Y. ruckeri* strain 26 were prepared in the standard way (see section 3.3.3.1). In one preparation the culture supernatant was removed by centrifugation and the bacterial cells resuspended in PBSa before carrying out

serial dilutions, while in the other case, the dilutions were performed without removal of the supernatant. Groups of 30 fish (7.3 g average weight) were injected i.p. with 10^3 or 10^4 c.f.u.. Two control groups, one injected with sterile BHIB and the other with the filtered culture supernatant were also set up. No significant differences occurred between the two challenge preparations ($p > 0.05$) after 2 weeks (Fig. 5.17). In addition, no mortalities occurred in either of the control tanks. Bacteriological sampling of survivors was not performed for this experiment, but antibody levels in serum from all the different groups of fish gave negative values at 2 weeks post challenge.

5.2.4 Factors affecting infection by bath immersion

Once the conditions to perform infection by i.p. injection had been standardised, a series of experiments was carried out in order to set up those for bath immersion. An infection established via a bath challenge may be considered a more natural way to study a disease event in fish, since it does not by-pass any of the natural barriers which could be avoided in a parenteral infection. The aim of this part of the work was to achieve a good compromise between the optimal conditions for virulence, minimum handling of the animals, and good reproducibility. Unless otherwise stated, the bacterial preparations for infection were produced in the same way as those for the i.p. injection (see section 3.3.3.4) but experiments were allowed to run for 4 weeks.

5.2.4.1 Effect of concentration of inoculum on infection by immersion

Adequate volumes of *Y. ruckeri* strain 26, resuspended in PBSa after centrifugation, were added to clean, oxygenated aquarium water held in a circular plastic container in order to achieve the desired concentration of bacteria. The final volume of water was identical for all the containers in each experiment. Fish were immersed in the bacterial suspension and, after the set time, returned to their tanks. The number of bacteria in the water was determined by viable counts before and, in some cases, after infection. All experiments were carried out in duplicate. Results of experiments to assess the possible correlation between bacterial dose and mortalities are presented in Table 5.13. No significant effect of inoculum size was observed in any of the experiments ($p>0.05$). Reproducibility of results was poor, except for experiment B. However, under these experimental conditions the range of dose studied did not correlate with the mortalities observed.

5.2.4.2 Effect of time of exposure on infection by immersion

The effect of exposure time on infection was studied in an identical system to that described in Section 5.2.4.1. Duplicate groups of 40 fish (13 g average weight) were exposed to the same amount of *Y. ruckeri* strain 26 (6×10^7 c.f.u. ml^{-1}) for 2, 5, 10 and 20 min. The mortalities recorded in all groups after 4 weeks were not significantly different (Fig 5.18) ($p>0.05$). Table 5.14 presents the results of the isolation of *Y. ruckeri* from groups of 10 fish survivors from each group. No significant differences were observed in the number of fish positive for *Y. ruckeri* in their kidneys 4 weeks post infection

($p > 0.05$). Whereas, faecal isolation of the bacterium increased concomitantly with length of exposure to the pathogen.

5.2.4.3 Effect of damage to the body surface of fish on infection by immersion

The effects of three different treatments of the surface of fish prior to a bath infection were studied. Duplicate groups of 60 fish (9 g average weight) were exposed to the following treatments before exposure to the pathogen; a) a two step hyperosmotic immersion, consisting of immersion of the fish in sea water for 10 min before transfer to a suspension of bacteria; b) causing physical damage to the surface of the fish by abrading one of the sites, along the lateral line, with a piece of dry plastic scouring material; c) mucus removal by immersing the fish in a 0.01% solution of dithiothreitol (Oxoid, Basingstoke) for 10 min prior to challenge. For dithiothreitol a preliminary experiment to determine the correct exposure time and concentration was performed in order to prevent toxicity problems. At the concentration used (0.01%) water became cloudy after 20 min exposure and fish appeared stressed. A solution of 0.05% proved to be lethal after 25 min. Therefore a 10 min exposure to 0.01% solution prior to challenge was chosen. Immediately after treatment (a, b or c) fish were infected by immersion using an identical system to that described in section 5.2.4.1. Fish were exposed to a suspension of approximately 1×10^8 c.f.u. per ml^{-1} (see section 3.3.3.4) for 20 min. Duplicated groups of non-treated controls were also infected. No

mortalities were recorded in the non-infected control groups after any of the treatments (a, b or c).

Abrasion produced not only much higher mortalities than any other group ($p<0.001$) but also a much earlier development of symptoms, with mortalities starting at day 3 post-challenge (Fig. 5.19). All other groups presented similar patterns of mortalities with no significant differences either within or between groups ($p>0.05$). Surviving fish were kept for a further 4 months and no extra loses due to *Y. ruckeri* were registered during this time. Bacteriological and serum samples were taken at days 6, 17, 24 and 31 days post inoculation (Table 5.15). It is interesting that recoveries were similar after 6 days for all four protocols used, even though at the time the samples were taken mortalities had only started in tanks where fish had been abraded. This pattern of presence of *Y. ruckeri* in kidney samples was, in fact, very consistent all through the experiment. Isolations of *Y. ruckeri* from faecal samples however, had a biphasic mode, appearing mainly in the second and fourth weeks. The second sampling (17 days) was carried out just when mortalities had stopped occurring in most groups (except for abraded fish, in which mortalities ceased 3 days before sampling). A higher level of faecal carriers was detected in fish sampled after 31 days compared to the previous sampling (24 days) but this may be due to the greater number of fish examined at 31 days. Serum samples screened by ELISA were negative (see section 3.3.4.6) for all treatments except abrasion and one group of the non-treated infected fish, which gave clear positive results at four weeks post-

infection (Table 5.15).

5.2.4.4 Effect of different infection by immersion techniques on mortality

A simplification of the protocol used previously for bath infection (see section 5.2.4.1), which would enable simultaneous handling of more groups of fish at the same time, and which would reduce the stress on the animals by reducing handling and netting, was devised and performed with duplicate groups of 50 fish (6.3 g average weight) as follows:

a) 25 ml of bacteria (strain 26) suspended in PBSa (see section 3.3.3.1), were added to 15 l of oxygenated aquarium water, to achieve 1 x 10^8 c.f.u. per ml^{-1}. Fish were transferred to the bacterial suspension for 30 min, after which time they were returned to a tank filled with 30 l of clean water.

b) 25ml of bacterial suspension in PBSa (as in above) were poured into the tanks, containing 15 l of water. The bacterial concentration was 8.5 x 10^7 c.f.u. per ml^{-1}. After 30 min, the water was restored at a flow rate of 0.6 l min^{-1}.

c) 1 l of a 5 h *Y. ruckeri* strain 26 culture in BHIB was poured into the tanks, containing 14 l of oxygenated water, to achieve 1.2 x 10^8 c.f.u. per ml^{-1}. After 30 min, water was restored at a fixed flow rate of 0.6 l min^{-1}.

The experiment was monitored for 4 weeks, after which time 10 fish from each group were sampled for the presence of *Y. ruckeri* in kidney and faeces. A summary of the results is presented in Table 5.16. There were no significant differences in mortalities either within groups or between groups ($p>0.05$). The results of isolation of *Y. ruckeri* from surviving fish also showed no clear differences between groups. This indicated that the simpler protocol, (c), could be used instead of (a) or (b) thereby reducing the handling of the fish and simplifying the preparation of the bacterial inoculum for infection.

Since the previous experiment gave reproducible results and high mortalities a further experiment on the same basis was performed. Fish were kept in the tanks, and infection was performed by adding either (a) *Y. ruckeri* strain 26 in its broth culture or (b) by adding it resuspended in PBSa as explained above. Two additional protocols were also tested, in which latex particles of 0.81 μm diameter (DIFCO) were incubated with 600 ml of each of the former preparations while gently shaking for 1 h at 30°C prior to challenge (c and d). Fish were exposed initially for 30 min to approximately 1×10^8 c.f.u. per ml^{-1} in 15 l of oxygenated aquarium water. Water flow was restored to the tank at a flow rate of 0.5 l min^{-1}. Duplicate groups of 40 fish (6 g average weight) were used and mortalities followed for 4 weeks.

During the randomization of fish groups in the tanks, two lots (a1 and b1) were allocated to a different part of the tank room (area 1), which is fed by

a different set of header tanks than the other groups. Although all care was taken in making sure that conditions were homogeneous, a marked "site effect" appeared, producing significant differences in the mortalities of the replicate tanks located in different parts of the tank room (Fig. 5.20) ($p<0.05$). When results from tanks fed from the same header tanks were analysed, no significant differences were found, either between replicates or when the four protocols were compared (Fig. 5.20). Although replicated groups of fish placed in a different position in the tank room (area 1) showed fewer losses than those in area 2; mortalities started in both areas between days 4 or 5 post infection as shown in Fig. 5.21. Therefore, it seems likely that addition of latex particles in the conditions tested did not increase uptake of the pathogen by the fish.

5.3 DISCUSSION

5.3.1 Growth conditions for *Y. ruckeri in vitro*

Pathogenicity is determined both by the bacterial genome and environmental conditions. A pathogenic microbe grown *in vitro* may not produce all the determinants of pathogenicity that are found when it is growing in animals during infection (Smith, 1984). This fact must be taken into account in work involving bacterial virulence. However, the rate of growth of a pathogen is also important in pathogenicity, since the host defence mechanisms would be more able to cope with a slow growing bacteria than with a fast one (Smith,

1989).

The study of the growth of *Y. ruckeri* in the laboratory established the initial criteria for inoculum production for infectivity experiments by providing information about the optimal conditions (i.e. maximum growth) during the logarithmic phase, in which cells are in the most homogeneous state. Brain heart infusion (BHI) was chosen as the laboratory medium since it gave good, consistent growth and has also been commonly used in work with other pathogenic enterobacteria (Pai and Destephano, 1982; Portnoy *et al.*, 1984; Simmons *et al.*, 1988). It is possible that preparation of the inoculum on a different medium could affect virulence although this was not investigated. McCarthy *et al.* (1983) noted that cultures of *A. salmonicida* grown in BHI appeared more virulent than those grown in TSB. Although the growth of *Y. ruckeri* strain 5 was lower under static conditions than in shaken cultures, its kinetics were similar. Therefore, static cultures were used because they were more convenient for the preparation of large numbers of broths. Most of the work done with *Y. ruckeri* has been conducted within a temperature range between 22 and 25°C, with 25°C being considered the optimum (Busch, 1983), although no experimental evidence has been given to support this statement. In this study optimal growth, during the exponential phase, occurred at 30°C and pH 7.5. This pH is very close to that recommended by Amend (1983) for bacterin production of *Y. ruckeri*. Although the pH value of the cultures changed concomitantly with bacterial growth, there was no attempt to maintain constant pH since only young cultures (5 h) needed to be harvested.

Y. ruckeri grew optimally on full strength BHIA (37 g/l), and this concentration was used for preparation of standard inocula. Such criteria for optimal *in vitro* growth were reassessed with *in vivo* experiments in order to modify any which could have a negative effect on virulence.

5.3.2 Factors affecting the virulence of *Y. ruckeri in vivo*

Increasing virulence by serial passage through experimental animals is a common practice in pathogenicity work (Smith, 1984). Busch and Lingg (1975), working with *Y. ruckeri* used a fourth passage for their infectivity experiments to maintain virulence. In this work bacteria recovered after seven serial passages in rainbow trout (*O. mykiss*) were used because up to this stage, mortalities were increasing and days to death were decreasing. This selection was carried out using groups of 5 fish and it is possible that virulence had reached a plateau before the seventh passage but the small number of fish used did not allow this to be discriminated. However, an increase in the virulence of the original strain (5) was accomplished with a difference of three logarithms between the LD_{50} before and after 7 passages. Such an increase did not seem to be related to a higher capacity of the passaged strain (26) to establish infection, since bacterial numbers in fish surviving infection seemed to be more related to the dose of inoculum than to the bacterial strain used. On the other hand, it should be pointed out that an alternative infection method such as immersion could perhaps reflect some differences between the two bacterial strains (5 and 26), since any acquired increase in capacity for colonization by the passaged strain would be shown

more easily. The higher antibody against *Y. ruckeri* detected in the serum of fish infected with the passaged strain has to be considered carefully, since this response was not observed in further experiments where the same bacterial strain was used. It could be argued that this was due to inherent differences between stocks of fish. Johnson *et al.* (1982a) and Cossarini-Dunier (1986) found no correlation between humoral (agglutinating) antibodies and protection.

Loss of virulence when a pathogen is grown on artificial media is a well known phenomenon (Smith, 1988). Aoki *et al.* (1984) reported that the virulence of *Vibrio anguillarum* increased after the third passage in ayu (*Plecoglossus altivelis*) but decreased after the thirteenth subculture in medium. Busch (1983) stated that virulence of *Y. ruckeri* was not lost by either subculturing or storage, but no data supporting this comment were presented. Experimental results from this work agree with part of this statement showing a maintenance of virulence levels after 6 consecutive subcultures. However, passaged bacteria stored in glycerol did have lower virulence when injected directly into fish. Further work on this effect of glycerol could be interesting since it is possible that bacteria "coated" with glycerol are able to establish a carrier state in the fish causing a very low number of mortalities. If this is the case, all traces of glycerol must be eliminated before *in vivo* testing of virulence. Contradictory information has been published about the effect of storage upon the virulence of *A. salmonicida*. Michel (1980) reported a reduction in this pathogen's virulence

when it was kept at -70°C, whereas McCarthy *et al.* (1983) detected no variation in strains maintained at -60°C. In the present study, neither storage time (1 year) nor temperature (-20°C to -70°C) affected the virulence of *Y. ruckeri* strain 26. This is an important finding which should enable comparative work to be carried out with the same strain at different times in different laboratories, since it eliminates a difficult to control variable from the experiments.

In vivo growth provides all the essential conditions for full expression of pathogenicity, and small differences in physiological conditions for the pathogen between *in vivo* and *in vitro* growth can produce huge differences in virulence (Smith, 1989). When *Y. ruckeri* was injected into fish directly from infected live kidney material, higher mortalities were produced than with its homogenous isolate cultured only once on BHIA, although mortalities were within the same order of magnitude. This fact should be taken into account when strict virulence studies are in progress, but from the perspective of standardization, culturing the bacteria at least once in laboratory medium is more convenient, allowing accurate bacterial counts, purity control and determination of growth parameters. More knowledge of *Y. ruckeri* metabolism is needed to maximise the conditions of *in vitro* culture and reduce differences with *in vivo* growth. The first approach could be the use of fish peptones in culture media which would probably mimic *in vivo* growth conditions better than most of the currently available laboratory media.

Although there are reports in the literature about differences in resistance to disease among fish stocks (Watkins *et al.* 1981; Okamoto *et al.* 1987), this fact is often not considered, and the susceptibility of infection of a particular fish population is not tested before carrying out challenge experiments. Most pathogenicity research and vaccine challenge studies using mammals, mainly rats and mice, is based on work carried out with pure inbred lines of animals. Unfortunately, very few pure lines of fish are available. Most workers have to obtain their stocks from commercial or government hatcheries. Genetic variability of fish is enormous, and fish farming practice increases such differences (Michel *et al.*, 1984). A considerable amount of work has been done with salmonids, and some proposals to overcome the problem have been suggested, such as the use of high numbers of fish and a good knowledge of the broodstock use at the farm in terms of number of fish and male:female ratio. (Michel *et al.*, 1984; Simon *et al.* 1981). For the present work, fish were always obtained from the same hatchery and kept under as similar conditions as possible. Experiments were carried out with as many fish as possible in each case and an LD_{50} was performed on each new batch after acclimatization, to assess the level of susceptibility to infection with *Y. ruckeri*. The LD_{50} values obtained were used to determine the dose of infection for further experiments.

Most statistical analysis for LD_{50} or ED_{50} tests, are designed as a full titration protocol on the basis that an accurate LD_{50} value is the final objective of the experiment. This necessitates the use of a large number of experimental

groups of animals to cover a wide range of doses in order to obtain from 0 to 100% mortalities. Such ideal conditions not would have been feasible if used systematically throughout in this work. Since the main aim of the LD_{50} experiments was to establish whether or not the fish stocks were susceptible to *Y. ruckeri*, and the approximate bacterial dose range necessary for further experiments. Similar results were obtained when analysis was performed by either the Reed and Muench or Probit analysis methods.

The significant resistance to *Y. ruckeri*, found in the "winter" stocks of fish, emphasised the necessity of carrying out the preliminary LD_{50} tests. The reason for such resistance remains unknown, but could be due to variation in a non-specific immune substance such as C-reactive protein (CRP), lysozyme, natural agglutinins, precipitins or antibody-like molecules. Increased levels of these molecules have been found in different fish species depending on stress, seasonal variations, photoperiod, breeding state, and other alterations in the environment which can modify the homeostasis status (Ingram, 1980; Ellis, 1989). Water temperature variations on the farm varied by only 0.5°C throughout the year, since it was fed by a well. On the other hand fish were always the same size when delivered to the laboratory holding facilities; therefore, a variation due to a developmental stage is unlikely. Photoperiod differences at the farm could perhaps have been related with such immunological status, or pre-exposure to an organism which could induce the synthesis of some of the above mentioned substances. Engstad *et al.*, (1990) for instance, found that ß-1, 3-glucan (M.glucan), from cell walls of

Saccharomyces cerevisiae, could increase the resistance of Atlantic salmon to several bacterial fish pathogens including *Y. ruckeri*. These authors suggested that this could be due to an activation of the non-specific immune system in the fish. The differences in susceptibility of the "winter" stock were observed not only in the mortality record but also in the recovery of the pathogen from survivor fish, with a lower percentage of fish positive for *Y. ruckeri* compared with data from fish of non-resistant stocks. This suggests that if performance of the pathogen in the host changes with different stocks, data from different experiments should only be compared where the same batch of fish had been used.

5.3.3 Factors affecting infection by i.p. injection

The effect of temperature upon immune response in fish has been studied in detail by Avtalion *et al.* (1973, 1980). Work carried out with salmonids also shows correlation of temperature with antibody production, proving that the synthesis of antibodies occurs at low temperatures but that different kinetics of production exist at various temperatures (Paterson and Fryer, 1974; Maisse and Dorson, 1976). In this study mortality data obtained with the fish stock C indicated a direct effect of the high temperature (18°C) on the progression of infection. However, in stock A and B, although a similar effect occurred it was not statistically significant.

Results of bacterial recovery indicated the existence of an equilibrium state, since at 10°C the pathogen seemed able to remain in the host in high

numbers but caused lower mortalities than at 13°C. Whereas at the higher temperature the host seemed able to eliminate the bacterium from its system, although was more susceptible to infection. This could be related to the apparent transition between carrier stages during winter periods and increased mortalities observed in fish farm population when water temperature rises in the spring time (Rodgers, 1987). The choice of 13°C for all further experiments was based on trying to simulate natural conditions as closely as possible. Although the disease process was more rapid at 18°C, this high temperature was considered outside the range usually encountered on British river-fed fish farms that undergo epizootics of ERM (Rodgers, pers. comm.).

ERM is a stress related disease, and outbreaks often happen after grading and handling fish (Rucker, 1966; Hunter *et al.*, 1980). Overcrowding of fish has been associated with the production and release of a pheromone-like immuno-suppressive factor (Perlmutter, *et al.* 1973). In this work a significant correlation between increasing numbers of fish, from 20 to 50, per tank and mortalities has been found. The apparent high mortalities obtained from the tank holding ten fish could be due to a random effect, since the probability of having either 1 or 3 mortalities amongst ten fish is probably the same. Moreover, to allow comparison among the different experimental groups of fish, data had to be treated as a percentage of cumulative mortality. In this case, small variations in the results from the group of 10 fish, produced comparatively high variations in the calculated percentage value. Therefore,

the potency of the test for a group of ten fish with no replicates was too low to allow proper discrimination of results. These findings indicate the danger of comparing results obtained from experiments in which different fish numbers and/or biomass in the tank have been used. Therefore, subsequently an identical number of fish of the same average weight were routinely used within the experiments.

The effect of size and/or age of fish on resistance to disease has been studied previously (Dorson and Torchy, 1981; Bootland *et al.*, 1990). In salmonids, size of fish seems to be more important than age in the development of the immune response (Johnson *et al.*, 1982a; Tatner and Horne, 1983, 1984). When ERM was used as a model, Rucker (1966) and Busch (1978) also pointed out a correlation between disease processes and fish size leading to ERM of epizootic proportions in fish smaller than 7.5cm, whereas in fish bigger than 12.5cm usually the chronic process developed. However, these observations were based on field studies. Under the experimental conditions used in this work no significant effect of fish size was observed, either within or between individual fish stocks within the range studied (6 g - 53 g), using i.p. infection.

The results obtained indicate that the temperatures of incubation used for *Y. ruckeri* growth (15°C, 22°C and 30°C) did not affect virulence within the range studied. Variations in the time of incubation gave some differences in mortalities although they were not statistically significant. However, when

results of mortalities and recovery of bacteria from survivors were considered together they indicated a higher virulence for cultures grown for only five hours at 30°C. Amend *et al.* (1983) have published some results regarding factors affecting the potency of bacterins, and their study indicated that there was no effect of time of incubation. However, the experimental set-up was markedly different from the one presented in this work. The time span used by Amend *et al.* (1983) (12 to 96 h) did not cover 5 h growth cultures. Since *Y. ruckeri* can be transmitted horizontally from fish via water (Busch, 1983), the bacterium might undergo several generations in low nutrient conditions. The change from the "rich" nutritional environment that the fish host provides, to that of the water may produce metabolical alterations in the pathogen which could affect its virulence. Experiments were performed to test whether medium concentration had any effect on the pathogen's performance *in vivo*. The pathogenicity of *Y. ruckeri*, grown under the conditions tested was not affected either by the dilution of the media or by the incubation conditions (shaken and static cultures). The capacity of the pathogen to survive in fish kidney was also not affected. However, it is interesting to point out that bacterial recovery from faeces varied concomitantly with media concentration, being higher in fish injected with bacteria grown on full strength BHI. This observation could help explain some aspects of ERM development in the farm environment, depending on whether or not the pathogen resides for periods in the dilute nutrient conditions of the river water. If the fish are densely stocked, horizontal transmission of the pathogen would be quicker and in this case *Y. ruckeri*

could be more capable of establishing the faecal carrier stage in a shorter period of time than in a less dense farm or indeed in the wild situation.

In summary, a series of conditions have been studied in order to determine whether or not they influence the infection process of *Y. ruckeri* in rainbow trout. This was performed as a progressive study. The results and conclusions from preceeding experiments were used in the design of the next experiment(s). Thirteen batches of fish were supplied on a regular basis throughout the study period and experiments were designed to use the fish from only one batch at any time. It has been shown that variation between batches could be quite significant, for example, two batches of fish were found to be too resistant to *Y. ruckeri* and had to be withdrawn. Of the thirteen batches, a further two were lost due to husbandary failure. This, together with the desire to use fish from the same batch in individual experiments imposed certain constraints on experimental design. These constraints particularly concerned the size and numbers of fish per experimental group. Within experiments, the groups had equal numbers of fish of the same size range. However, between experiments the number of fish per group varied as a result of the number available from the batch being used. Such variations were accepted since no attempt of comparing specific data from different experiments was intended. Virulence, measured only in terms of mortalities produced, proved to be an insufficient way to determine which protocol was most suitable. However, when considered in conjunction with bacteriological data enable the results to be interpreted more accurately

and this helped in the establishment of the standard protocol challenge. Also, a practical approach was followed to decide which should be the conditions used in future, to provide easy reproducibility and accurate quantification of the pathogen including purity and metabolic states. A diagrammatic summary of the standard protocol for intraperitoneal challenge is shown in Fig. 5.22. The background information obtained from this work was used in further studies on development of an immersion challenge for *Y. ruckeri*.

5.3.4 Factors affecting infection by bath infection

Y. ruckeri has been used by various authors to perform immersion challenges in a relatively homogeneous way (Bullock *et al.*, 1976; Hunter *et al.*, 1980; Knittel, 1981; Johnson *et al.*, 1982; Amend *et al.*, 1983; Busch, 1983). In most cases, bacteria were grown for 24 hours at 23-25°C on TSB. Comparison of results obtained by the various authors is difficult because the length of exposure to the bacteria and the dose used varied considerably. The apparent independence between bacterial dose and mortalities in this study could be due to a phenomenon of "saturation", and maximum mortalities may have occurred because all sites on the fish capable of taking in the pathogen were occupied. When viable counts were performed on the bacteria present in the tank before and after fish had been immersed, there were no significant differences (data not shown). Similar results were obtained when the effect of exposure time on mortalities was studied. Tatner and Horne (1983) also reported that uptake of *V. anguillarum* vaccine by rainbow trout

was not affected by an exposure time longer than 10 seconds. However, in the current study, the apparent positive correlation between length of exposure and establishment of the pathogen in the intestine of the fish is interesting, suggesting that longer exposure times to the pathogen led to development of a higher number of carrier fish.

The effect of modifications to the body surface has been studied mainly for vaccine work in efforts to improve antigen uptake. In early work hyperosmotic infiltration (HI), using one or two steps, was the chosen model (Antipa and Amend, 1977; Croy and Amend, 1977; Antipa *et al.,* 1980; Alexander *et al.*, 1981; Bowers and Alexander, 1981; Tatner and Horne, 1983). The use of mucus removal agents and physical damage of the skin by abrasion has been preferred for the study of live bacteria uptake (Cipriano, 1982; McCarthy *et al.*, 1983). In the present study, the negligible effects observed for HI and dithiothreitol on mortalities could be due either to a lack of influence on the uptake itself or to a negative effect produced by stressing the fish. Despite the existing reports on the effect of HI on the increased antigen uptake, it has also been proved that entry of bovine serum albumin (BSA) into rainbow trout decreased when fish were stressed (Fender and Amend, 1978). Although skin abrasion had a much clearer effect, producing the highest mortalities of all treatments, it was decided not to use this for further work since it was considered that the disease development occurring after this treatment was unnaturally rapid, being more similar to those found when a parenteral injection was used. The aim of developing an

immersion protocol was to try to simulate conditions of natural infection as closely as possible. Additionally, the abrasion treatment proved to be impractical when a large number of fish were to be used. Nevertheless, the higher mortality observed when abrasion was used has implications in the fish farm environment. Tanks constructed of materials which allow extensive skin damage would be more likely to encourage ERM infection. The reason why serum antibody levels against *Y. ruckeri* were detected only in the groups of fish which had been abraded cannot be explained as a result of a relationship with levels of exposure to the pathogen since the results of bacterial recovery from kidney and faeces, showed a very similar pattern for all treatments. Perhaps the more direct route of entry of the pathogen into the fish following abrasion may have stimulated a different immune response from that observed with other routes. Since fish were not challenged a second time and no more mortalities were recorded after the third week post infection, it was not possible to examine whether or not such antibody levels were correlated with protection of fish against *Y. ruckeri*. Cyclical shedding of the pathogen by fish has been reported by Busch and Lingg (1975). In the present study, a biphasic pattern in the recovery of the bacterium from faecal samples was also observed.

The presence of BHIB or latex particles in the inoculum affected neither mortalities nor bacterial recovery and no visible signs of stress of the fish were observed. By contrast, Hodgkinson *et al.* (1987), found that *A. salmonicida* uptake by rainbow trout was greater when the bacteria had been

incubated with latex particles. They also reported that the isolation of the pathogen from skin throughout a period of 24 hours post-challenge occurred only when latex particles were in the bacteriological medium. The results of these authors are not directly comparable with those described here, since the time scale for the bacteriological testing differed, and no mortalities occurred as a result of their challenge. The effect of the fish tank position on the reproducibility of results was very marked. Thus the variations in the results due to an uncontrolled area-effect could be more significant than those produced by the experimental variable under study. This indicates that care should be taken in the design and use of aquarium facilities.

These experiments led to the development of a standard protocol for immersion challenge. A diagrammatic representation is shown in Fig. 5.22.

Fig. 5.1: *In vitro* growth conditions for *Y. ruckeri* strain 5

Effect of shaking

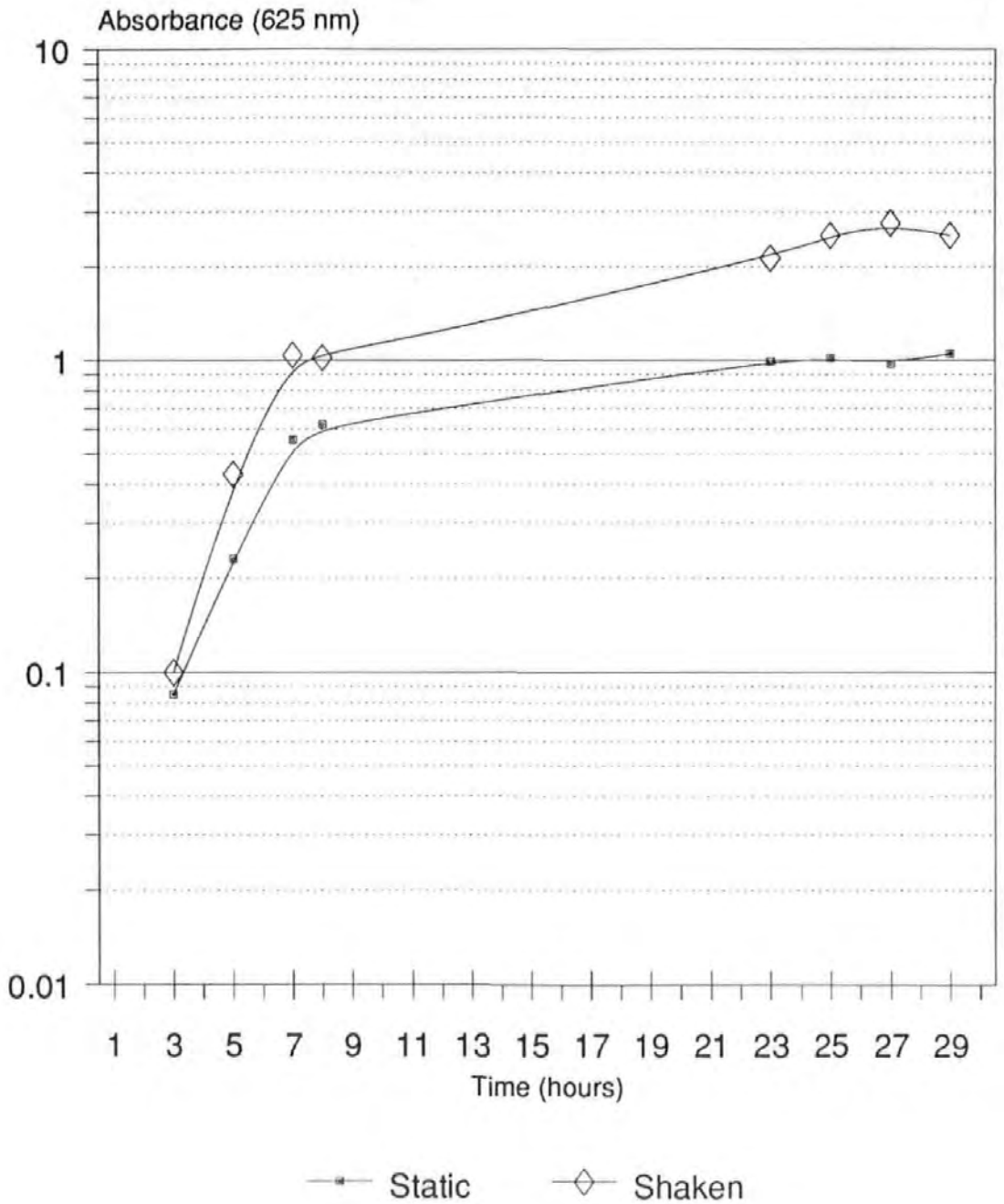
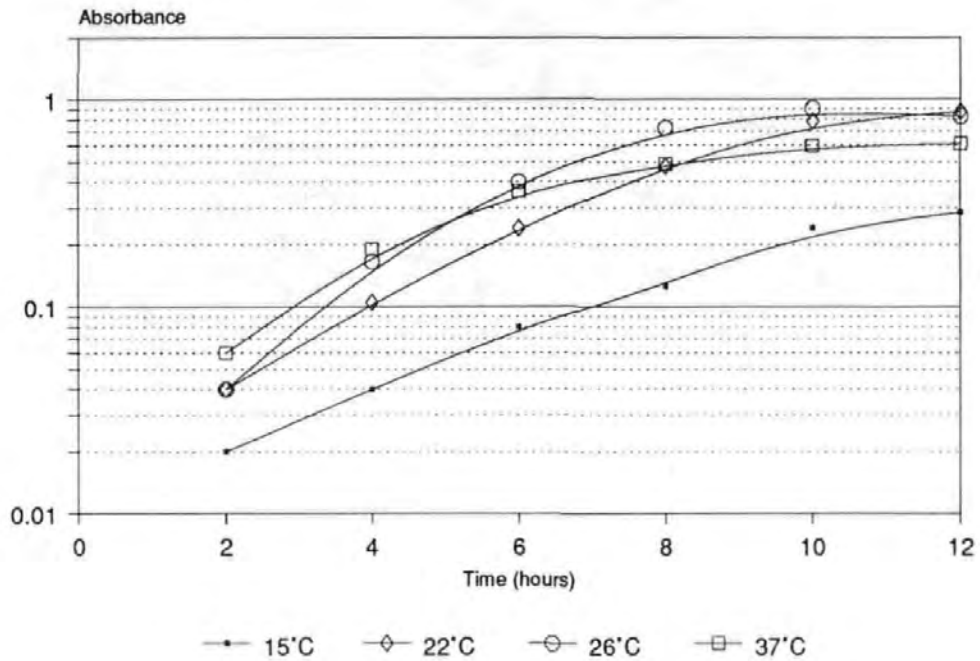


Fig. 5.2: *In vitro* growth conditions for *Y. ruckeri* strain 5

Effect of temperature on growth



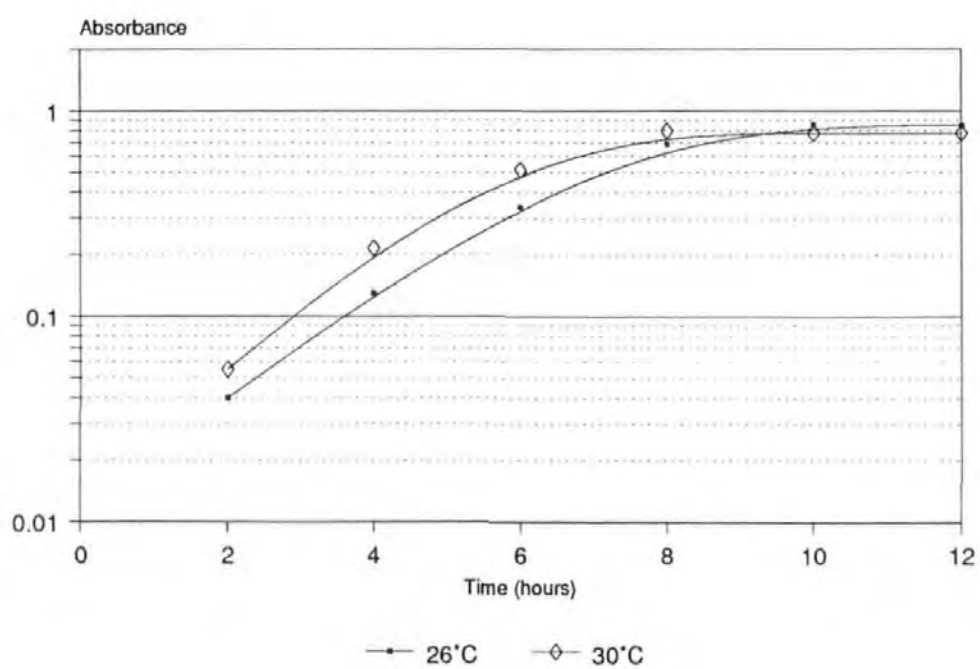
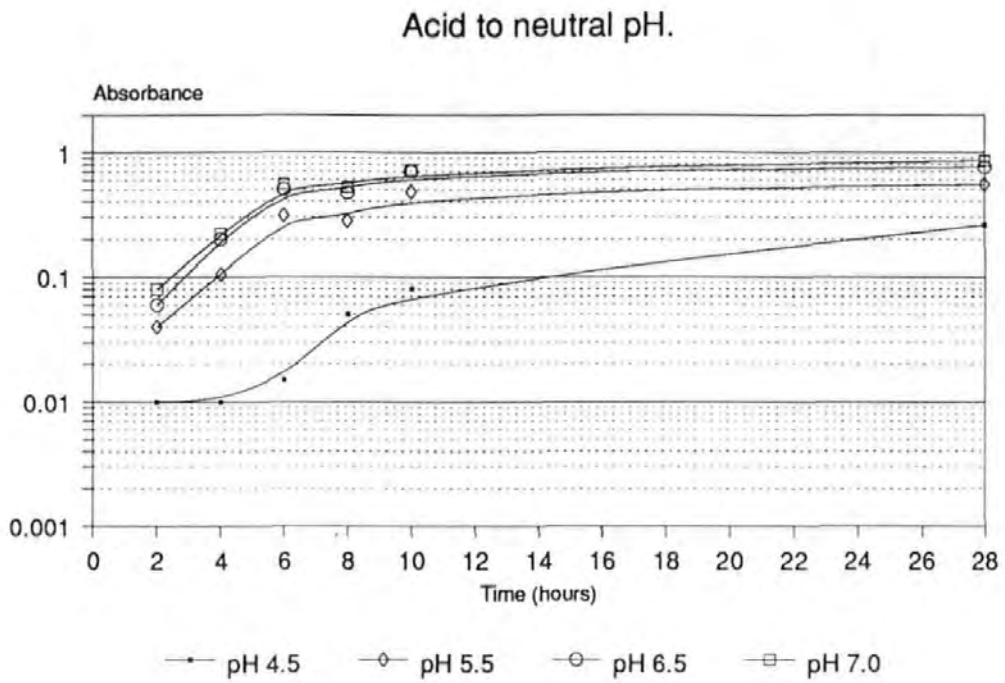


TABLE 5.1: pH CHANGES IN BHIB MEDIA AFTER AUTOCLAVING AND CULTURE AT 30°C.			
BEFORE AUTOCLAVING	AFTER AUTOCLAVING (BEFORE INOCULATION)	AFTER 10 h OF GROWTH	AFTER 27 h OF GROWTH
4.5	4.5	4.2	4.5
5.5	5.5	4.7	4.7
6.5	6.5	5.2	5.5
7.0	6.9	5.75	6.0
7.5	7.3	5.9	6.3
8.5	8.3	6.4	6.85
9.5	8.8	7.6	7.9
10.5	9.5	8.7	8.65

Fig. 5.3: *In vitro* growth conditions for *Y. ruckeri* strain 5

Effect of pH



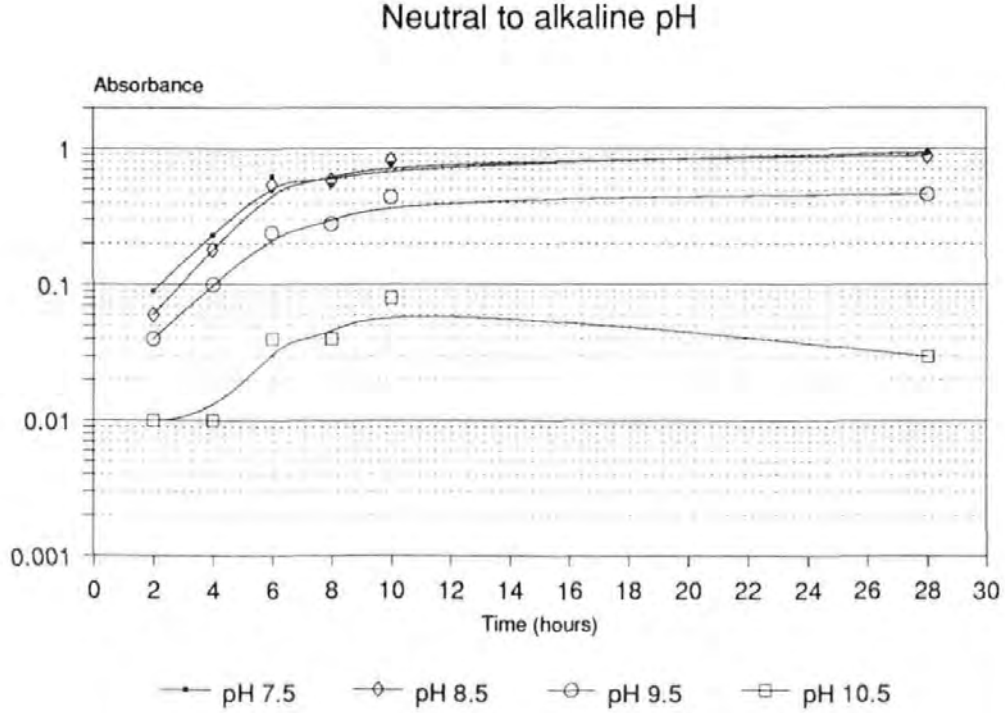
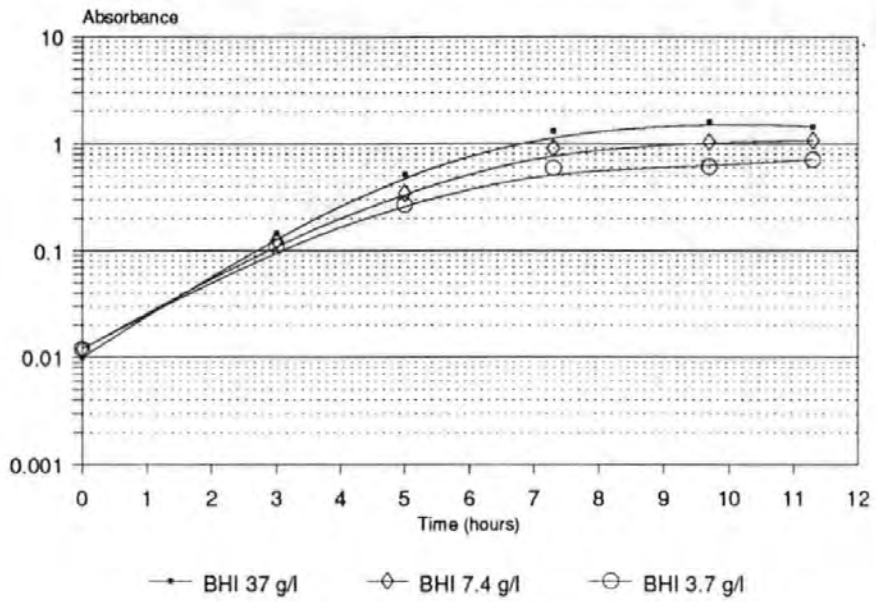


Fig. 5.4: *In vitro* growth conditions for *Y. ruckeri* strain 5

Effect of BHI concentration on growth



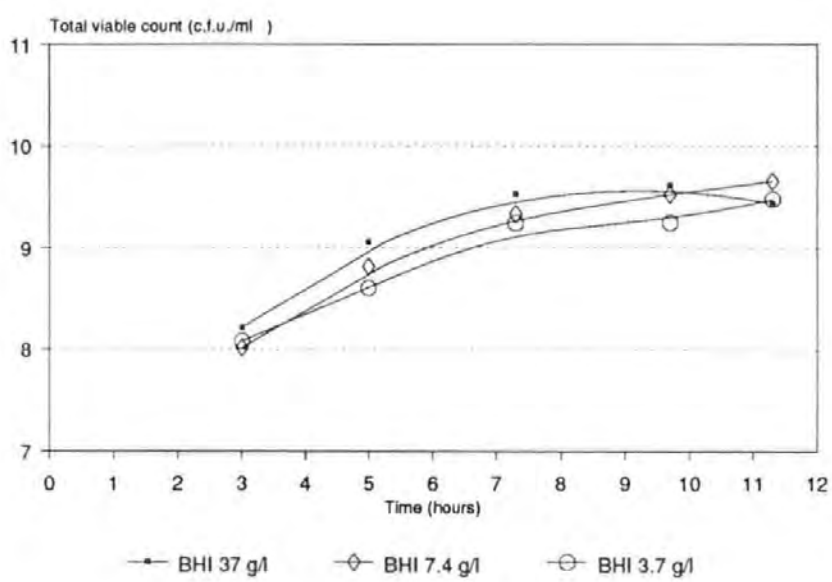
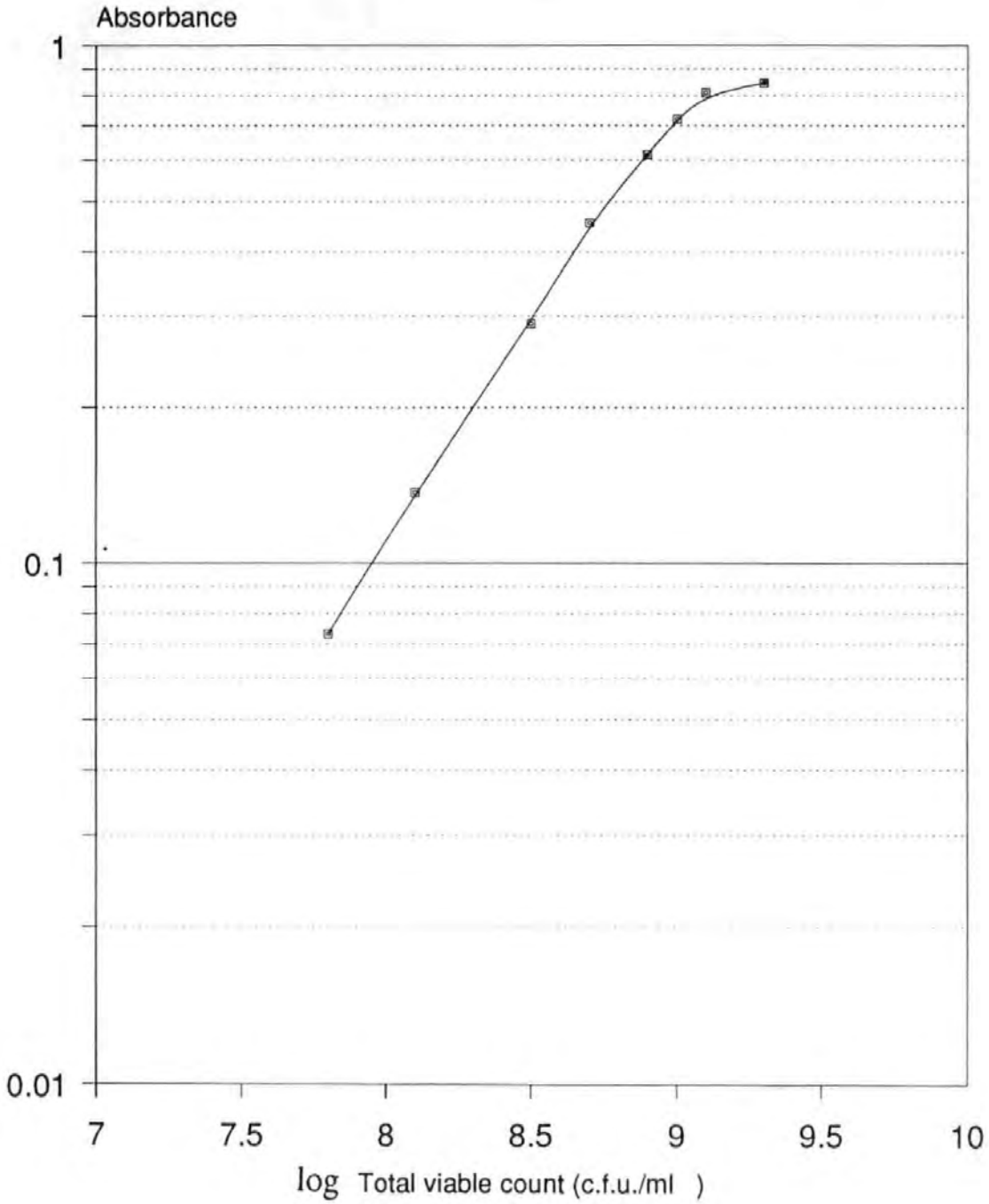


Fig. 5.5: Relationship between absorbance and viable count (strain 5)



$$\log \text{c.f.u.} = 7.9163 + 1.6202 \times A_{625}\text{nm} \quad (r = 0.9865)$$

Fig. 5.6: Effect of *in vivo* passage on selection for increased virulence of *Y. ruckeri*

(a) mean weight of fish (5 g) inoculum size = 10^7 c.f.u. per fish
(b) mean weight of fish (5 g) inoculum size = 10^5 c.f.u. per fish
(c) mean weight of fish (160 g) inoculum size = 10^7 c.f.u. per fish
(d) mean weight of fish (160 g) inoculum size = 10^5 c.f.u. per fish

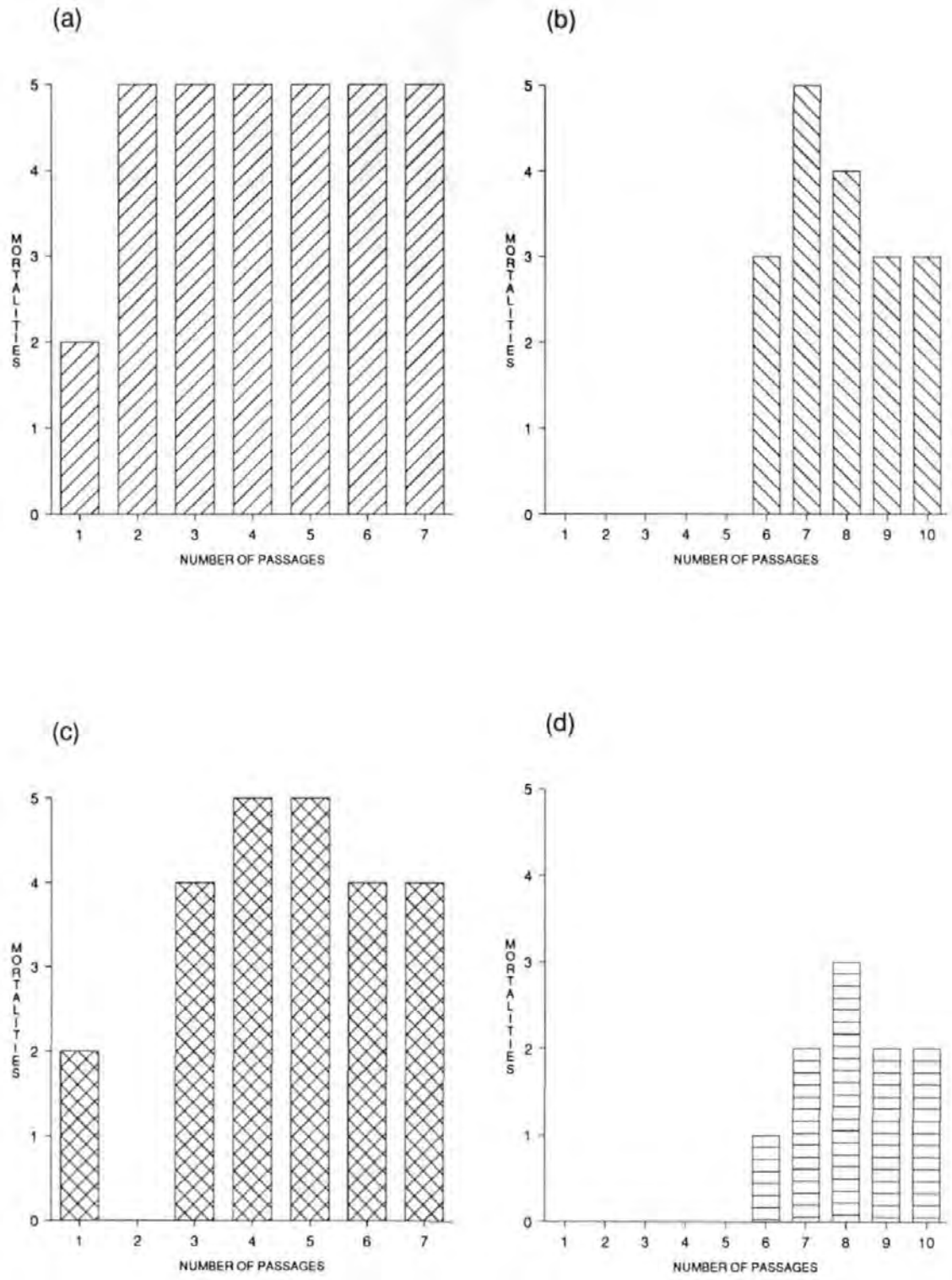


Fig. 5.7: Effect of *in vivo* passage on virulence

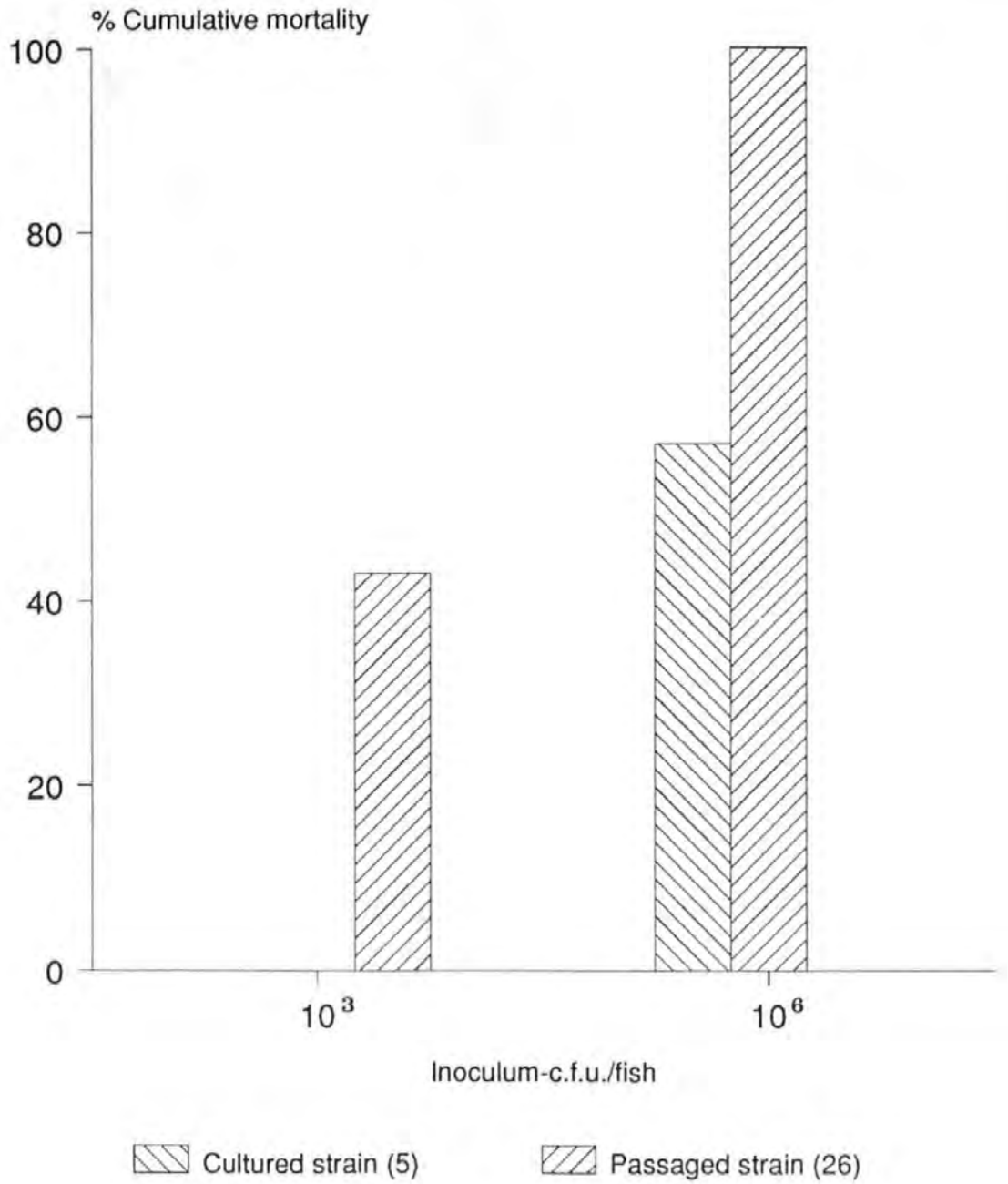


TABLE 5.2: RECOVERY OF *Yersinia ruckeri* FROM FISH SURVIVING FOLLOWING I.P. INJECTION WITH PASSAGED AND NON-PASSAGED ISOLATES

	Cultured strain (5)		Passaged strain (26)
Inoculum (cfu per fish)	**1×10^6**	**1×10^3**	**1×10^3**
Kidney % fish positive for *Y.ruckeri*[1]	100	100	100
Relative bacterial growth (sv)	60	43	50
Faeces % fish positive for *Y.ruckeri*[1]	$80^{(a)}$	$0^{(b)}$	$10^{(b)}$
Relative bacterial growth (sv)	37	-	6.7
Serum presenceof antibodies (see section 3.3.4.6)	+/-	-	+++

[1] 10 fish per group.

N.B. Values in the same row with different superscripts(^{a} or ^{b}) were significantly different ($P < 0.001$).

Fig. 5.8: Effect on virulence of a single subculture of *in vivo* passaged kidney material

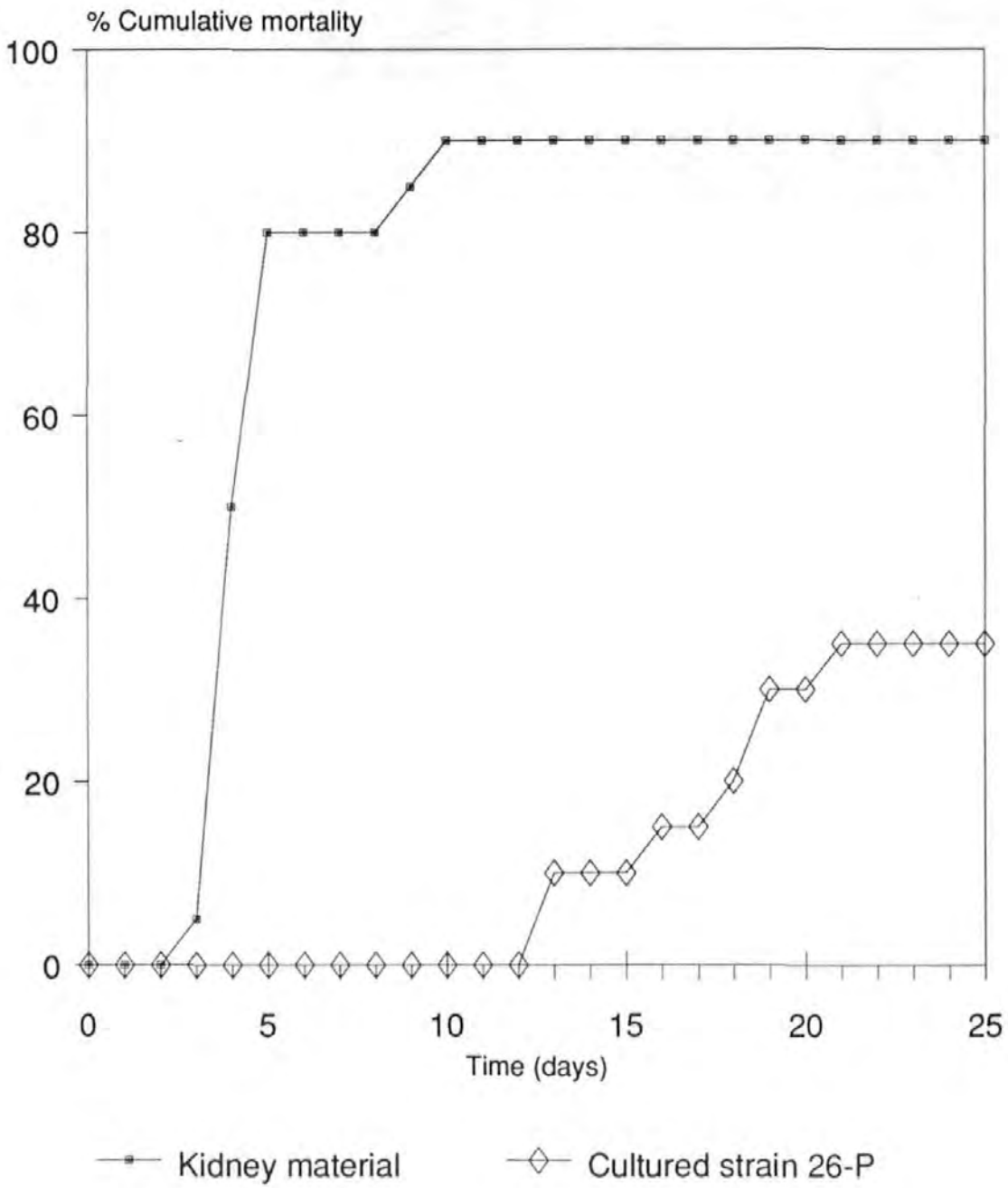


Fig. 5.9: Effect of repeated subculturing on virulence

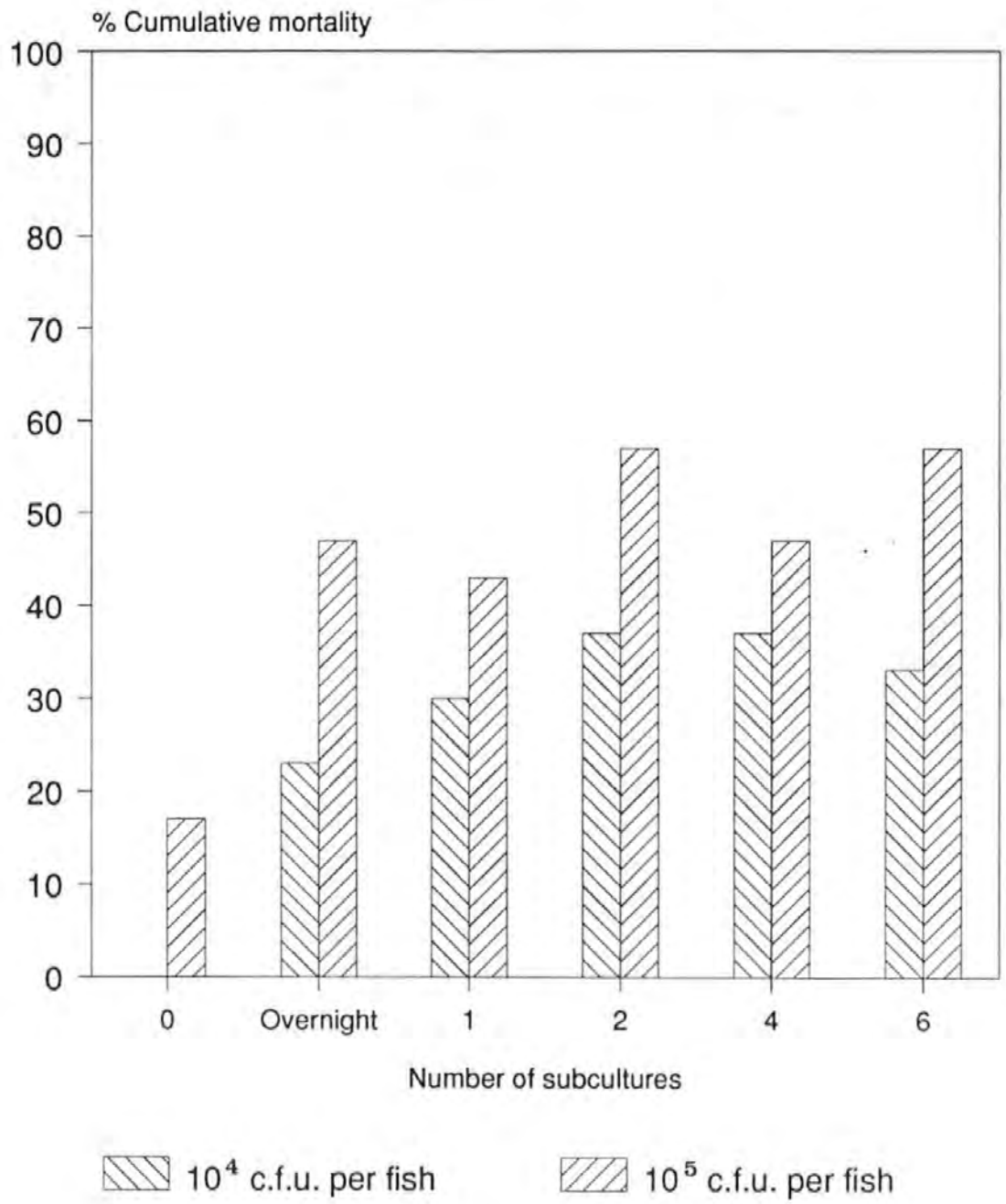


TABLE 5.3: EFFECT OF REPEATED SUBCULTURING ON VIRULENCE						
Number of Subcultures	**6**	**4**	**2**	**1**	**O/N**	**0**
Inoculum (cfu per fish)	10^4	10^4	10^4	10^4	10^4	$4x10^4$
% cumulative mortality[1]	33	37	37	30	23	0
Kidney (TSA) % fish positive for *Y.ruckeri*[2]	90	100	90	100	100	90
Relative bacterial growth (sv)	43	33	30	33	47	33
Faeces(ROD) % fish positive for *Y.ruckeri*[2]	0	0	10	0	40	50
Relative bacterial growth (sv)	-	-	10	-	13	20

[1] 30 fish per group [2] 10 fish per group

TABLE 5.4: EFFECT OF STORAGE TIME ON VIRULENCE						
Y.ruckeri **isolate number**	**75**			**26**		
Inoculum (cfu per fish)	$8x10^3$	$8x10^2$	$8x10^1$	$4x10^3$	$4x10^2$	$4x10^1$
% cumulative mortality[1]	27	13	10	43	3	7
Kidney (TSA)						
% fish positive for *Y.ruckeri*[2]	100	90	100	100	100	100
Relative bacterial growth (sv)	57	50	47	50	50	50
Faeces(ROD)						
% fish positive for *Y.ruckeri*[2]	20	10	0	10	10	10
Relative bacterial growth (sv)	6.7	3.3	-	6.7	6.7	3.3

[1] 30 fish per group [2] 10 fish per group

TABLE 5.5: VIRULENCE OF *Y. ruckeri* FROM DIFFERENT STOCKS OF RAINBOW TROUT				
Fish Lot	**No of Fish Used/Group**	**Average Weight**	**Reed and Muench**	**Probit**
A	15	6.4	2.4×10^3	4.5×10^3
B	15	12.0	1.6×10^3	1.26×10^3
C	15	28.0	9.7×10^2	1.6×10^3
D	15	6.3	4.3×10^3	3.16×10^3
E	30	9.6	6.25×10^3	1.0×10^3
F	15	10.0	-	3.16×10^3
G	15	6.3	2.8×10^3	5.0×10^2
H	15	57.0	1.47×10^3	1.0×10^3
I	15	5.0	-	$2.0 \times 10^{6*}$
J	20	6.0	2.77×10^3	3.16×10^3
K	30	9.0	3.0×10^5	$3.16 \times 10^{5*}$

* Late Winter- Early Spring Stocks.

Fig. 5.10: Susceptibility of different fish stocks to laboratory infection

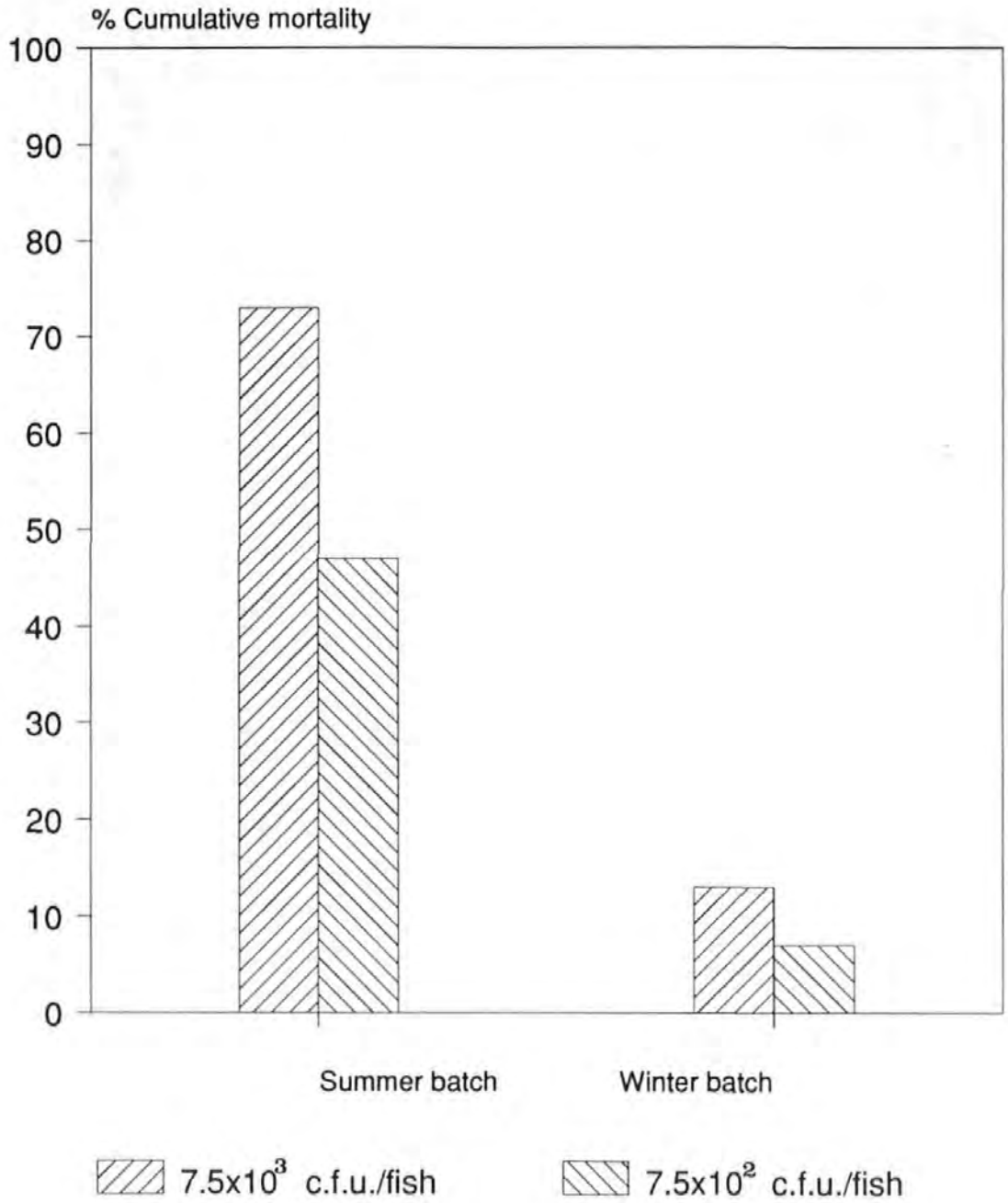


TABLE 5.6: SUSCEPTIBILITY OF DIFFERENT FISH STOCKS TO *Y.ruckeri*.				
Fish stock	**Summer stock**		**Winter stock**	
Inoculum (cfu per fish)	**$7.5x10^3$**	**$7.5x10^2$**	**$7.5x10^3$**	**$7.5x10^2$**
% cumulative mortality[1]	73	47	13	6.7
Number of fish sampled	4	7	10	10
Kidney(TSA)				
% fish positive for *Y.ruckeri*	100	86	50	70
Relative bacterial growth (sv)	53	52	50	70
Faeces(ROD)				
% fish positive for *Y.ruckeri*	60	57	10	0
Relative bacterial growth (sv)	33	24	6.7	-

[1] 15 fish per group.

Fig. 5.11: Effect of water temperature on infection by i.p. injection with *Y. ruckeri* (strain 26)

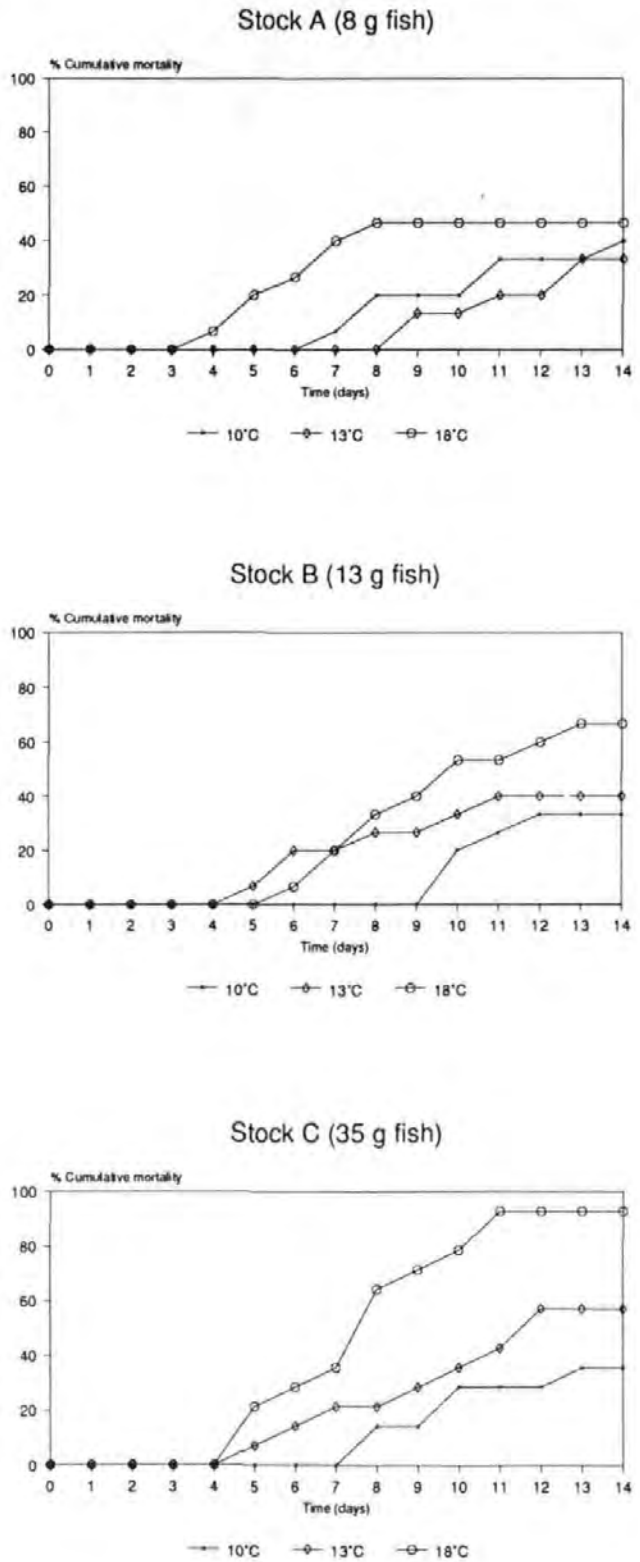


TABLE 5.7: EFFECT OF WATER TEMPERATURE ON BACTERIAL RECOVERY FROM SURVIVOR FISH AFTER I.P. INFECTION WITH *Y.ruckeri* (STRAIN 26).

Fish stock (average weight grammes)	Stock A (8g)		Stock B (13g)		Stock C (35g)	
Water temperature	**10°C**	**13°C**	**10°C**	**13°C**	**10°C**	**13°C**
Number of fish	10	10	9	9	7	4
Kidney(TSA)						
% fish positive for *Y.ruckeri*[2]	100	100	100	89	100	100
Relative bacterial growth (sv)	47	37	59	52	67	42
Faeces(ROD)						
% fish positive for *Y.ruckeri*[2]	10	0	44	11	57	21
Relative bacterial growth (sv)	3.3	-	29	7.4	43	25

Data from 18°C is not presenteddue to the low number of survivor fish in the group.

Fig. 5.12: Effect of stocking density on infection by i.p. injection

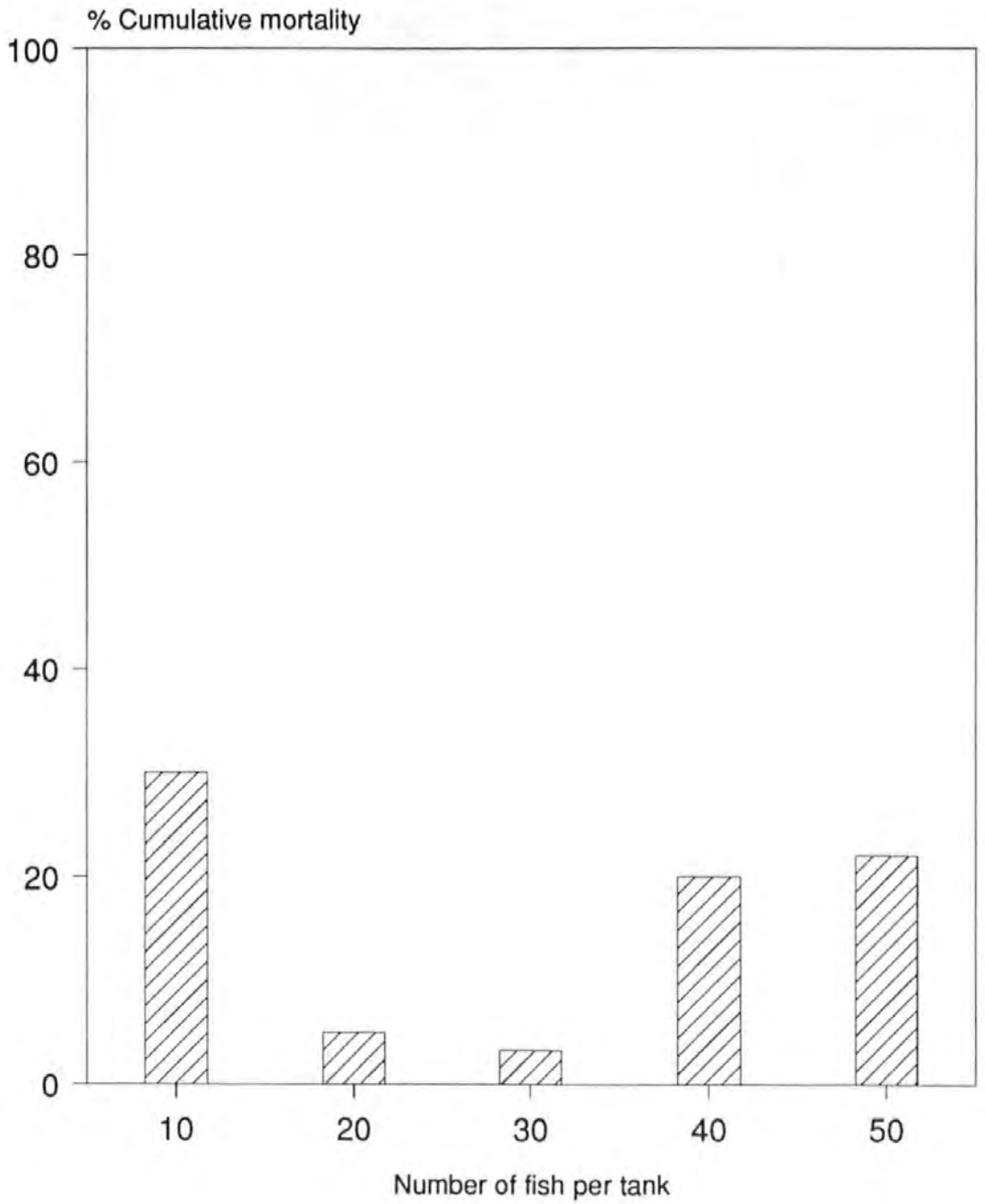


Fig. 5.13: Mortality of fish of different sizes at different doses

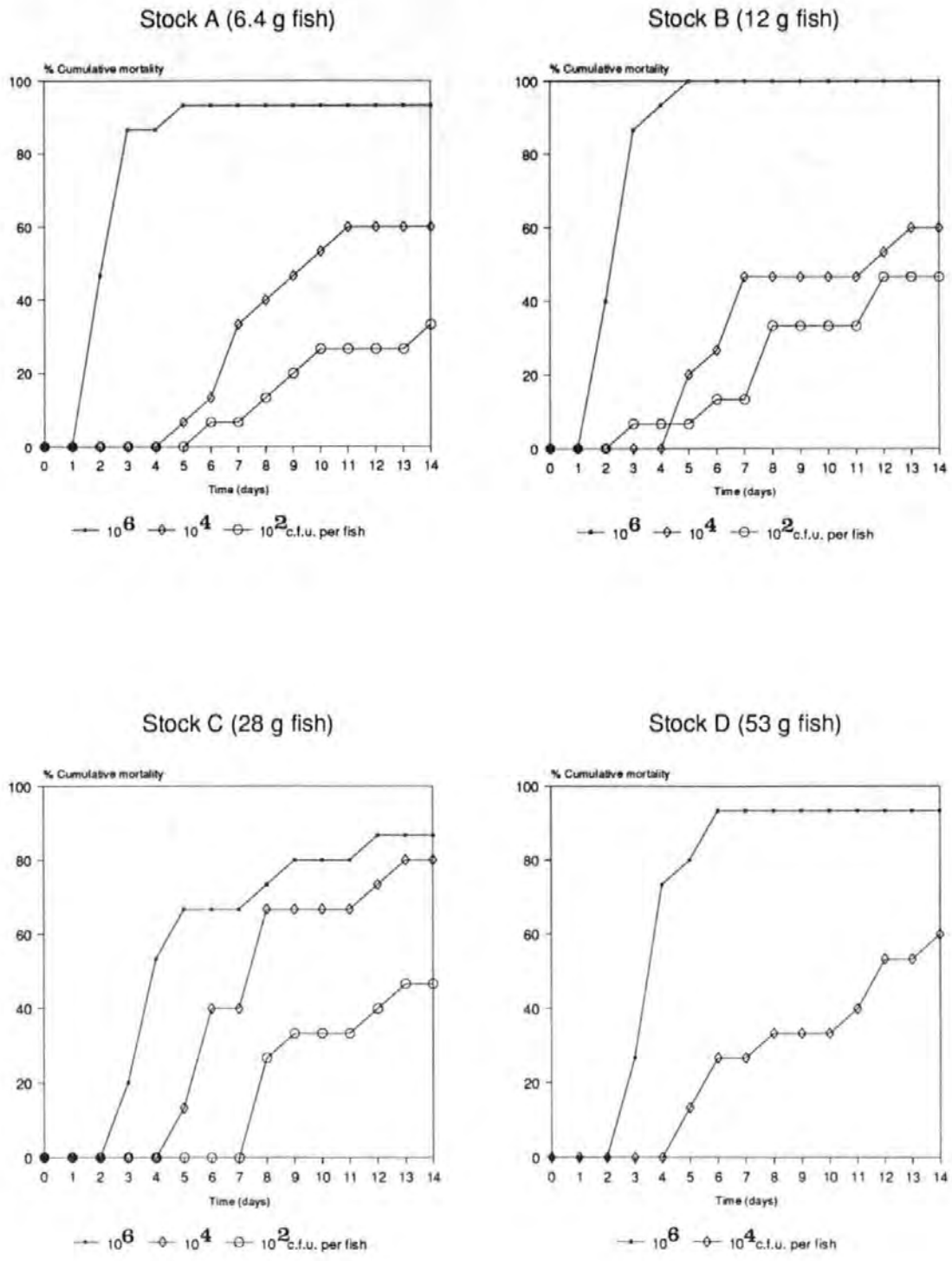


TABLE 5.8: LD_{50} VALUES FOR FOUR GROUPS OF RAINBOW TROUT INFECTED (I.P.) WITH *Y.ruckeri* STRAIN 26			
Fish Stock	**Average Weight**	**Reed and Muench**	**LD_{50}Probit**
A	6.4	2.4×10^3	4.5×10^3
B	12	1.6×10^3	1.26×10^3
C	28	9.7×10^2	1.6×10^3
D	53	-	1.5×10^4

TABLE 5.9: EFFECT OF FISH SIZE ON THE RECOVERY OF *Y. ruckeri* FROM SURVIVOR FISH							
Fish Stock (average weight)	**Stock A (6.4g)**		**Stock B (12g)**		**Stock C (28g)**		**Stock D (53g)**
Inoculum (cfu per fish)	**$2.3x10^4$**	**$2.3x10^2$**	**$2.3x10^4$**	**$2.4x10^2$**	**$2.3x10^4$**	**$2.3x10^2$**	**$2.3x10^4$**
Number of fish sampled	**6**	**9**	**6**	**7**	**3**	**9**	**6**
Kidney (TSA)							
% fish positive for *Y.ruckeri*	100	100	100	100	100	100	100
Relative bacterial Growth (sv)	39	55	61	62	44	39	61
Faeces(ROD)							
% fish positive for *Y.ruckeri*	17	33	83	75	33	11	67
Relative bacterial Growth (sv)	55	15	39	44	33	4	63

Fig. 5.14a: Effect of incubation temperature on virulence of *Y. ruckeri* (strain 26) Inoculum approx. 10^4 c.f.u. per fish

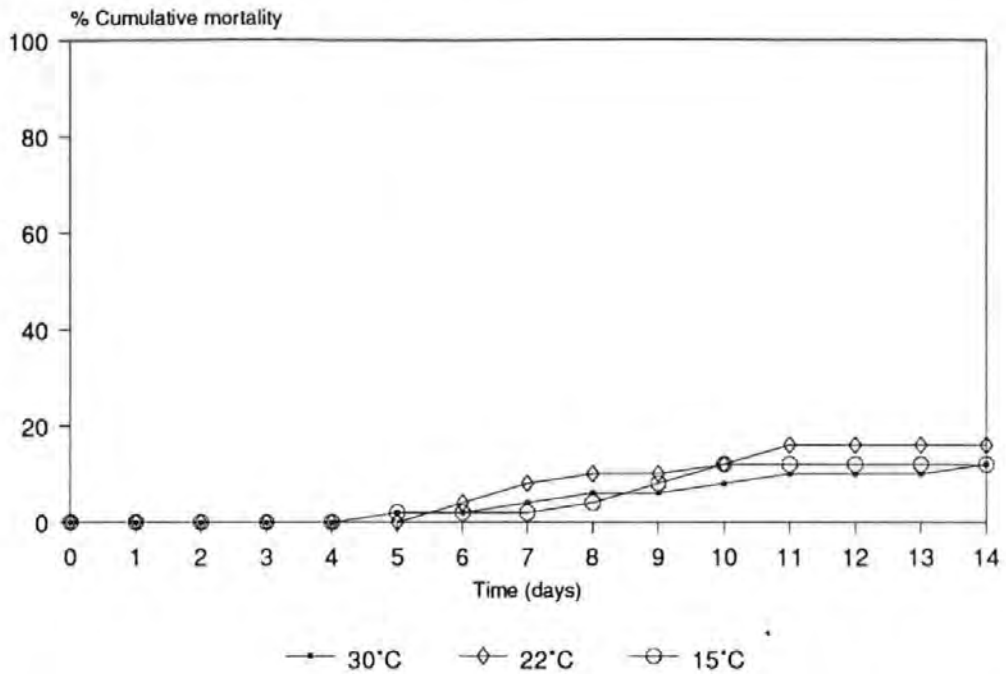


Fig. 5.14b: Effect of incubation time on virulence of *Y. ruckeri* (strain 26) Inoculum approx. 10^4 c.f.u. per fish

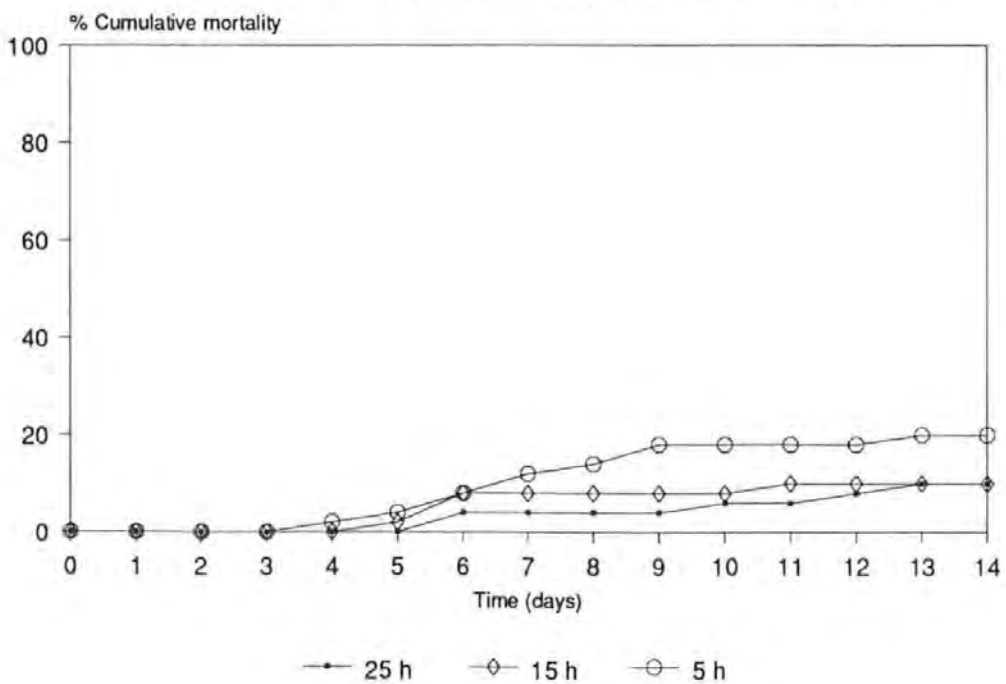


TABLE 5.10: EFFECT OF TIME AND TEMPERATURE OF INCUBATION OF THE INOCULUM ON I.P. INFECTION WITH *Y.ruckeri* (STRAIN 26).

	INCUBATION TEMPERATURES (°C)			INCUBATION TIMES (h)		
PROTOCOL	30	22	15	25	15	5
Inoculum (cfu per fish)	$8x10^3$	$1x10^4$	$2x10^4$	$1.5x10^4$	$1x10^4$	$6x10^3$
% cumulative mortality	14	16	12	10	10	20
2 weeks post-infection - 10 fish/group						
Kidney (TSA)						
% fish positive for *Y.ruckeri.*	100	100	100	100	100	100
Relative bacterial growth (sv)	10	43	43	40	40	40
Faeces(ROD)						
% fish positive for *Y.ruckeri.*	40	30	30	0	20	50
Relative bacterial growth (sv)	17	10	10		7	17
4 weeks post-infection - 10 fish/group						
Kidney (TSA)						
% fish positive for *Y.ruckeri.*	100	100	100	90	80	100
Relative bacterial growth (sv)	33	33	33	30	27	33
Faeces(ROD)						
% fish positive for *Y.ruckeri.*	0	0	0	0	30	20
Relative bacterial growth (sv)	-	-	-	-	23	10

TABLE 5.11: EFFECT OF TIME AND TEMPERATURE OF INCUBATION OF THE INOCULUM ON VIRULENCE *Y. ruckeri* (STRAIN 26)

Protocol	Reed and Muench	LD_{50} Probit
A 15 Hours @ 30°c	1.0×10^6 c.f.u per fish	8×10^5 c.f.u per fish
B 15 Hours @ 25°c	1.3×10^6 c.f.u per fish	1×10^6 c.f.u per fish
C 5 Hours @ 30°c	4.5×10^5 c.f.u per fish	4×10^5 c.f.u per fish
D 5 Hours @ 25°c	5.0×10^5 c.f.u per fish	5×10^5 c.f.u per fish

Fig. 5.15: Effect of concentration of BHI growth medium on virulence.

Inoculum 2 x 10^3 c.f.u. per fish

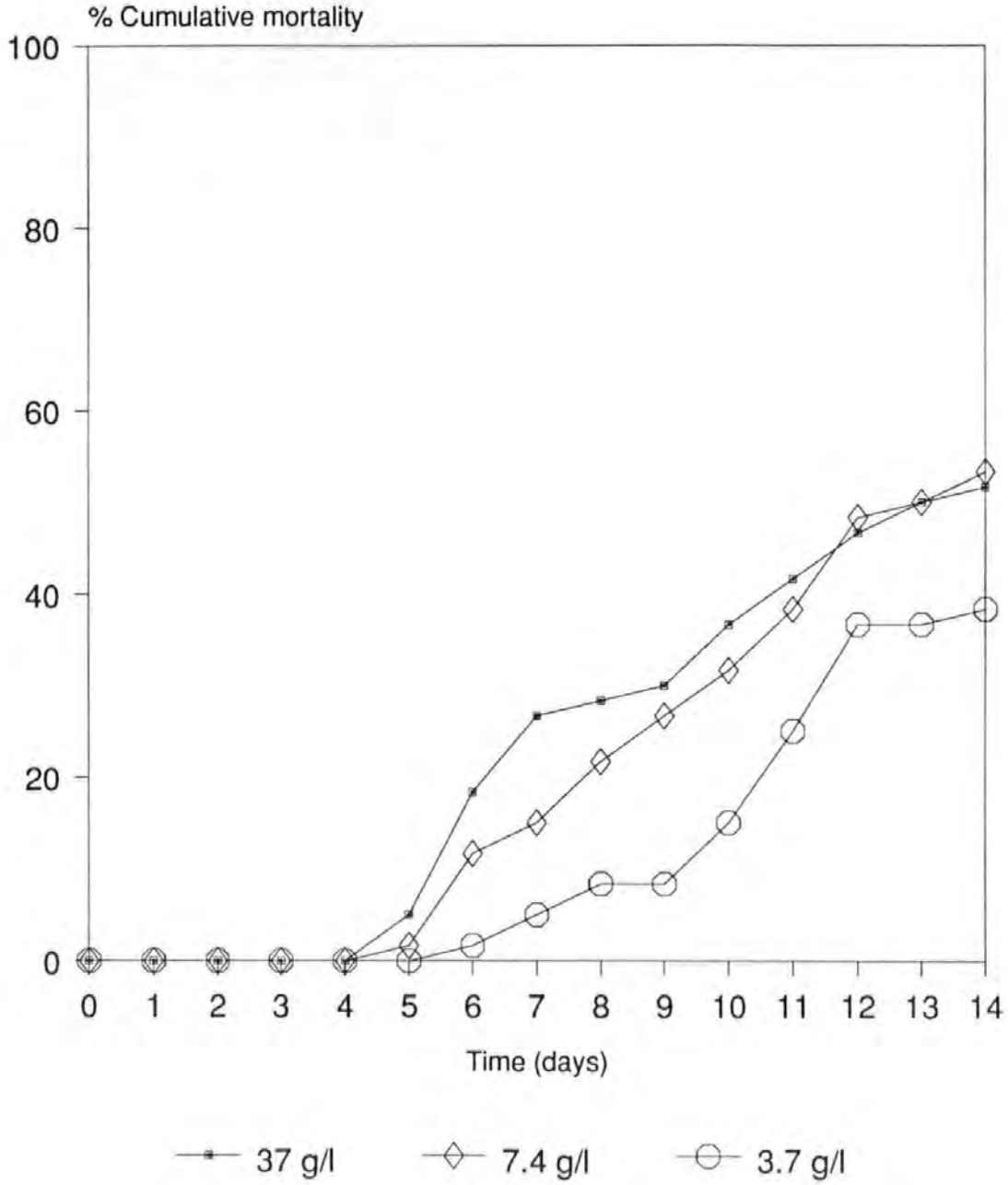


TABLE 5.12: EFFECT ON VIRULENCE AND BACTERIAL RECOVERY OF CONCENTRATION OF THE MEDIA FOR INOCULUM PREPARATION

Protocol	37 g/l shaking	37 g/l static	7.4 g/l Static	3.7 g/l Static
Inoculum (cfu per fish)	1.5×10^4	1.4×10^4	2.5×10^4	2.4×10^4
% cumulative mortality	38	52	53	38
Kidney (TSA)				
% fish positive for *Y. ruckeri*.	100	80	100	90
Relative bacterial growth (sv)	60	50	53	43
Faeces(ROD)				
% fish positive for *Y. ruckeri*.	40	50	20	0
Relative bacterial growth (sv)	17	27	7	-

Fig. 5.16: Effect on virulence of conditions of culture of the inoculum (approx. 10^3 c.f.u. per fish).

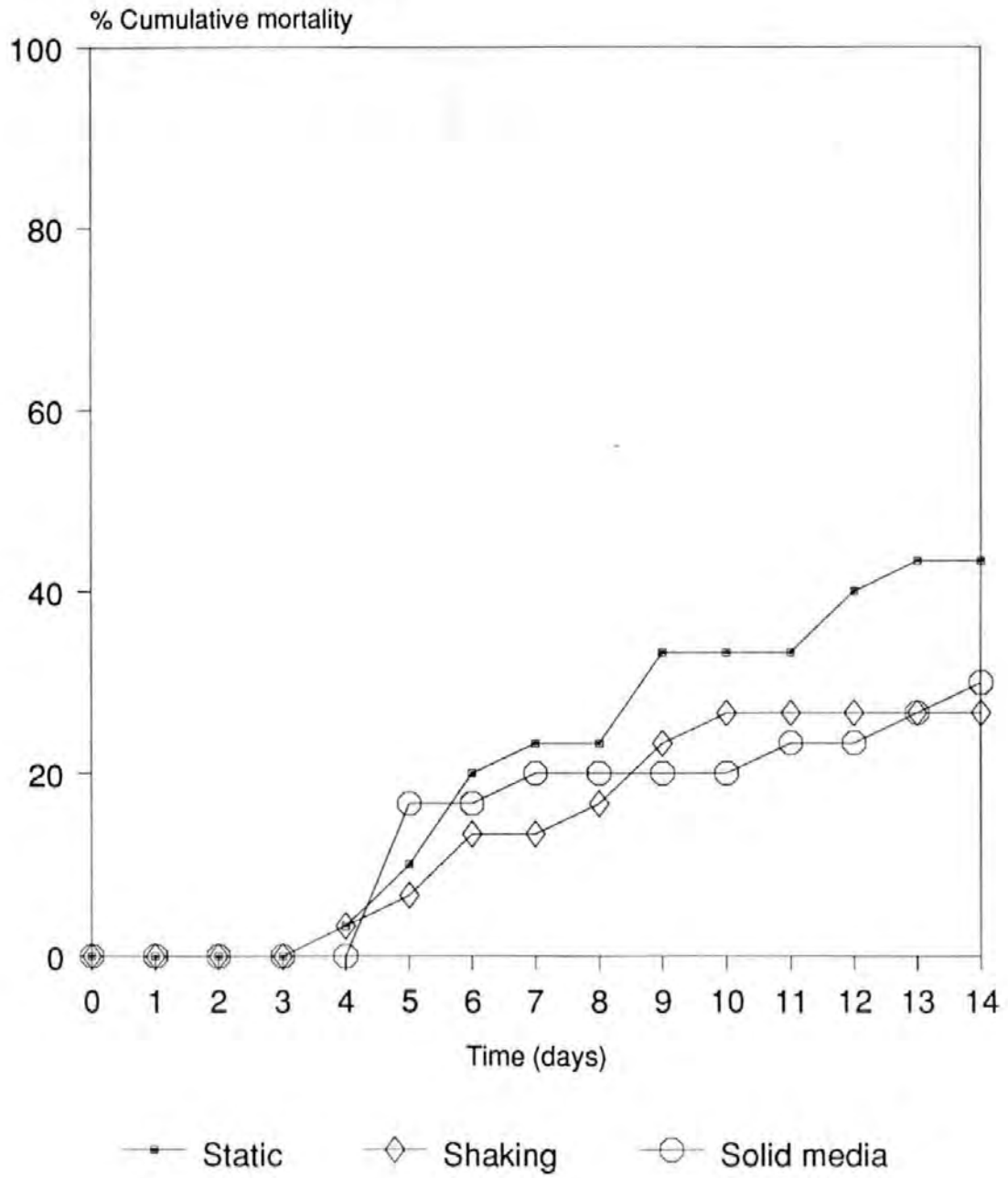


Fig. 5.17: Effect on virulence of the presence of culture supernatant in the inoculum

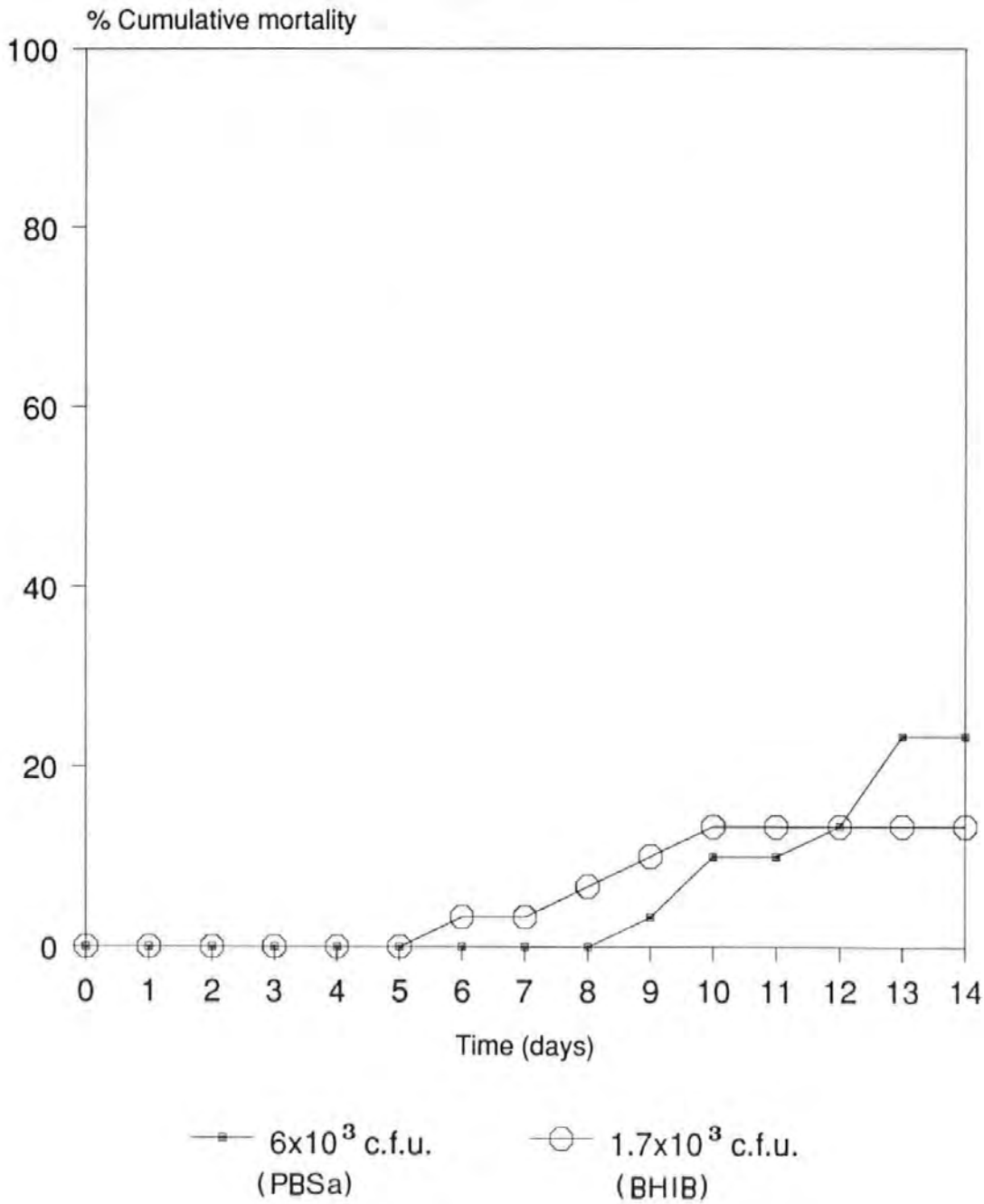


TABLE 5.13: EFFECT OF CONCENTRATION OF INOCULUM ON MORTALITIES ON INFECTION BY IMMERSION

	% Cumulative mortalities					
Inoculum		**10^8 cfu per ml^{-1}**		**10^7 cfu per ml^{-1}**		**10^6 cfu per ml^{-1}**	
Experiment A	15 fish/tank 15g fish 2 min. bath	20	7	7	7	27	13
Experiment B	15 fish/tank 5g fish 2 min. bath	NP		20	20	7	7
Experiment C	50 fish/tank 6.3g fish 5 min. bath	6	10	20	20	16	4

NP: Not Performed.

Fig. 5.18: Effect of time of exposure on infection by immersion

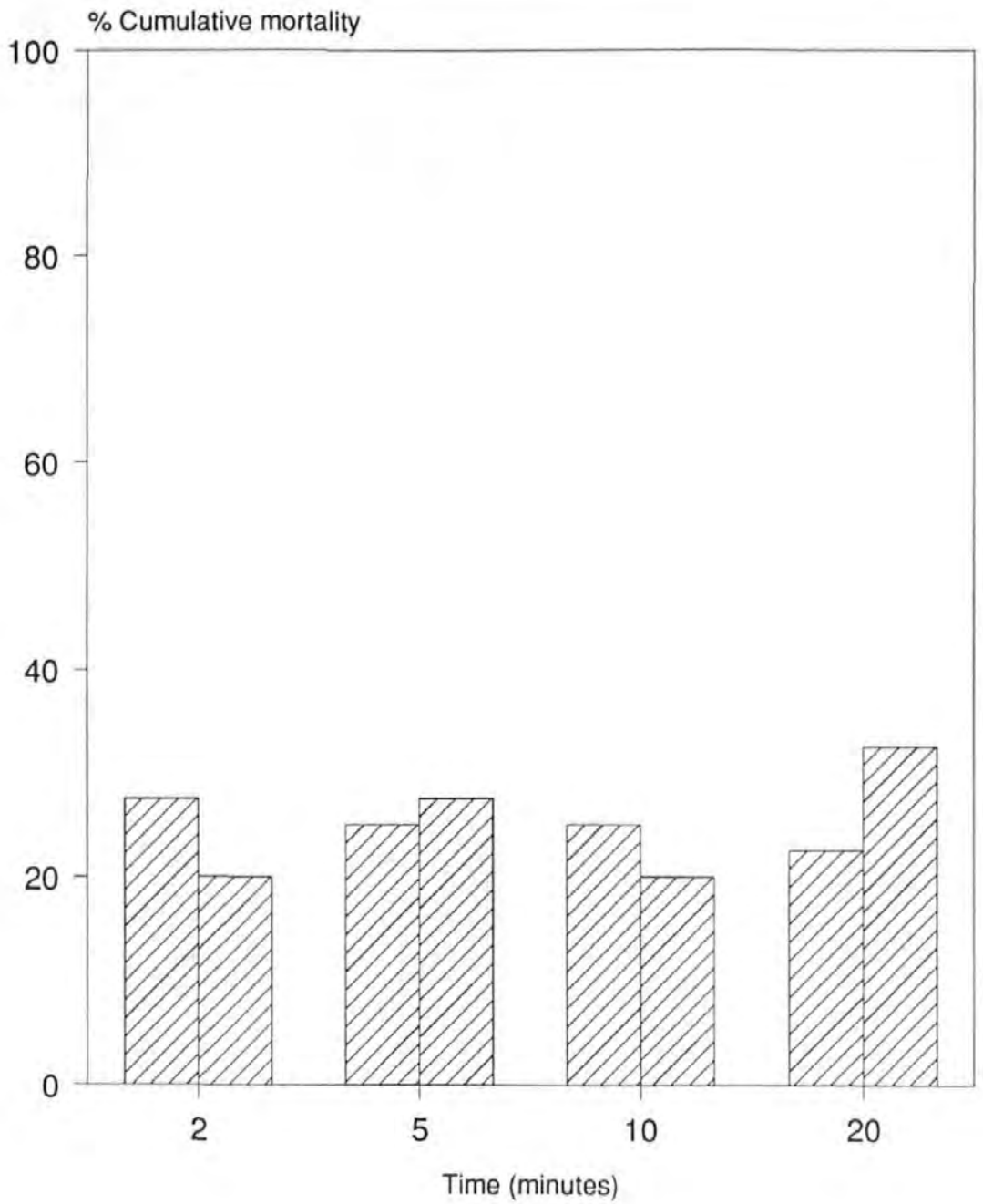


TABLE 5.14: EFFECT OF TIME OF EXPOSURE ON BACTERIAL RECOVERY AFTER INFECTION BY IMMERSION

Time	2 min		5 min		10 min		20 min	
Kidney (TSA)								
% fish positive for *Y. ruckeri*.	80	80	100	80	90	100	90	100
Relative bacterial growth (sv)	27	27	33	30	37	33	30	37
Faeces(ROD)								
% fish positive for *Y. ruckeri*.	0	20	20	40	20	50	80	60
Relative bacterial growth (sv)	-	7	13	37	17	47	77	60

Fig. 5.19: Effect of skin damage of the body surface of fish followed by infection by immersion

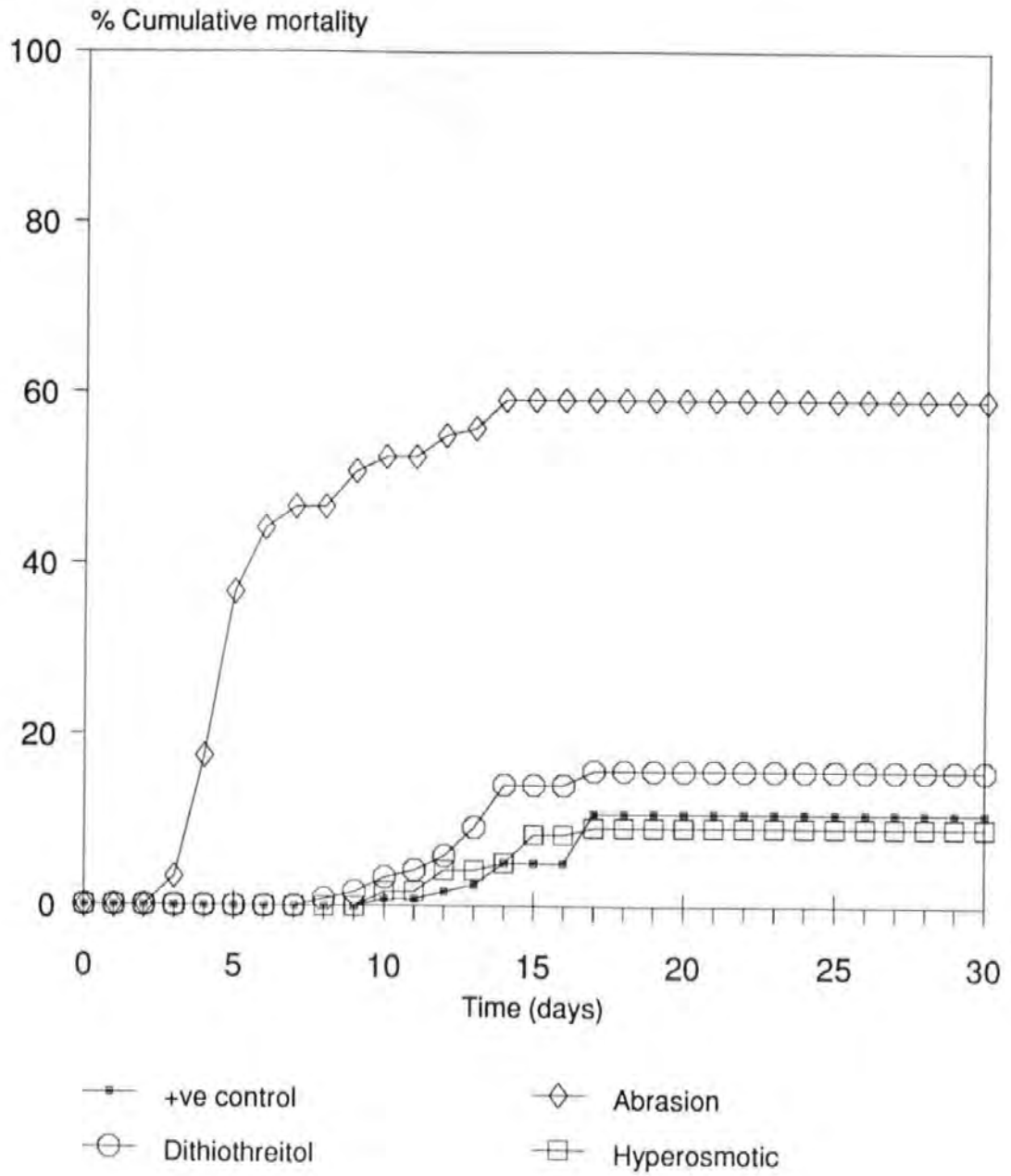


TABLE 5.15: EFFECT OF SKIN DAMAGE TO THE BODY SURFACE ON BACTERIA RECOVERY AFTER INFECTION BY IMMERSION

PROTOCOL	Positive Control		Abrasion		Dithiothreitol		Hyperosmotic	
Inoculum (cfu/ml)	$1x10^8$	$1x10^8$	$1x10^8$	$1x10^8$	$2x10^8$	$2x10^8$	$2x10^8$	$9x10^7$
6 days post inoculation								
Kidney (TSA)								
% fish positive for *Y. ruckeri*	100	100	100	100	100	100	100	100
relative bacterial growth (sv)	44	55	78	44	55	55	67	67
Faeces(ROD)								
% fish positive for *Y. ruckeri*	33	0	33	0	0	0	0	0
relative bacterial growth (sv)	11	-	11	-	-	-	-	-
Antibody (P/N)	-	-	-	-	-	-	-	-
17 days post inoculation								
Kidney (TSA)								
% fish positive for *Y. ruckeri*	100	100	100	100	100	100	NS	NS
relative bacterial growth (sv)	67	33	67	56	67	33		
Faeces(ROD)								
% fish positive for *Y. ruckeri*	67	67	33	33	33	100	NS	NS
relative bacterial growth (sv)	44	22	22	22	11	44		
Antibody (P/N)	-	-	-/+	-/+	-	-		
24 days post inoculation								
Kidney (TSA)								
% fish positive for *Y. ruckeri*	100	100	100	100	100	100	100	100
relative bacterial growth (sv)	33	33	33	67	33	44	33	44
Faeces(ROD)								
% fish positive for *Y. ruckeri*	0	0	0	0	0	33	0	0
relative bacterial growth (sv)	-	-	-	-	-	11	-	-
Antibody (P/N)	-	-	-/+	-/+	-	-	-	-
31 days post inoculation								
Kidney (TSA)								
% fish positive for *Y. ruckeri*	90	100	100	100	100	100	100	90
relative bacterial growth (sv)	33	33	37	33	33	33	33	33
Faeces(ROD)								
% fish positive for *Y. ruckeri*	20	10	11	0	10	20	0	0
relative bacterial growth (sv)	7	3	7	-	3	13	-	-
Antibody (P/N)	-	+	+	+	-	-	-	-

TABLE 5.16: EFFECT OF DIFFERENT INFECTION BY IMMERSION TECHNIQUES ON MORTALITY AND BACTERIAL RECOVERY

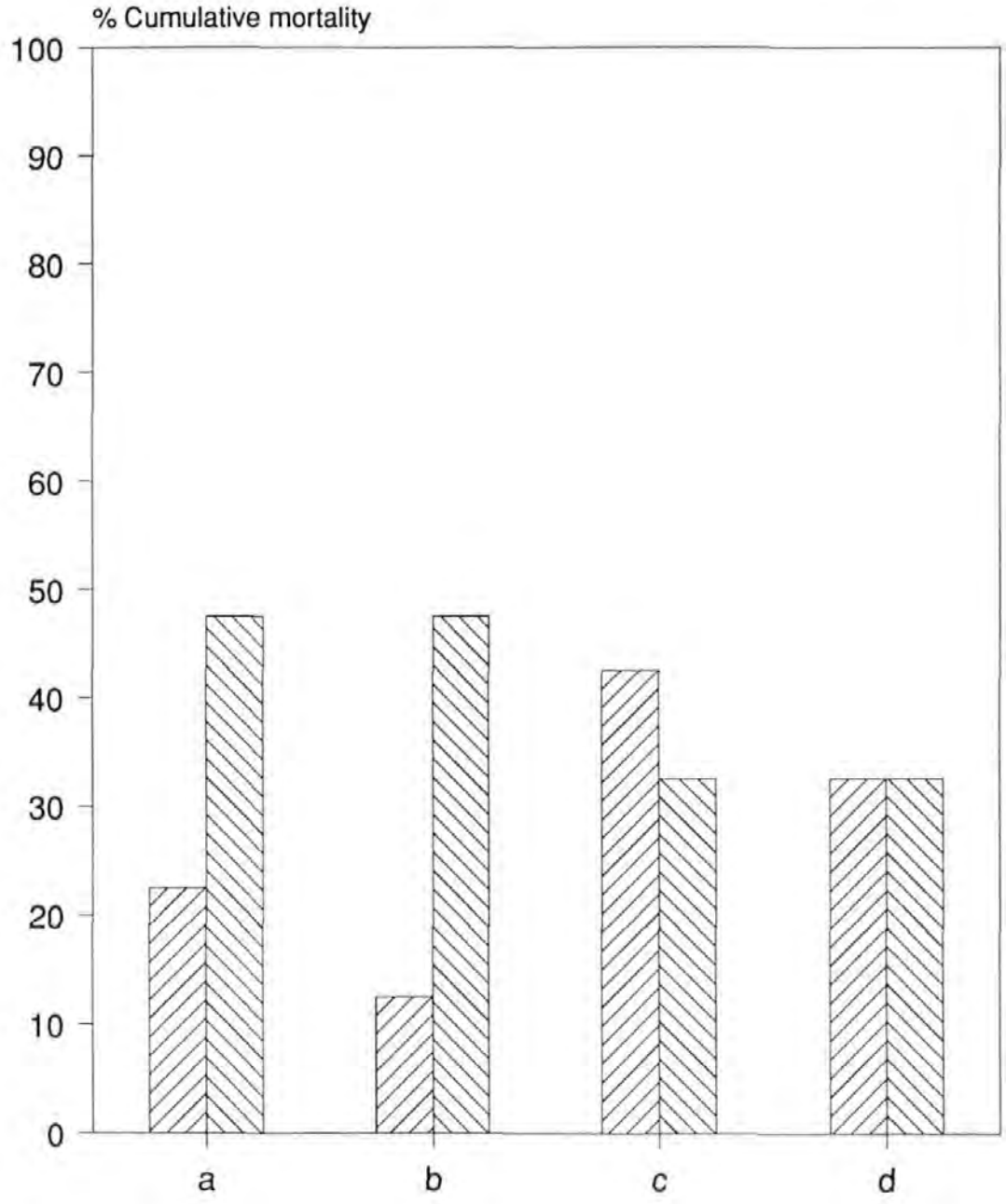
Protocol	a		b		c	
% cumulative mortalities	28	24	32	44	32	36
Kidney (TSA)						
% fish positive for *Y.ruckeri*	90	90	70	90	100	100
Relative bacterial growth (sv)	33	30	23	40	40	37
Faeces(ROD)						
% fish positive for *Y.ruckeri*	60	30	20	10	70	20
Relative bacterial growth (sv)	20	10	7	3	23	7

a: Bacterium suspended in PBSa. 30 min. ($1x10^8$ cfu/ml).

b: Bacterium suspended in PBSa. 30 min. exposure($8.5x10^7$ cfu/ml) + dilution (0.61 l/min).

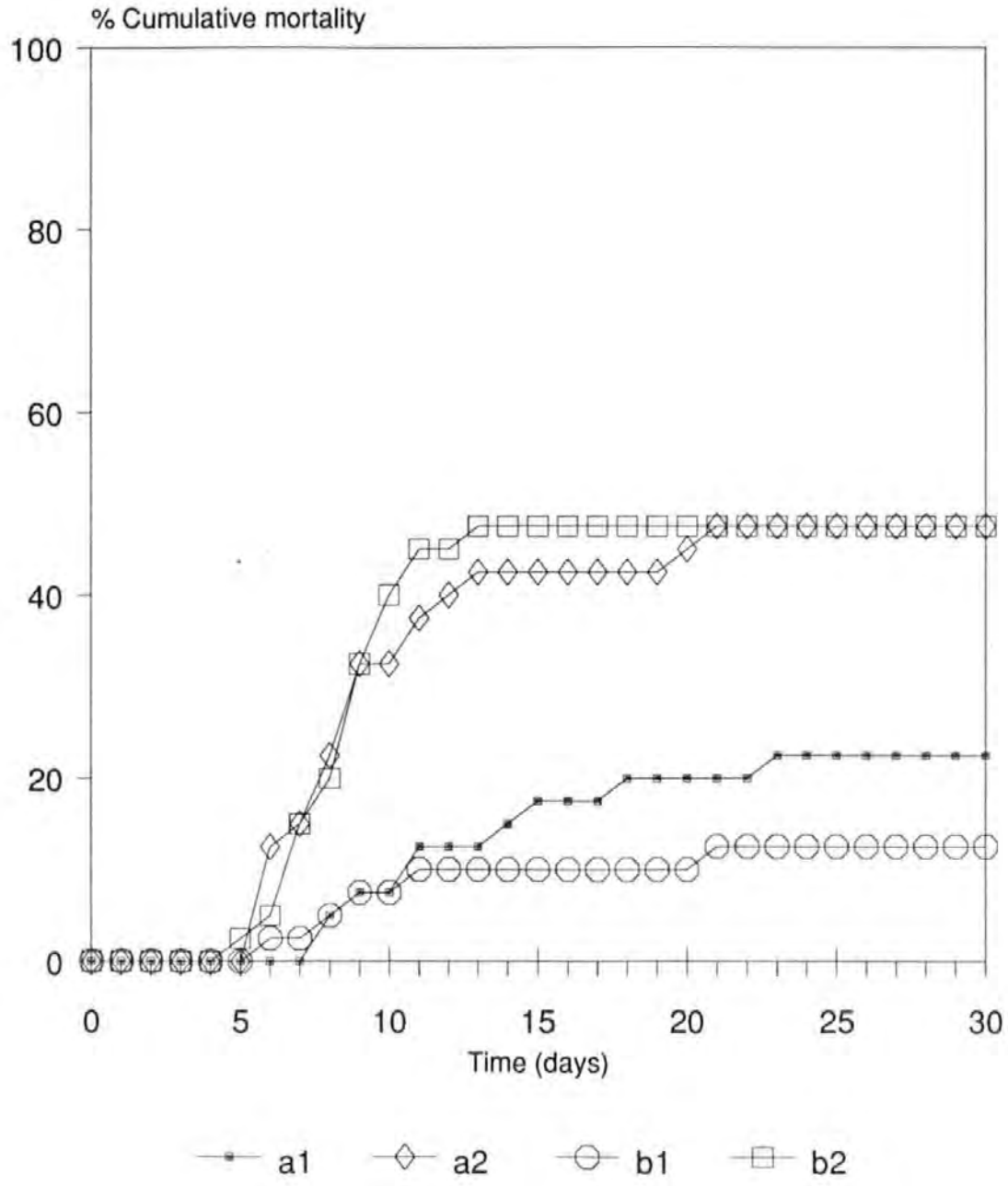
c: Bacterium suspended in BHIB. 30 min. exposure($1x10^8$ cfu/ml) + dilution (0.61 l/min).

Fig. 5.20: Effect of suspending medium and addition of latex particles to inoculum on mortality following infection by immersion



(a) *Y. ruckeri* suspended in BHIB
(b) *Y. ruckeri* suspended in BHIB + latex
(c) *Y. ruckeri* suspended in PBSa
(d) *Y. ruckeri* suspended in PBSa + latex

Fig. 5.21: Effect of position in tank room on infection by immersion



a_1 *Y. ruckeri* suspended in BHIB in area 1
a_2 *Y. ruckeri* suspended in BHIB in area 2
b_1 *Y. ruckeri* suspended in PBSa in area 1
b_2 *Y. ruckeri* suspended in PBSa in area 2

FIG. 5.22: SUMMARY OF CONDITIONS FOR INFECTION OF RAINBOW TROUT WITH *Y. ruckeri*

- Determination of standard growth conditions for *Y. ruckeri in vitro*

- Selection for increased virulence of *Y. ruckeri*

- Study of factors affecting virulence

- Intraperitoneal infection model. Study of:

  - Pathogen's variables
  - Fish variables
  - Environmental variables

- i.p. infection conditions for maximum virulence and reproducibility:

  - Homogeneous fish stock (preliminary LD_{50} test)
  - Inoculum preparation: 37 g/l BHI, 5 h, 30°C, static.

- Infection by immersion model.

CHAPTER 6

EFFECT OF DIETARY VITAMIN E LEVELS ON INFECTION WITH *Y. ruckeri*

6.1 INTRODUCTION

Tocopherols are a family of compounds with biological vitamin E activity, of which α-tocopherol is the one with the highest biological activity. Although the principal function of vitamin E is as an intracellular antioxidant, it has a number of other roles (Putnam and Comben, 1987). During the last decade it has been demonstrated that vitamin E has an important role in immunity to infectious diseases, and numerous reports have been published on the effects of dietary vitamin E on the immune response of birds and mammals. Reviews by Nockels (1979), Corwin and Gordon (1982) and Panush and Delafuente (1985) provide essential information in this respect, although the mechanism of the activity of vitamin E on the immune response has not yet been clarified. Vitamin E deficiency can result in a wide range of conditions in different species of animals. The primary lesion usually involves a breakdown in the structure of cell walls. Muscle tissue is frequently affected. Haemorrhage results when blood vessels are involved, but the predominant deficiency syndromes vary according to species. It is also frequently reported that an individual animal, or a small number in a larger group, has suffered signs of vitamin E deficiency while the others in the group remained

apparently healthy (Putnam and Comben, 1987).

During the last two decades work on fish nutrition has progressed substantially. Studies on the vitamin E requirement for the main farmed fish species have been published. Often such requirements were studied in conjunction with the relationship between levels of vitamin E and selenium and/or with the influence of the level in unsaturated fatty acids on the fish requirement for vitamin E (Aoe *et al.*, 1972; Cowey *et al.*, 1981; Cowey *et al.*, 1983; Hung *et al.*, 1976; Hung *et al.*, 1981; Murai and Andrews, 1974; Watanabe *et al.*, 1977; Watanabe *et al.*, 1981). A summary of the vitamin E deficiency signs in various fish species has been presented by Tacon (1985).

Only recently has the involvement of nutrition with the fish immune response been studied and only a few reports have been published (Blazer and Wolke 1984a-b; Hardie *et al.*, 1990; Lall *et al.*, 1987; Landolt, 1989). This area of work is promising, mainly for those fish diseases which are proving difficult to control by means of vaccination or chemotherapy, such as bacterial kidney disease (BKD) or furunculosis caused by *Renibacterium salmoninarum* and *Aeromonas salmonicida* respectively. Improvements in diet formulation with regard not only to growth and food conversion rates but also immunological performance, together with adequate husbandry management could alleviate the devastating effects of such diseases in farmed fish. In order to carry out this kind of work, suitable *in vivo* models are needed. The infection and immersion challenge protocols with *Y. ruckeri* developed for rainbow trout in

this project were used as *in vivo* models in which dietary vitamin E was the variable under study.

6.2 RESULTS

6.2.1 Experimental schedule

Week 1 - Fish adaptation to diets (3 laboratory prepared, 3 commercial)

↓

Week 12 - Fish transferred to field station

↓

Week 20 - Fish transferred back to laboratory (3 laboratory diet, 1 commercial)

↓

Week 22 - Infection by immersion (6 tanks/diet).

- Post infection sampling at weeks: 1 (12 fish/diet); 2 (18 fish/diet); 3 (18 fish/diet); 7 (30 fish/diet) and 12 (15 fish/diet).

Parallel infection by injection experiment (Fish always at the laboratory).

Week 17 - Infection (i.p.) (2 diets, 2 tanks/diet).

- Post infection sampling at weeks: 1 (5 fish/diet); 3 (5 fish/diet); 4 (10 fish/diet) and 15 (5 fish/diet).

The experiment was finished at week 33 (i.e., week 12 after infection by immersion and week 15 after infection by i.p. injection).

6.2.2 Haematological and biochemical parameters in experimental fish

Analyses of vitamin E content of the livers (Table 6.1), showed that the deficient group had attained their lowest levels of vitamin E within 14 weeks of starting the experimental diets. The fish were therefore reflecting their dietary vitamin concentration when they were bath challenged with the virulent bacterium (strain 26) at week 22. Analyses at week 28 (Table 6.2) were not significantly different from week 14. At the end of the experiment (week 33), the levels of vitamin E in the livers of the remaining fish were obtained (Table 6.3). Values of tocopherol were very similar in fish challenged either by injection or by immersion. When levels of vitamin E in livers of infected fish were compared against uninfected controls, differences were found only in the group fed with the intermediate dose (86 mg vitamin E kg^{-1}) which had lower values in infected fish than in controls. Such a difference was significant both for fish which had been infected by i.p. injection ($p < 0.001$) and for fish infected by immersion ($p < 0.02$)(Table 6.3).

At week 28 the classic vitamin E deficiency symptoms of significantly reduced haematocrit values, increased erythrocyte fragility and plasma pyruvate kinase were also apparent (Table 6.2). The liver vitamin C concentrations indicated low levels in fish on the commercial diet (Table 6.2). This had also been observed at week 18 when livers from 5 fish from each group were analysed for vitamin C. The fish fed commercial diets with 81, 123 and 158 mg vitamin E had livers containing 23.3 ± 0.4, 12.6 ± 1.0 and 12.0 ± 1.1 μg vitamin C g^{-1} respectively. This compared with 145.9 ± 16.7, 142.1 ± 1.8 and 133.5 ± 5.3 μg

vitamin C g^{-1} for fish on the prepared diets that contained increasing levels of vitamin E. There was no apparent correlation between liver concentrations of vitamins E and C in the trout. Interestingly, at week 28, the level of vitamin C for fish fed on the 806 mg of vitamin E per kg^{-1} diet, was significantly lower ($p<0.01$) than the other two laboratory prepared diets (Table 6.2).

6.2.3 Histological Studies

Histological studies on trout after 28 weeks on the diets showed significant changes in the deficient group. Spleens exhibited marked degrees of histological change compared with spleens in the other groups (Fig. 6.1). These changes included haemosiderosis accumulation of lipofuscin. Skeletal muscle showed focal areas of dystrophic fibrilar degeneration leading to the proliferation of fibroblasts and necrosis of muscle bundles (Fig. 6.2). Where necrosis had occurred, the breakdown tissues were seen to be phagocytosed within macrophage cells. There were aggregates of these cells interspersed within the interstitial spaces. Hepatocyte vacuolation and cytoplasmic inclusions were the consistent histological changes in the deficient group, whereas only occasional livers in other groups showed any change (Fig. 6.3). Only the deficient livers reacted positively to Schmorl's method for lipofuscin. No macroscopic symptoms were associated with the described pathologies.

6.2.4 Infectivity experiments

6.2.4.1 Field Trial

This was intended to provide a natural challenge, but after eight weeks no fish had been found, from weekly sampling, to contain detectable bacteria. *Yersinia ruckeri* was also not detectable in the tank water.

During the weeks that the fish were held at the field station, a small number of mortalities occurred. In fish fed with laboratory diets, the cumulative mortalities were 10 (7 mg kg^{-1} diet), 3 (86 mg kg^{-1} diet), and 0 (806 mg kg^{-1} diet). In fish fed with the commercial diets the mortalities were 0 (81 mg kg^{-1} diet, 8 (123 mg kg^{-1} diet) and 36 (158 mg kg^{-1} diet). These mortalities were not correlated with *Y. ruckeri* infection since the bacterium was not isolated from either fish or water samples. The only microscopic symptom observed amongst the examined fish during the field study was some exophthalmia. The experiment was therefore modified and the fish were returned to the aquaria facilities where they were quarantined for two weeks and bacteriological and serological tests were undertaken to confirm that there had been no previous exposure to *Y. ruckeri*. Because of limitations on tank space in the aquarium, the experiment was continued with four treatments: the commercial diets containing 123 and 158 mg vitamin E kg^{-1} being discontinued.

6.2.4.2 Bath infection

Trout (150) from each of the four remaining dietary groups transferred back to the aquarium, were divided into tanks of 25 fish i.e. six tanks per dietary treatment. The challenge was performed in half-filled tanks containing 15 l of aerated water. The fish were exposed to *Y. ruckeri* at 6.17 x 10^7 cfu ml^{-1} for 60 min, after which time the water level was restored to 30 l and the flow rate adjusted to 0.5 l min^{-1}. Bacterial numbers in the water were determined after one and 60 min of the challenge in each tank. Fish were sampled at 1, 2, 3, 7 and 12 weeks.

Bath exposure to pathogenic strain 26 of *Y. ruckeri* resulted in mortalities in all four dietary groups (Fig. 6.4). The least mortalities were recorded in trout on the highest dose of vitamin E (806 mg). Both the log rank test and the chi-square test indicated that such mortalities were significantly different ($p<0.01$) from those recorded on fish fed on the experimental diet containing 86 mg kg^{-1} vitamin E, but not significantly different ($p>0.05$) from those in the fish fed with either the commercial diet or the laboratory diet containing 7 mg kg^{-1} vitamin E.

6.2.4.3 Intraperitoneal infection

A parallel experiment was carried out on fish which had not been taken to the field station but had been held continuously in the aquarium throughout the course of the experiment. Ninety trout from each of two groups fed at 86 or 806 mg vitamin E kg^{-1} prepared diets were kept in 30 l tanks at 45 fish

per tank. After 17 weeks of adaptation to the respective diets the fish were injected i.p. with 9.8 x 10^3 c.f.u. of strain 26 suspended in 0.15M NaCl. Five fish were sampled from each dietary group at weeks 1, 3, 4 and 15. Comparable controls for i.p. and bath challenge groups were also included. Fish fed with the higher vitamin E (806 mg kg^{-1}) diet showed fewer deaths (31%) compared with fish fed on the 86 mg kg^{-1} vitamin E diet (43%), although both groups reached their total mortalities within 20 days (Fig. 6.5).

Mortality curves were calculated as the average of 6 replicate tanks for the bath immersion and 2 tanks for the i.p. injection. In either case the differences in mortalities within groups were not significant ($p > 0.05$). It can be seen that there were more deaths in the injected groups than in the comparable dietary groups exposed to the pathogen by immersion (Fig. 6.4 and 6.5).

6.2.4.4 Antibody levels

Serum antibody production in the trout exposed to *Y. ruckeri* by bath challenge or i.p. injection, was the only test of immunocompetence examined. Using the sensitive ELISA method, serum from fish on all the diets gave similar positive results at week 12 following bath exposure. No differences due to diet were observed and the ANOVA test showed a difference due only to the time after challenge with only the last sample, at week 12, being significantly different from the others (Table 6.4). Antibody levels also increased with time after fish had been injected (Table 6.5), although with the

data available, no significant differences were observed due to either diet or time post infection.

In the last serum sample taken (week 12 or 15) fish in both infection experiments showed ELISA positive/negative values close to 2, suggesting the presence of serum antibodies against *Y. ruckeri*.

6.2.4.5 Bacteriology results

Viable bacteria persisted, up to week 12, in the kidney of all groups of fish infected with *Y. ruckeri* by immersion. The number of fish from which the pathogen could be isolated was similar during the first 3 weeks of the experiment, both from faecal and kidney samples. At week 7, significant differences between groups were observed ($p < 0.05$), with the group of fish fed with the diet containing 806 mg kg^{-1} vitamin E showing higher carriage in both types of samples. By the end of the experiment (week 12) *Y. ruckeri* was undetectable from the lower intestine in all groups (Fig. 6.6). The relative growth of the bacteria (score value, sv) on the plates followed a similar pattern to that mentioned above, with a steady decrease over 12 weeks (Fig. 6.6).

Results from trout injected i.p. (Fig. 6.7) showed that *Y. ruckeri* was also present in kidney samples of both groups of fish (86 and 806 mg kg^{-1} vitamin E) during the 15 weeks of the experiment, with all fish sampled during the first four weeks positive for the pathogen. In addition no isolation of the

pathogen occurred in faecal samples after the first week. The relative growth (sv) of *Y. ruckeri* from kidney material decreased from week 1 (Fig. 6.7) to no detectable bacteria by week 3.

6.3 DISCUSSION

The diets used in this experiment were formulated to measure the effect of tocopherol (vitamin E) requirements in the absence of complicating factors such as a variable polyunsaturated fatty acid (PUFA) intake or a selenium deficiency. Under these conditions, tocopherol deficiency did not lead to any reduction of the growth rate of rainbow trout (data not shown). This agrees with the findings in Atlantic salmon (Hugh *et al.*, 1976) and rainbow trout (Cowey *et al.*, 1981; Cowey *et al.*, 1983; Silas *et al.*, 1981). Also, Hardie *et al.*, (1990), found no differences in growth when they studied the effect of vitamin E on Atlantic salmon using a diet composition almost identical to the one used in this experiment. Reduction in weight gain has been correlated with tocopherol deficiency in carp (Watanabe *et al.*, 1977) and rainbow trout (Watanabe *et al.*, 1981), when fish had been fed diets containing herring oil triglycerides.

The liver was the tissue selected for the analysis of vitamin E because, together with the kidney it reflects the fish dietary status for α-tocopherol more closely than heart, muscle, gills, brain or adipose (Cowey *et al.*, 1983).

Comparing the values obtained in this study with those obtained by Cowey *et al.*, (1981), it can be inferred that fish given vitamin E at levels of 7 mg kg^{-1} were depleted. Transfer of the fish to the farm did not influence the level of vitamin E depletion.

The haematological and biochemical parameters tested at week 28 produced results consistent with those reported by Cowey *et al.*, (1983); Hugh *et al.*, (1976) and Woodall *et al.*, (1984). The last authors pointed out that some of the erythrocyte changes noted in the tocopherol-deficient fish, such as decrease in size, increased percentage of juvenile forms and increased fragility, suggest that anaemia results from premature erythrocyte breakdown rather than from a deficiency of blood-forming elements or enzyme co-factors.

The differences observed between the levels of vitamin C measured in the liver of fish fed on the commercial diets and the laboratory prepared diets were highly significant. This could be explained by the commercial scale of fish food manufacture and the usually inadequate storage of dry pellets which greatly reduces the concentration of labile components such as vitamin C. Food manufacturers make allowances for this problem and supplement their diets with overdoses of the most labile factors. The laboratory diets were prepared on a small scale and frozen immediately after production allowing the preservation of the vitamin C levels. Some work has been carried out relating to the fish immune response with dietary levels of vitamin C, showing

the beneficial effect of overdoses of vitamin C on the diet as a potentiator of the fish immune response (Durve and Lovell, 1982; Mazik *et al.*, 1987 and Navarre and Halver, 1989). No obvious correlation between the levels of vitamin E and C in the livers was observed, although this should be interpreted with caution since comparison between the two groups of diets is difficult due to the different formulations of the diets used. When the concentrations of vitamin C among fish fed on laboratory prepared diets were compared, the levels of vitamin C were lower in the livers of fish fed with the 806 mg kg^{-1} vitamin E diet. *In vitro* studies (McCay, 1985) have shown a relationship between the levels of vitamins C and E indicating that ascorbate is used first in a reactive oxygen situation and that tocopherols do not appear to become active until all the ascorbate present has been utilised. Although this theory has to be validated with *in vivo* studies, a study in pigs has reported that changes in ascorbate concentrations in serum and muscle tissues are associated with vitamin E and/or selenium deficiency. However, such dynamics are not yet fully understood (Putnam and Comben, 1987).

Previous histological studies on the effects of vitamin E deficient diets in fish have indicated a number of histological changes, including exophthalmia and ascites (Woodall *et al.*, 1964), severe anaemia (Woodall *et al.*, 1964), accumulation of hepatic ceroid (Millikin, 1982), muscular dystrophy (Murai and Andrews, 1974; Watanabe and Takashima, 1977; Cowey *et al.*, 1984), visceral pigment accumulations and retinal degeneration (Blazer and Wolke, 1983). In this study, whereas only a tentative histological examination was

made, the pathological changes denoting muscular dystrophy, haemolytic anaemia and accumulation of lipopigments in the fish fed the vitamin E deficient diet were similar to those described above and therefore, indicated that there was a diet-related change in this sample. The other samples, however, showed no significant changes related to the diets.

The mortalities recorded during the field trial in fish fed on commercial diets were not correlated with the vitamin E level of the diets. Other differences due to diet formulations could be responsible for such differences. However, mortalities amongst fish fed on laboratory diets were correlated with the vitamin E content. Watanabe *et al.*, (1981) reported mortalities of 40% occurring only among rainbow trout (*O. mykiss*) fed on vitamin E depleted diets, but in that case depleted fish were showing slower growth rates due to a lack of appetite. In the results presented here, the small number of mortalities in each group during the experiment and the large number of environmental variables occurring in the field trial make the establishment of any positive conclusions difficult and any interpretation should be treated with caution.

The results of fish mortalities obtained from both infectivity experiments, i.p. and immersion, were consistent, showing a higher mortality rate in the group of fish fed with the laboratory prepared diet containing 86 mg kg^{-1} vitamin E when compared with the diet containing 806 mg kg^{-1} vitamin E. This could be interpreted as a positive effect of excess level of vitamin E in the diet.

However, mortalities did not follow a linear pattern correlating with dietary vitamin E content, since both the vitamin E depleted and the commercial diet produced lower mortalities than the diet with an intermediate level of vitamin E. In the case of the commercial preparation, such results could be due to other unknown factors in this diet, which could have a synergistic effect on the immune response. There is no obvious explanation of the results obtained with the low level vitamin E diet. The results shown in Fig. 6.4 present the mortalities during a period of 30 days, since most of the mortalities occurred in the first four weeks post infection. Fish receiving the depleted diet, however, showed an extended mortality pattern and by the end of the experiment the differences in mortality between the depleted and intermediate level diets had been reduced. Statistical analyses of these results was done by the chi square test and by the log-rank method (Reto *et al.*, 1977). The second method is a more sophisticated analysis which estimates the probability of a given individual being alive at a certain time during the experimental period, instead of considering only the final mortality value. Both tests produced equivalent results.

Hardie *et al.*, (1990) showed significant differences between the mortalities recorded in Atlantic salmon fed on laboratory diets with 7 mg kg^{-1} vitamin E and a commercial diet containing 327 mg kg^{-1} vitamin E after bath immersion with a virulent strain of *Aeromonas salmonicida*. However, no results on a intermediate level of vitamin E were presented.

Direct comparison of the results obtained in the present study using live virulent bacteria with those of other workers is not possible, since most work has been concerned with immunizing fish with different killed antigen preparations of *Y. ruckeri* (Blazer and Wolke, 1984). Therefore, in other published work the effects of the interaction between host and pathogen can not be expressed. Very little is known about the mechanisms of pathogenicity of *Y. ruckeri* and which factors could reduce full expression of its virulence. The vitamin E depleted fish observed in this experiment, showing signs of anaemia, tissue disruption and compromised cell membrane integrity, most probably provide a very different environment for a pathogen than healthy, non-depleted fish. Therefore, it is speculated that the mortality results obtained from this work could reflect a complex interaction between the two entities involved in the disease process, the details of which are not yet fully understood. Besides tocopherol, a number of enzyme systems also function to prevent or limit initiated tissue damage by free radicals. Adaptative changes including release of glutathione peroxidase and superoxide dismutase may be involved under low vitamin E conditions (Cowey *et al.*, 1981). Such enzymes could affect, in an antagonistic mode, the production and/or expression of the pathogen's virulence factors, producing the biphasic mortality effect observed among the groups of fish fed with the three diets. In reviews on the effects of dietary vitamin E on the immune response in humans (Panush and Delafuente, 1985) and chickens (Nockels, 1979), biphasic dose-dependent responses to vitamin E have been described.

The results obtained from the study of the humoral immune response using the indirect ELISA technique agree with those of Lall *et al.*, (1987), where no differences in the humoral immune response of Atlantic salmon to *A. salmonicida* were found due to variations of dietary vitamin E. Using the same fish-pathogen model, Hardie *et al.*, (1990) found that only complement function was affected in vitamin E depleted salmon. In contrast, Blazer and Wolke (1984), studying the effects of vitamin E deficiency in rainbow trout, found that fish fed a deficient diet had significantly reduced responses in almost all immunological assays including the humoral response to *Y. ruckeri* measured as agglutination titres in immune serum. However, these authors did not find deficiency signs within 24 weeks in rainbow trout fed on a diet containing no vitamin E or synthetic antioxidants. Such conflicting results could be solved in the future by including more immunological tests in this kind of study, especially tests for non-specific and cell-mediated immunity, which are probably of greater importance in fish. In addition, dietary treatments may affect overall blood volume or general protein (including immunoglobulin) synthesis, which could have a distorting effect on results (Landolt, 1989).

The dynamics of recovery of *Y. ruckeri* from infected fish were very different depending on the route of delivery of the pathogen. Fish infected by immersion presented a more gradual pattern of bacterial recovery, supporting growth in the lower intestine for a longer time period (up to 7 weeks), in contrast with the quicker clearance occurring in injected trout. The kidney,

by contrast, supported a similar amount of growth (score value) during the first 3-4 weeks, with all fish harbouring the pathogen. These differences between the infectivity models show the importance of the experimental design in providing information about host-pathogen relationships and it should be given more consideration when direct comparison with events occurring in field studies are under investigation. The results obtained in this work are comparable with those described by Busch and Lingg (1975) although when the bacterial dynamics are studied, the relative recoveries of the pathogen from the intestine and kidney are fundamentally different. This is not surprising, since those authors worked with a different model based on the establishment of the carrier state and therefore, their challenge doses were lower. They also used a non-selective bacteriological medium to examine their intestinal samples.

The influence of the amount of vitamin E on bacterial recovery does not seem to be of much importance, when the i.p. challenge results are considered, whereas fish infected by immersion seem to be able to support more growth of the pathogen when they have been fed with the highest dose of vitamin E.

TABLE 6.1: CONCENTRATION OF VITAMIN E IN LIVERS FROM RAINBOW TROUT ON DIFFERENT DIETS

	VITAMIN E (mg/Kg) IN PREPARED DIETS.			VITAMIN E (mg/Kg) IN COMMERCIAL DIETS.		
WEEKS AFTER START FEEDING.	7	86	806	81	123	158
WEEK 6	26.8±3.4 (n = 10)	75.3±5.5 (n = 10)	558±84 (n = 10)	–	123±15 (n = 10)	–
WEEK 9	9.5±1.4 (n = 10)	407±126 (n = 10)	411±171 (n = 10)	96.3±10.6 (n = 10)	101.5±9.6 (n = 10)	100.1±12 (n = 10)
WEEK 14	2.4±0.2 (n = 10)	64.1±7.7 (n = 10)	1289±94 (n = 10)	58.7±6.7 (n = 10)	222±25 (n = 10)	96.9±11.5 (n = 10)
WEEK 18	4.5±0.4 (n = 10)	81.3±7.3 (n = 10)	1717±157 (n = 10)	98.3±24.5 (n = 10)	176±31 (n = 10)	73.7±8 (n = 10)
WEEK 19	36±0.4 (n = 10)	97.6±12.8 (n = 10)	1321±14.2 (n = 10)	108.5±15.4 (n = 10)	238±25 (n = 10)	122.1±15 (n = 10)
WEEK 21	3.2±0.8 (n = 10)	92.9±15.6 (n = 10)	1993±258 (n = 10)			132±24 (n = 10)
WEEK 23	4.6±1.0 (n = 12)	40.5±6.7 (n = 12)	1304±131 (n = 12)			95±12 (n = 12)
WEEK 24	4.3±0.3 (n = 18)	28.3±2.1 (n = 18)	1279±221 (n = 18)			80±16 (n = 18)
WEEK 25	3.6±0.3 (n = 18)	34.6±2.9 (n = 18)	1593±132 (n = 18)			86±8.6 (n = 18)
WEEK 28	3.1±0.21	58.5±5.61	1323±140			91.8±8.05
WEEK 33	9.2±1.2	82.9±91	2285±223			97.4±9.2

Values are mean +/- S.E.μg vitamin E /g liver. ()= number of fish.

Fish moved to farm week 12, back to laboratory week 20 and infected by immersion week 22.

TABLE 6.2: ANALYSIS OF RAINBOW TROUT BLOOD AND TISSUES AFTER TWENTY-EIGHT WEEKS ON DIFFERENT DIETARY LEVELS OF VITAMIN E

Diet	Haematocrit	Erythrocyte Fragility	Plasma Pyruvate Kinase	Liver Vitamin E	Liver Vitamin C
	%	% Haemolysis	μ moles NADH/min /min plasma	μ E/g wet weight liver	μ C/g wet weight liver
7.2 mg E/kg diet	19.4 ± 1.86[a]	25.9 ± 3.30[a]	11.62 ± 2.94[a]	3.1 ± 0.21[a]	108.7 ± 9.83[a]
86.4 mg E/kg diet	34.6 ± 1.69[b]	9.8 ± 0.50[b]	0.94 ± 0.12[b]	58.5 ± 5.61[b]	129.3 ± 8.25[a]
805.7 mg E/kg diet	32.6 ± 1.56[b]	8.2 ± 0.31[b]	0.94 ± 0.19[b]	1323 ± 140[c]	60.5 ± 3.03[b]
80.9 mg E/kg diet*	37.5 ± 0.99[b]	6.3 ± 0.99[b]	1.20 ± 0.19[b]	91.8 ± 8.05[d]	11.2 ± 0.71[c]

Values represent mean ± S.E. of 15 fish

Values in the same column with different superscripts were significantly different $p<0.01$

* Commercial diet

TABLE 6.3: CONCENTRATION OF VITAMIN E IN LIVERS FROM RAINBOW TROUT ON DIFFERENT DIETS VALUES AT WEEK 33.				
EXPERIMENT	**INFECTION BY INJECTION.**		**INFECTION BY IMMERSION**	
Diets (mg vit E./Kg)	**Negative Controls**	**Infected Fish**	**Negative Controls**	**Infected Fish**
7	--	--	8.9 ± 0.8 (5)	9.2 ± 1.2 (15)
86	134 ± 10.4 (5)	83.6 ± 10.8 (5)	140.0 ± 20.2 (3)	82.9 ± 9.1 (9)
806	2465 ± 141 (5)	2470 ± 422 (5)	2726 ± 481 (5)	2285 ± 223 (15)
81(*)	--	--	116.2 ± 28.2	97 ± 0.2

(*)= Commercial diet.

Values are mean ± S.E. μg Vitamin E g^{-1} liver

( ) = number of fish

Fig. 6.1: Sections of spleen from rainbow trout after 28 weeks on laboratory diets. Perl's Prussion blue.

A: Normal spleen from rainbow trout fed on 86 mg kg^{-1} vitamin E (75x).

B: Normal spleen from rainbow trout fed on 86 mg kg^{-1} vitamin E (300x).

C: Spleen from rainbow trout fed on 7 mg kg^{-1} vitamin E (deficient) (75x). Note the severe pigment accumulation.

D: Spleen from rainbow trout fed on 7 mg kg^{-1} vitamin E (deficient) (300x). Note the increase in pigmented materials denoting haemosiderin and accumulation of pigment.

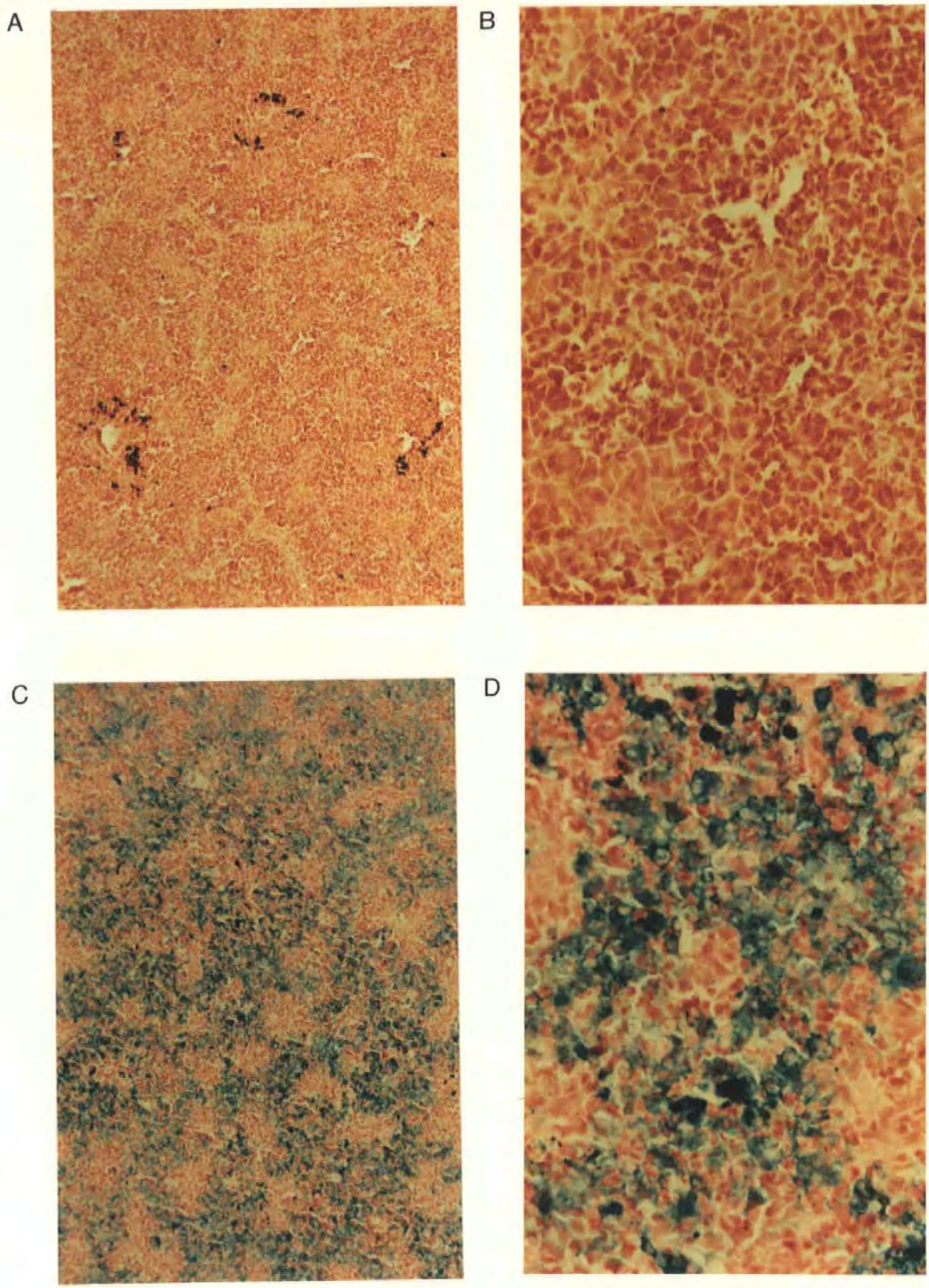
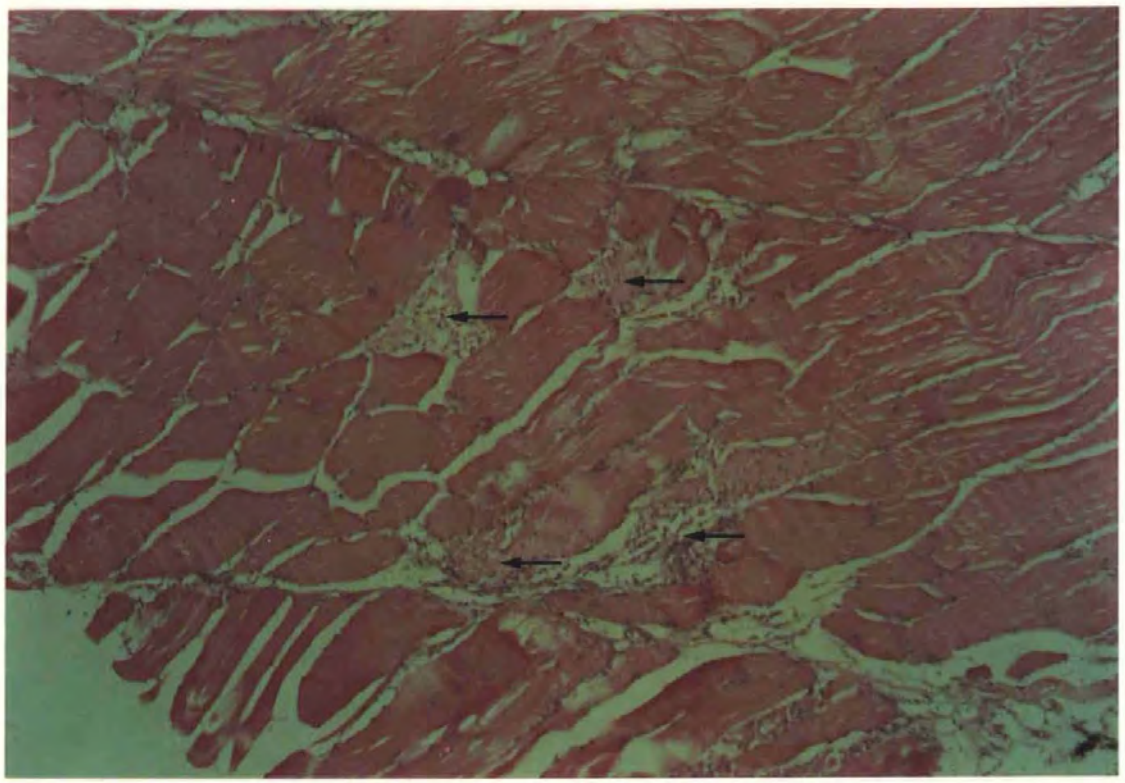

A
B
C
D

Fig. 6.2: Sections of skeletal muscle of rainbow trout after 28 weeks on vitamin E deficient diet (7 mg kg^{-1} diet). H&E.

6.2A: Note the focal areas (arrowed) of fibrilar degeneration and necrosis (75x).

6.2B: Detail (300x) of focal area of necrosis.

A



B

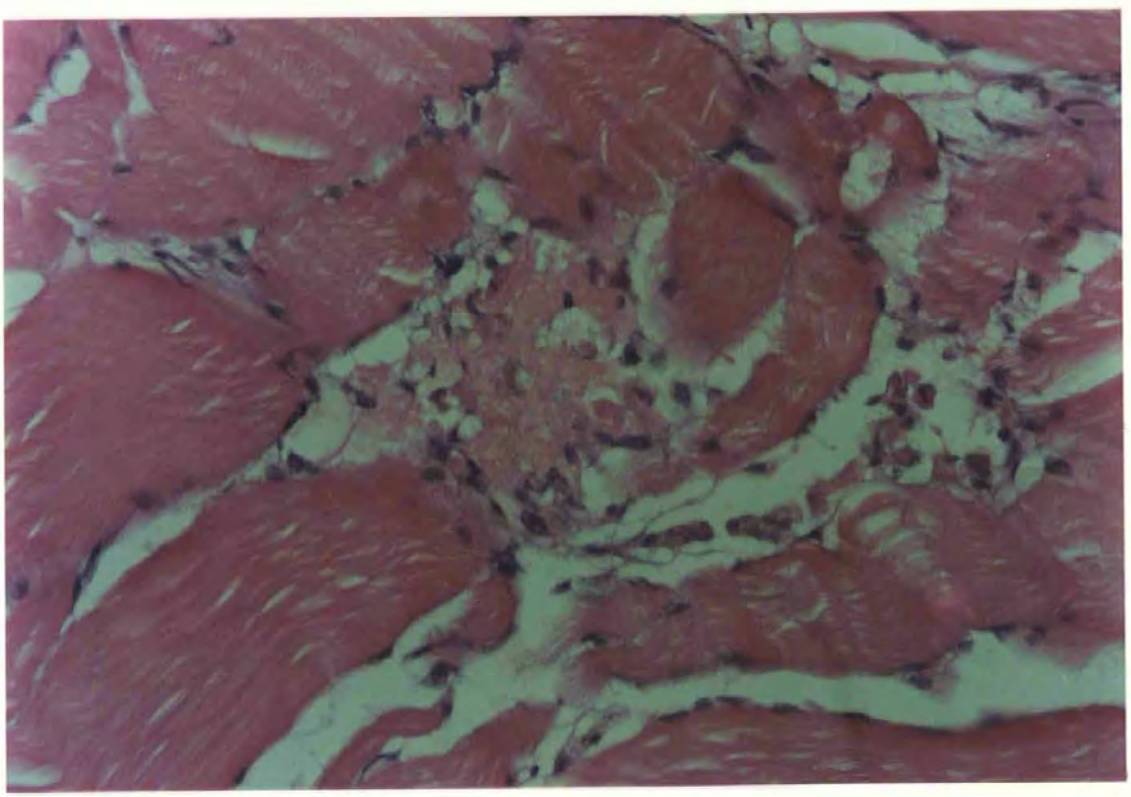


Fig. 6.3: **Liver of a rainbow trout fed on 7 mg kg^{-1} vitamin E (deficient) for 28 weeks (300x). H&E. Note the hepatocyte vacuolation and cytoplasmic inclusions (arrowed), due to the removal of lipid by the embedding technique.**

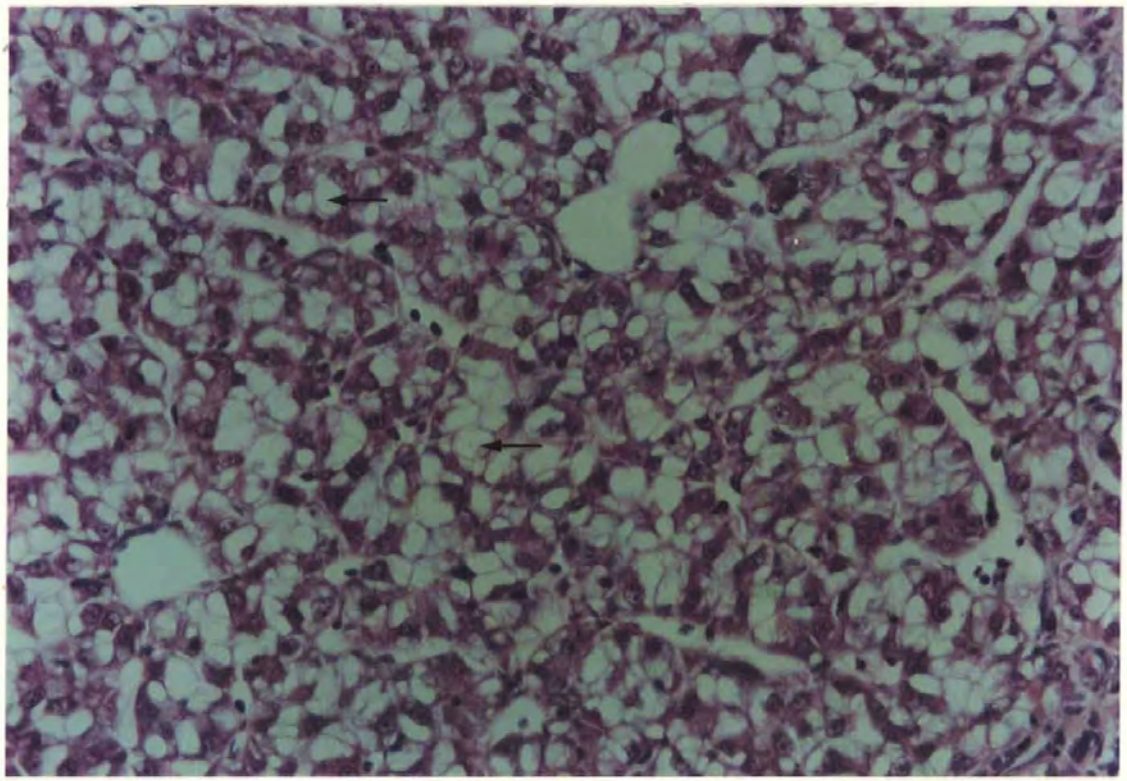


Fig. 6.4: Mortalities following infection by immersion with *Y. ruckeri*, in fish fed different levels of dietary vitamin E

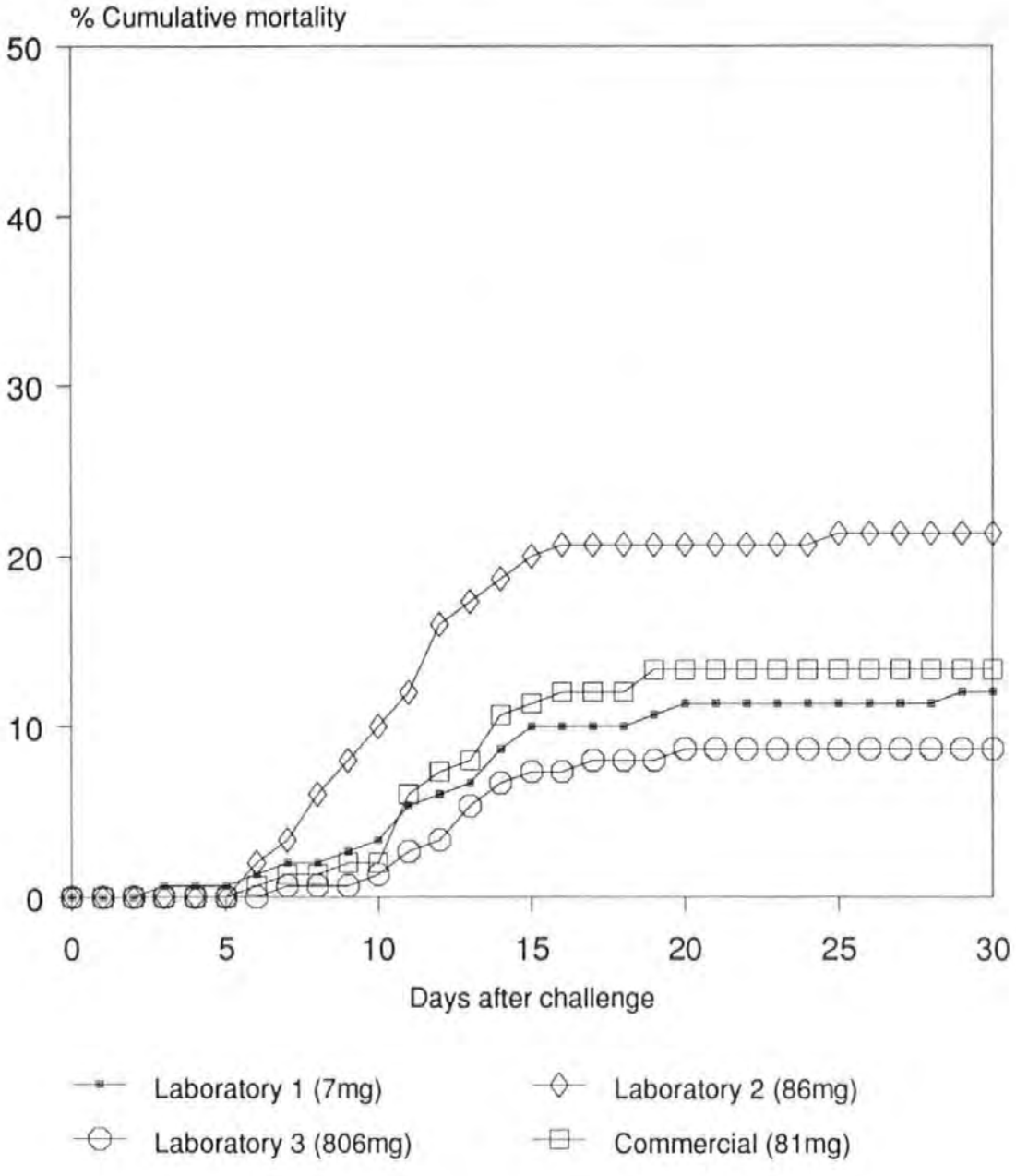


Fig. 6.5: Mortalities following infection by injection with *Y. ruckeri*, in fish fed different levels of dietary vitamin E

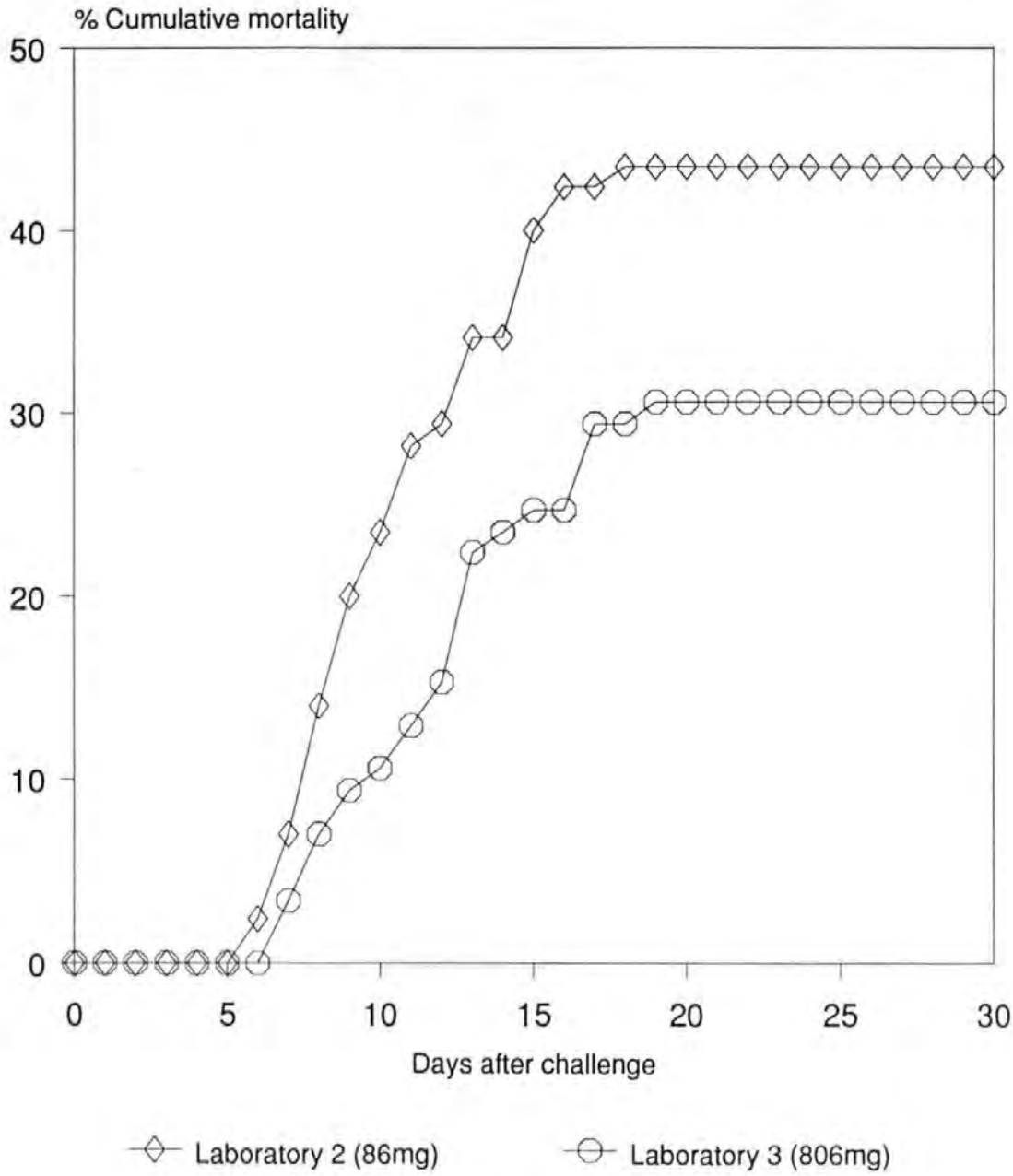


TABLE 6.4: SERUM ANTIBODY LEVELS IN FISH FED WITH DIETARY VITAMIN E, FOLLOWING INFECTION BY IMMERSION

	DIETS (mg vitamin E /Kg)			
WEEK POST INFECTION	**7**	**86**	**806**	**81(#)**
1	0.98±0.32	1.14±0.30	0.78±0.29	1.09±0.19
2	1.22±0.39	1.31±0.27	1.56±0.27	1.19±0.11
3	1.10±0.15	1.20±0.26	1.43±0.23	1.06±0.13
7	1.80±1.01	1.33±0.45	1.10±0.05	1.46±0.29
12	2.85±1.15	2.71±0.71	1.76±0.24	1.61±0.41

(#) = Commercial diet.

Data obtained as : Sample value (mean) / Negative control value (mean).

(Samples are considered positives when the value of the ratio is equal to or higher than two).

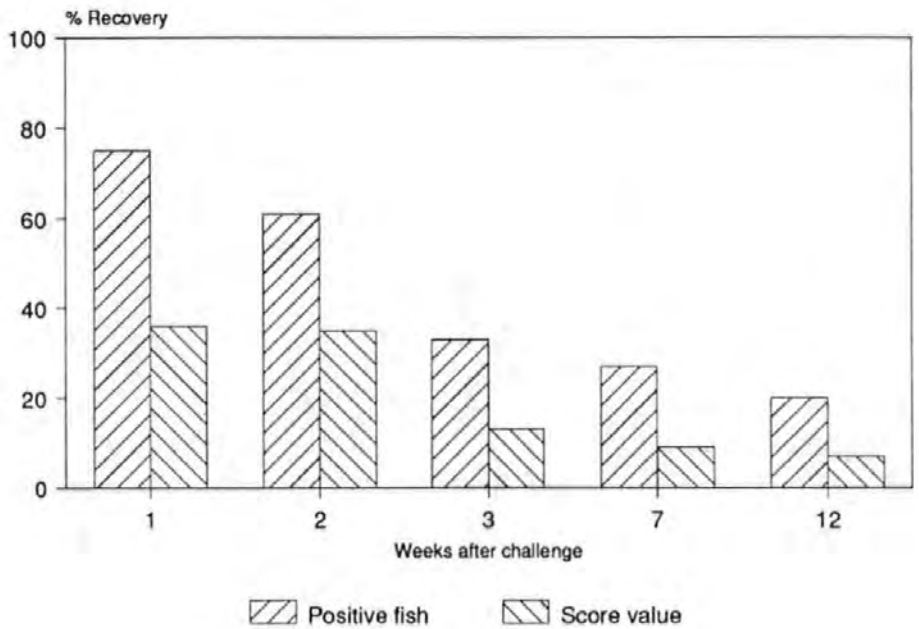
TABLE 6.5: SERUM ANTIBODY LEVELS IN FISH FED WITH DIETARY VITAMIN E, FOLLOWING INFECTION BY INJECTION.

	DIETS (mg vitamin E/Kg)	
WEEK POST INFECTION.	**86**	**806**
1	1.22	1.29
3	1.67	1.98
4	1.48	1.92
15	1.98	3.17

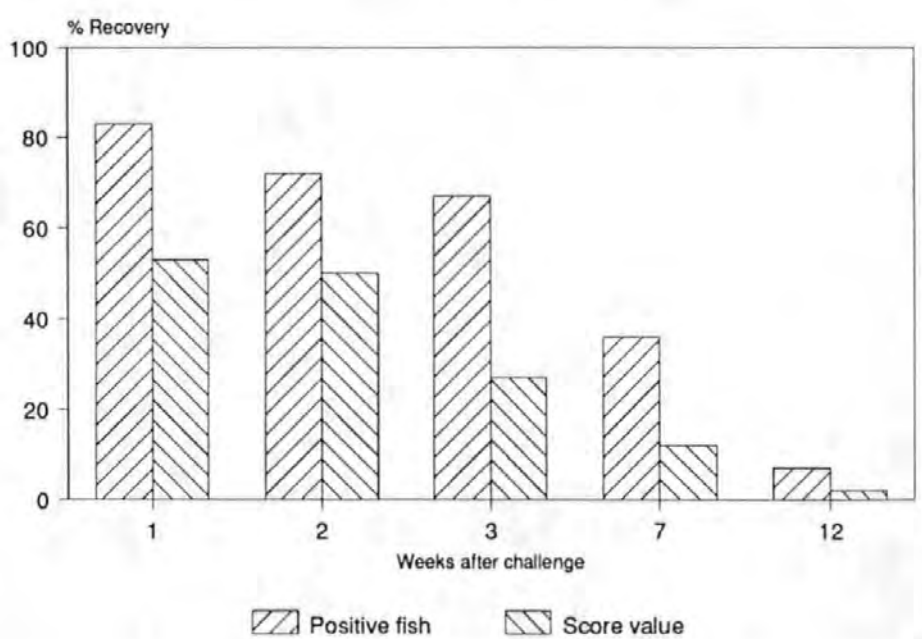
Data obtained as : Sample value / Negative control value .
(Samples are considered positives when the value of the ratio is equal to or higher than two).

Fig. 6.6a: Recovery of *Y. ruckeri* from the kidney of fish infected by immersion. Effect of dietary vitamin E levels.

Laboratory diet 1 (7 mg)

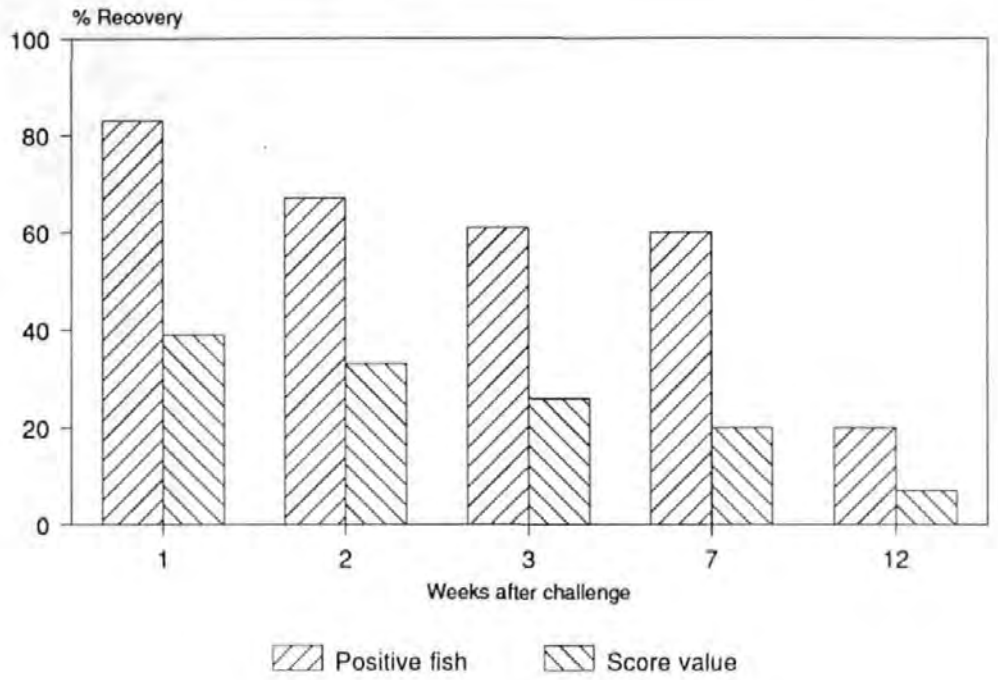


Laboratory diet 2 (86 mg)



Recovery of *Y. ruckeri* from the kidney of fish infected by immersion
Effect of dietary vitamin E levels.

Laboratory diet 3 (806 mg)



Commercial diet (81 mg)

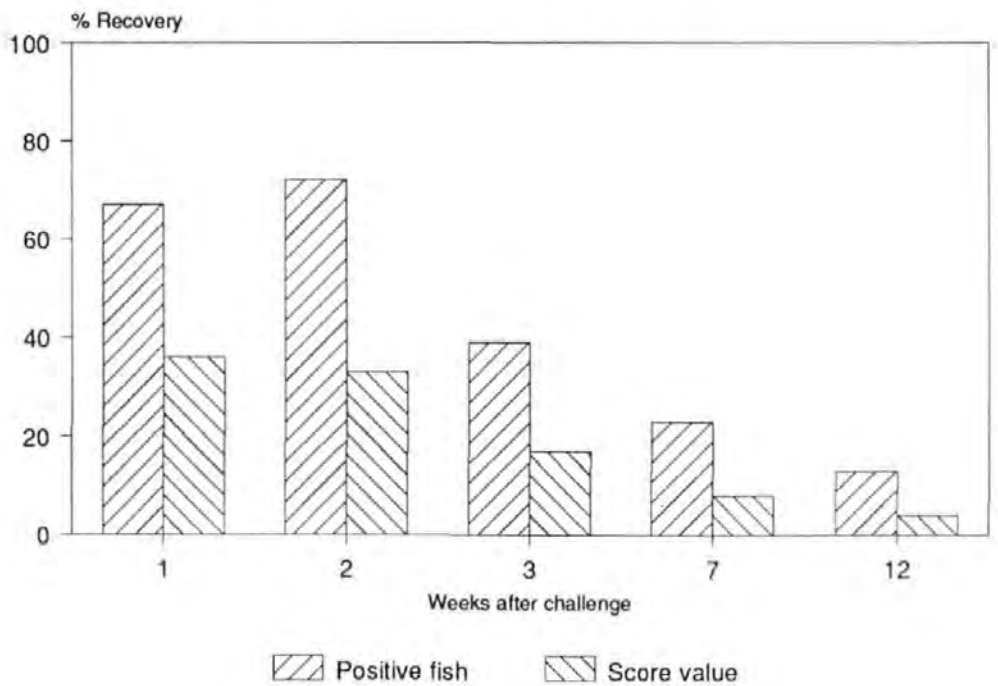
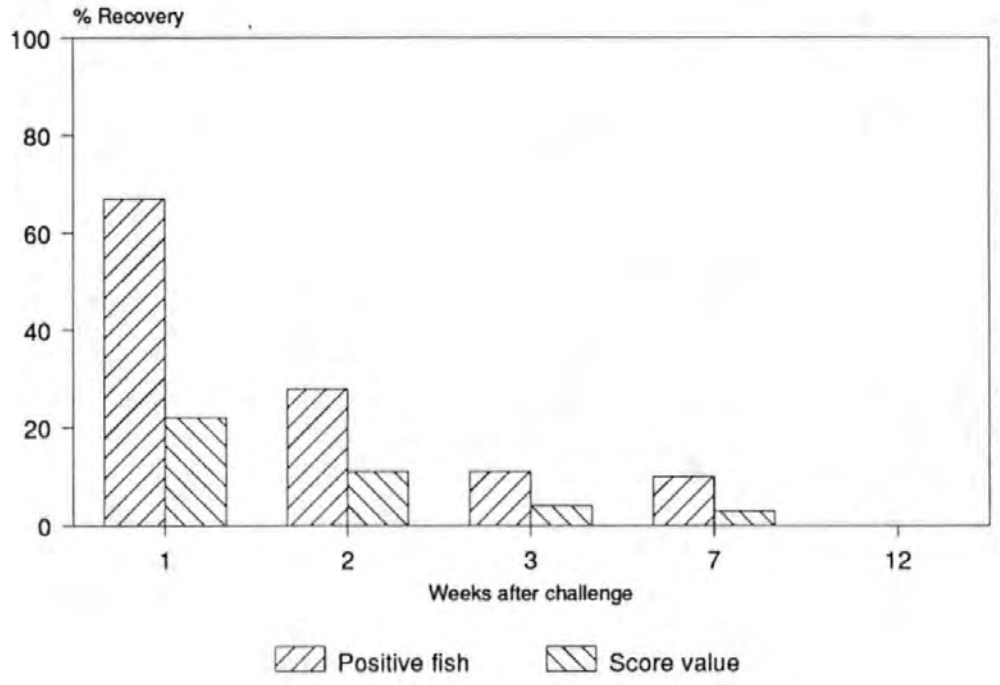
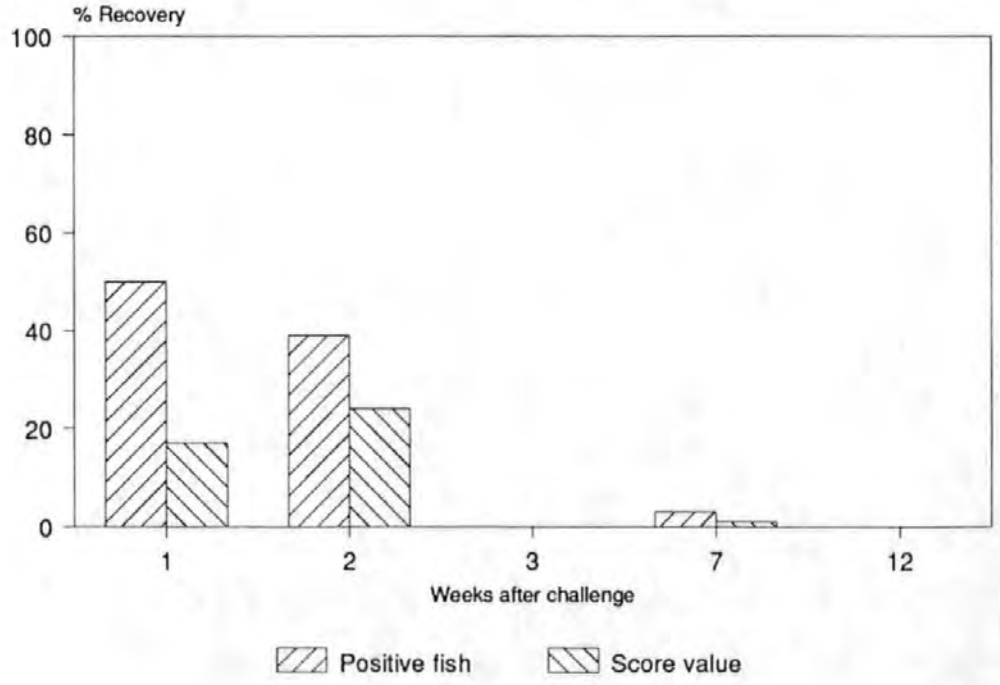


Fig. 6.6b: Recovery of *Y. ruckeri* from the faeces of fish infected by immersion. Effect of dietary vitamin E levels.

Laboratory diet 1 (7 mg)

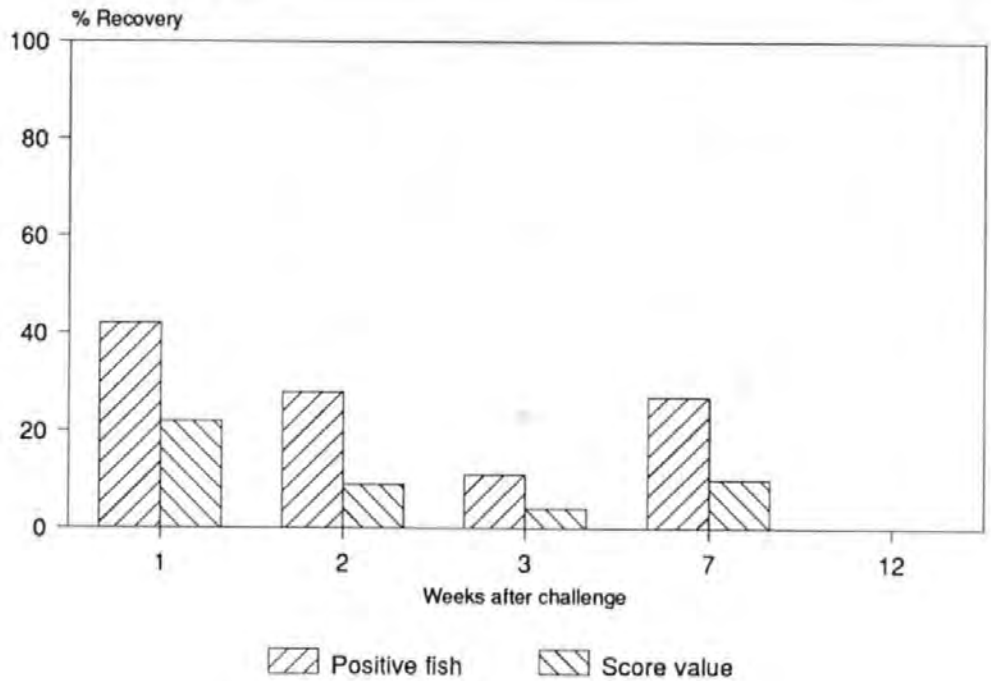


Laboratory diet 2 (86 mg)



Recovery of *Y. ruckeri* from the faeces of fish infected by immersion
Effect of dietary vitamin E levels.

Laboratory diet 3 (806 mg)



Commercial diet (81 mg)

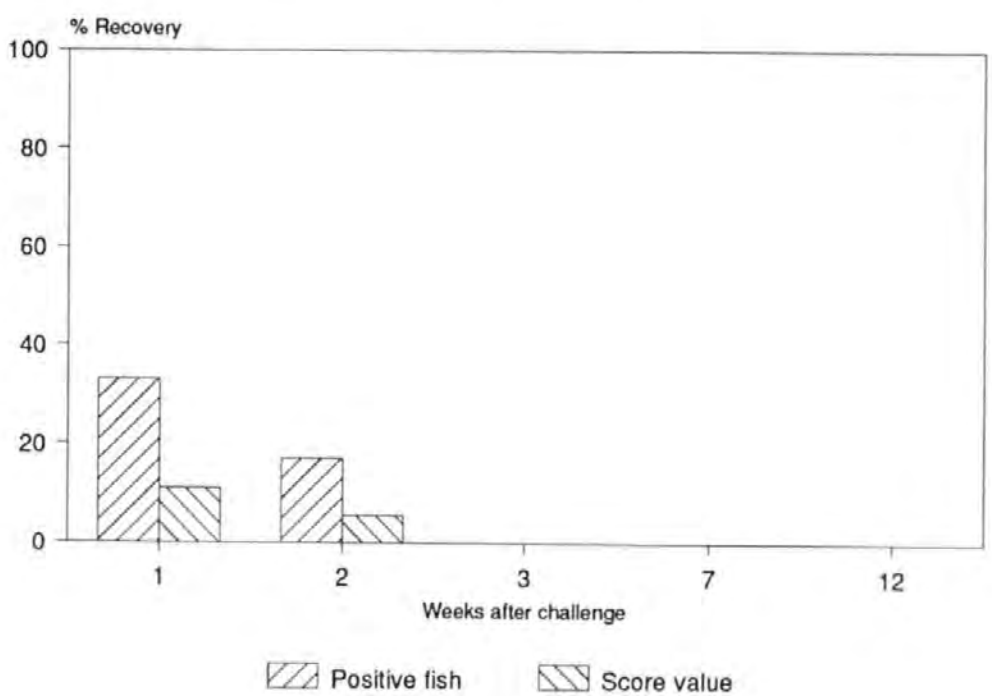
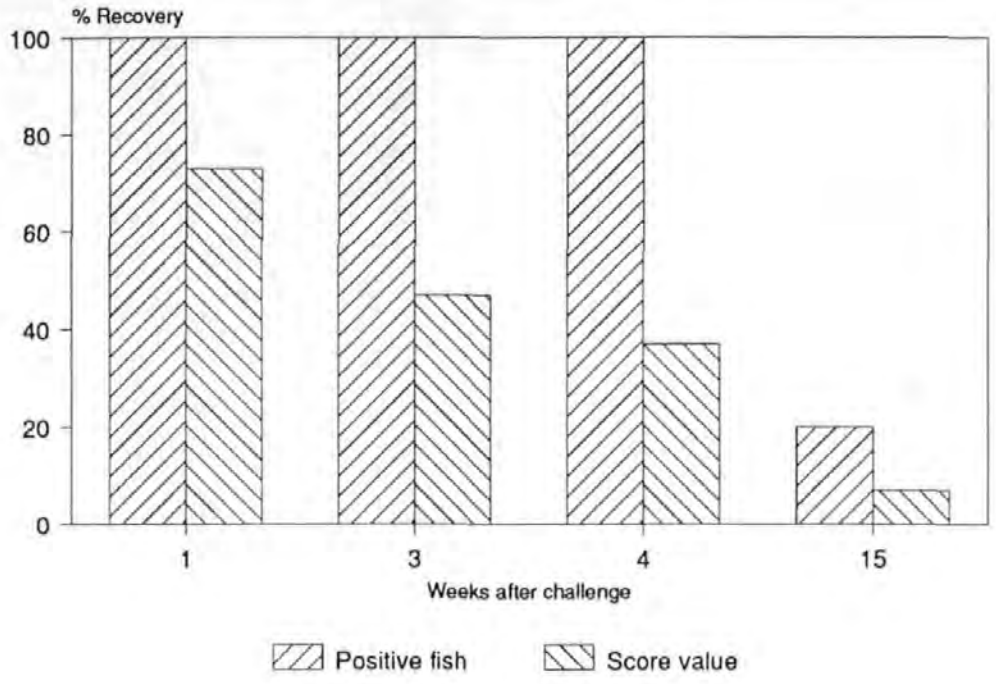


Fig. 6.7: Recovery of *Y. ruckeri* from kidney of fish i.p. injected. Effect of dietary vitamin E levels.

Laboratory diet 2 (86 mg)



Laboratory diet 3 (806 mg)

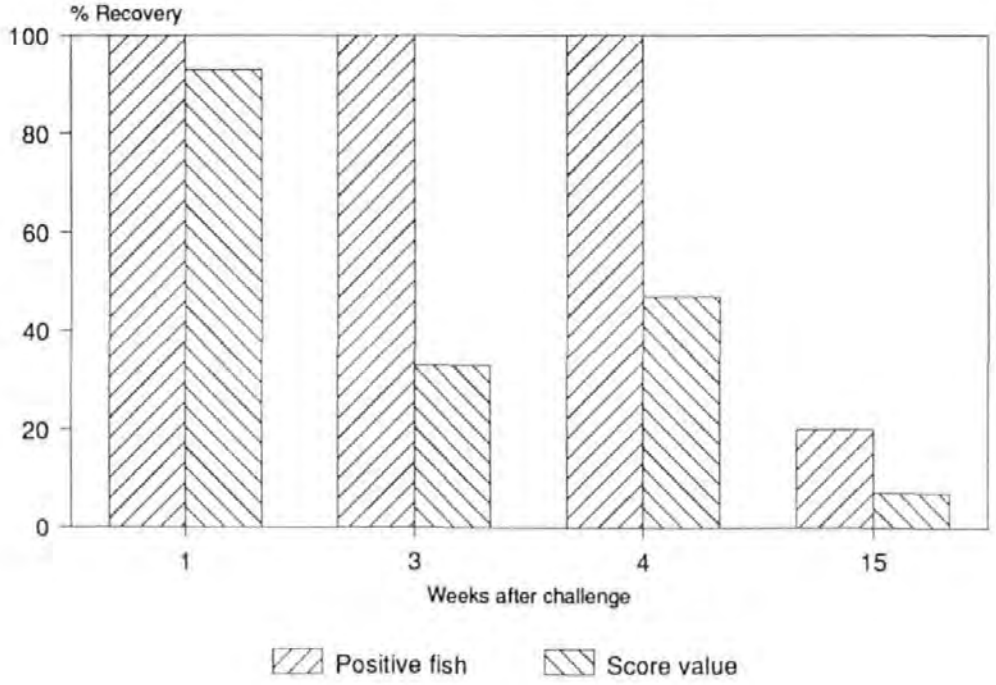
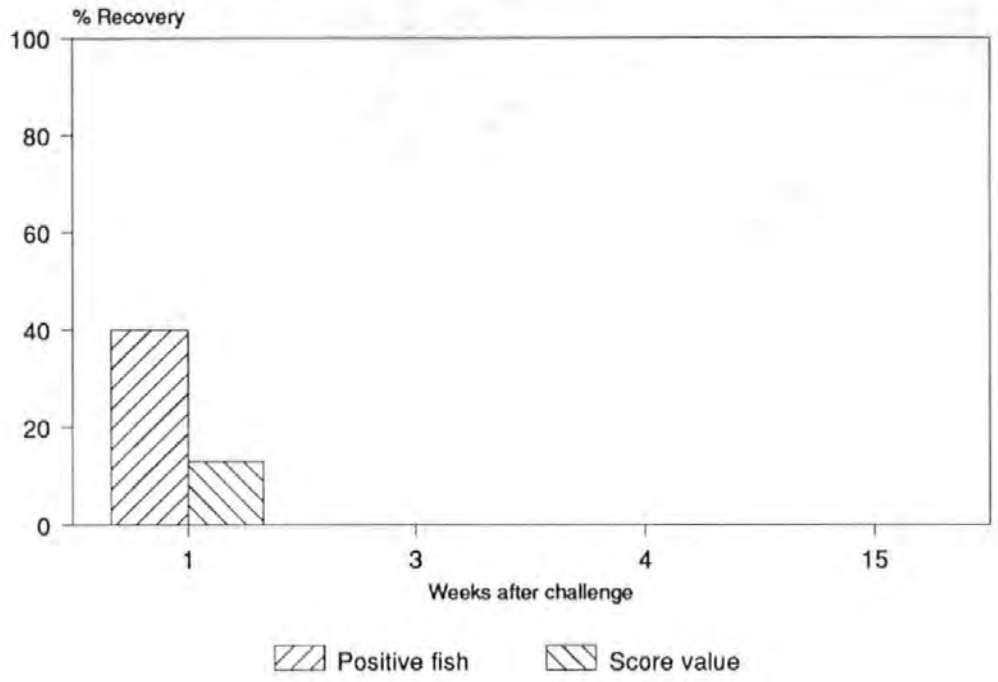
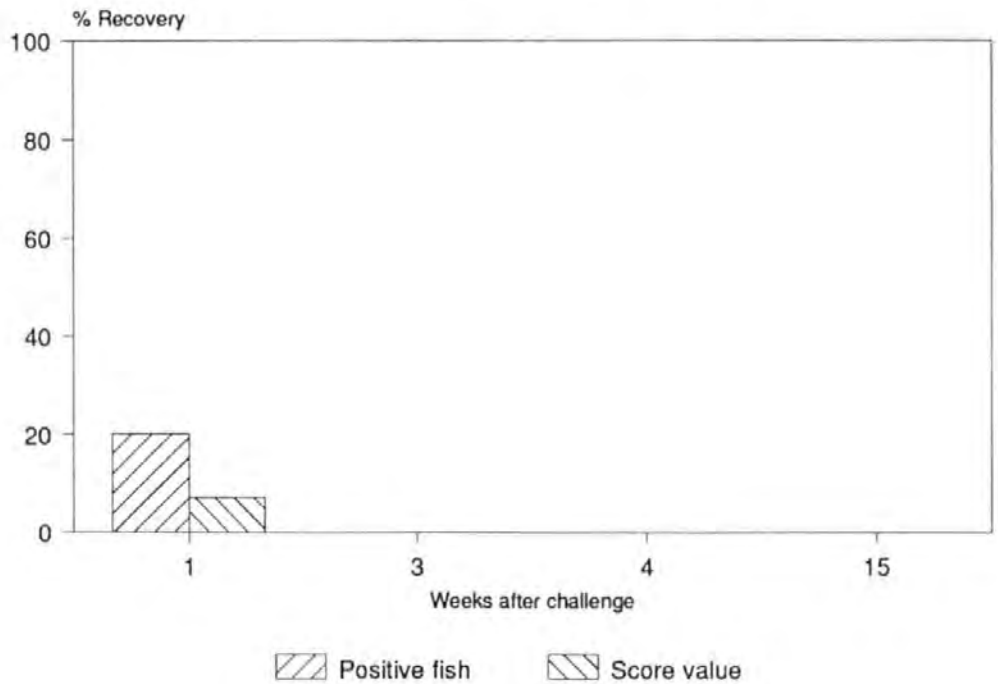


Fig. 6.8: Recovery of *Y. ruckeri* from faeces of fish i.p. injected Effect of dietary vitamin E levels.

Laboratory diet 2 (86 mg)



Laboratory diet 3 (806 mg)



CHAPTER 7

INVESTIGATION OF POTENTIAL VIRULENCE FACTORS OF *Y. ruckeri*

7.1 INTRODUCTION

Y. ruckeri has been recognised as a fish pathogen since the 1950's (Ross *et al.*, 1966; Rucker, 1966) and despite the considerable work carried out by various research teams, the virulence mechanisms of this pathogen remain a question mark. Three possible reasons could be involved in the lack of knowledge of *Y. ruckeri* pathogenicity mechanisms. The most significant reason could be the fact that since vaccination against ERM proved successful soon after the disease was described (Klontz and Anderson, 1968), less work has been carried out on the pathogenicity of *Y. ruckeri* compared with other fish pathogens. The second could be due to the fact that many studies carried out with *Y. ruckeri* were made in comparison with other *Yersinia* species, including human pathogens (Romalde *et al.*, 1988; Stave *et al.*, 1987). However, such comparisons can be considered invalid because *Y. ruckeri* is almost certainly unrelated to these species (Bercovier and Mollaret, 1984) (Section 4.1).

Plasmid involvement in virulence has been suggested (Cook and Gemski, 1982; Stave *et al.*, 1987) since the description by Cook and Gemski (1982) of the presence of a 70Mdal plasmid in the serotype I strain of *Y. ruckeri*.

Similar size plasmids are also present in other representatives of the genus *Yersinia* (Portnoy and Falkow, 1981) and a clear correlation between plasmid presence and virulence has been reported in these species. It is known today that despite the similarity in size of the plasmid present in *Y. ruckeri* serotype I strains, and that carried by other *Yersinia* enteropathogens, DNA-homology work has proved that both plasmids, are not related (Guilvout *et al.*, 1988).

Finally, most work performed with *Y. ruckeri* has been done with the assumption that representative strains of serotype O1 were virulent for rainbow trout, without performing *in vivo* work in parallel to prove it. In the present work, an *in vivo* challenge study was initially carried out in order to standardize parenteral and immersion infections under laboratory conditions (Chapter 5). Such standardized challenge was used to assess the virulence levels of various *Y. ruckeri* strains. As a result of this work a hitherto unidentified virulence factor of *Y. ruckeri* was found.

7.2 RESULTS

7.2.1 Comparative study of cell components of *Y. ruckeri* passaged and non-passaged strains

On the basis that sequential *in vivo* passage of *Y. ruckeri* strain 5 in rainbow trout led to increased virulence of the pathogen (strain 26), a comparative study between both isolates was performed. Whole cell lysates, LPS,

periplasmic proteins and extracellular products (ECP) of both strains were subjected to SDS-PAGE. Chromosomal and plasmid DNA were extracted and examined on agarose gels. A series of cell monolayers were used to perform cytotoxicity assays with live cells and their ECP's.

7.2.1.1 Electrophoretic studies of cell extracts

Whole cell profiles of *Y. ruckeri* strains 5 and 26 were compared after growth on agar media (48 h, 25°C, BHIA) and in liquid media (18 h, 25°C, BHIB). Some differences were observed in the protein profiles in the range of 45 and 29 kD (Fig. 7.1). Such differences corresponded with variations in culture conditions, and they occurred in both strains 5 and 26. Cells of strains 5 and 26, grown under similar conditions including shaken and static broth cultures, showed profile variations depending also on the way that cells had been produced (Fig. 7.2) but not on passage *in vivo*. The effect of temperature (15°C and 23°C) on whole cell profiles was also studied. The influence of laboratory media on the synthesis of proteins produced only *in vitro* was avoided by reisolation of *Y. ruckeri* strain 26 from the kidney of a clinically infected moribund fish onto agar media produced from an extract of the same kidney material (section 3.2.3.4). This isolate was named strain 26-F1. When cells of *Y. ruckeri* strains 5, 26 and 26-F1, grown at 15 and 23°C (only 15°C for strain 26-F1), were compared on SDS-PAGE, similar patterns were presented, independent of the temperature of growth, the passage status or the growth media used (Fig. 7.3). Isolate 26-F1 was harvested in very small quantities, and although its sample was more dilute than those of strain 5 and

26, no obvious extra bands appeared in its lane, nor were any of the main bands present in the other isolates missing. Kidney extracts from a non-infected fish, were run simultaneously as a protein contamination control of 26-F1 cells. The profile of periplasmic proteins of strains 5 and 26 was also similar, as shown in lanes 6 and 8 of Figure 7.4.

The effect of temperature of growth (15 and 23°C) and passage (strains 5 and 26) on LPS profiles was also studied. Figure 7.5 shows the LPS patterns obtained after proteinase-K digestion of the cells. Typical ladders of a smooth LPS appeared in all samples, showing no obvious differences due to passage *in vivo* of the cells. A more dense core region was present in the samples grown at 23°C, although a cell concentration effect could account for this.

When chromosomal and plasmid DNA obtained from *Y. ruckeri* strains 5 and 26 by caesium chloride-ethidium bromide equilibrium ultra-centrifugation were compared (section 3.4.8), no differences in the number of bands obtained from both, or in their migration on agarose gels, were observed (not shown).

Culture supernatants of *Y. ruckeri* strain 26 both neat, and concentrated with ammonium sulphate (65%) were analysed on SDS-PAGE. No bands of proteins were detected either in Coomassie blue or silver stained gels.

Concentration of the extracellular products (ECP) of strain 26, by the cellophane overlay technique, (section 3.4.3.7) was performed. Duplicate samples of the ECP were subjected to SDS-PAGE, followed by silver staining and immunoblotting using rabbit antiserum raised against strain 26. No bands were detected in the concentrated supernatant fraction of either the acrylamide gel or Western blot. Strain 26 was also grown under calcium limiting conditions (section 3.4.3.7) and supernatants were concentrated by precipitation with ammonium sulphate (40%) and run on SDS-PAGE. As in previous experiments, no protein bands were detected after Coomassie blue staining, independent of the concentration of calcium in the media.

7.2.1.2 Possible cytopathic effects (CPE) of *Y. ruckeri*

Cells of strains 5 and 26 and their culture supernatants were studied on a series of fish and mammalian cell monolayers (section 3.4.1). No cytopathic effects were observed in any of the combinations tested in either mammalian or fish cell lines.

7.2.2 Comparative Study of the Virulence of *Y. ruckeri* Serotype O1 Strains

Since the comparative study carried out with the passaged, increased virulence isolate 26 and its parental strain 5 did not lead to any conclusive evidence about the virulence mechanism/s of *Y. ruckeri* in rainbow trout, an alternative approach to investigate the bacterium's pathogenic factors was undertaken. Other representatives of the serotype O1 strains were brought into the study and both their *in vivo* effect in rainbow trout and their main

cell components were studied.

7.2.2.1 *In vivo* study of *Y. ruckeri* serotype O1 strains

A series of sequential passages through fish as described in section 3.3.3.3 were performed using *Y. ruckeri* strain 19. Very few mortalities occurred (1/5) even when extremely high doses of bacteria were injected i.p. (10^9 c.f.u. per fish). After four serial passages through fish, an experiment to compare the *Y. ruckeri* pair of strains 5 and 26 and the new pair 19 and 19-P was performed by injecting (i.p.) different groups of 5 fish (39 g average weight) with a series of 10-fold dilutions (from 10^7 to 10^4 c.f.u. per fish) of each of the four above mentioned strains. While only one fish survived from those injected with strains 5 and 26, no fish died after injection of either strain 19 or 19-P. Also, very few bacteria could be reisolated from the kidney of those survivor fish, and none from their faeces. In similar experiments using *Y. ruckeri*, strains 5, 7, 26, 30 and 48 which were injected i.p. into groups of 5 rainbow trout, the results shown in Table 7.1 were obtained.

Y. ruckeri strains 7 and 19 were tested 3 times in rainbow trout in parallel with strain 26 and similar results were obtained. *Y. ruckeri* strain 26 was reisolated from the kidney of all fish surviving injection whereas in fish injected with avirulent strains 7 and 19, recovery was lower (0 to 60%) but this was dose dependent. No positive isolations from faeces occurred.

Antisera against *Y. ruckeri* strains 7, 19 and 26 were raised in rainbow trout

using approximately 10^9 formalin killed whole cells. Antiserum titres against their homologous strains were determined four weeks after immunization. Titres of antiserum obtained against strain 26 were lower (1/16) than those against strains 7 and 19 (1/256).

7.2.2.2 Comparison of cell components of virulent and avirulent *Y. ruckeri* serotype O1 strains

Soluble whole cell proteins, LPS and periplasmic protein from strains tested "*in vivo*" were compared using SDS-PAGE. LPS of strains 19 and 26 were compared (Fig. 7.6) and no obvious differences between the two proteinase K digests (LPS) could be observed. Periplasmic proteins of *Y. ruckeri* strains 5, 7, 19, 26 and 48 also produced similar profiles (Fig. 7.4). Whole cell lysates of virulent (5, 26, 30, 48) and avirulent strains (7, 19) were compared (Fig. 7.7). Although some differences amongst strains could be observed (shown by arrows) such differences did not apparently correlate with the virulence of the strains, since *Y. ruckeri* strain 30 (virulent) differed both from strains 26 (virulent) and 7 (avirulent).

Western blotting was used to compare heated (100°C, 5 min) and unheated cell extracts of strains 5, 7 and 48. The blot of unheated extracts of strains 5 and 48, showed the presence of an unusual band, apparently unrecognised by the antiserum, in a position occupied by proteins of a molecular weight of approximately 120 kDa. Such a band was absent in the extract of strain 7 (Fig. 7.8).

When SDS-PAGE gels of unheated preparations were examined (before staining) an opaque band appeared in the tracts of strains 5 and 48 but not in 7 (Fig. 7.9). The band was not observed in samples which had been heated, and was therefore termed heat-sensitive factor (HSF).

7.2.2.3 Comparative study of HSF^+ and HSF^- serotype O1 strains

Unstained SDS-PAGE gels of cell extracts of a further 23 strains were examined to screen for presence of the HSF. Results (Table 7.2) show that all but 5 strains tested possessed HSF. In order to determine if there was a correlation between the possession of HSF and virulence in *Y. ruckeri* serotype O1, seven strains from different geographical origins were selected to be tested for virulence *in vivo*. Four strains were HSF^+ (2, 5, 9, 35) and three HSF^- (19, 22 and 39). Strains 5 and 19 were used as controls, since from previous work it was known that they were virulent and avirulent respectively. Both i.p. and immersion infection were performed. For i.p. infection, groups of 5 fish (5g average weight) were injected with 0.1ml of 10 and 100-fold dilutions of each strain suspension (approximately 1×10^9 c.f.u. ml^{-1}). For bath challenge, duplicate groups of 10 fish (12 g average weight) were exposed for one hour to water containing approximately 3.6×10^7 c.f.u. ml^{-1}.

The mortalities after 2 weeks for the i.p. injection challenge and after 4 weeks for the bath challenge are presented in Table 7.3. All fish injected with HSF^+ strains died after inoculation with either 10^6 or 10^7 c.f.u. per fish,

between the third and seventh days post-challenge, whereas all fish challenged with HSF$^-$strains survived. When fish were challenged by bath immersion, mortalities were again only observed in fish infected with HSF$^+$ strains, and no mortalities were recorded within one month after exposure to HSF$^-$ strains. Since all fish injected with HSF$^+$ strains died, no comparative study of the recovery of the pathogen with HSF$^-$ injected fish could be carried out; therefore, such comparison could only be done with data from bath challenge fish. Kidneys and faeces from the survivor fish from each tank were sampled for *Y. ruckeri*. *Y. ruckeri* was isolated from the kidney of fish infected with any of the HSF$^+$ strains, whereas only one fish among all those infected with HSF$^-$ strains had detectable bacteria in the kidney. The pathogen was detected in the faeces of fish from three of the four infected with HSF$^+$ strains. No detection occurred in any case of the groups infected with HSF$^-$ *Y. ruckeri*. Results are shown in Table 7.4. The possible correlation of HSF with the presence of another cell component/s, was studied by resolving cell sonicated supernatants on SDS-PAGE. An example of the obtained results is presented in Fig. 7.10. The profile of heated (5 min, 100°C) and unheated sonicated supernatants of two HSF$^+$ strains (2 and 5) and two HSF$^-$ (19 and 39) are shown. There were interstrain differences (arrowed), although no obvious correlation with the HSF character could be ascribed to them.

7.2.3. Characterization of the heat sensitive factor (HSF)

7.2.3.1 Staining of HSF present on SDS-PAGE

Having demonstrated the apparent importance of HSF in virulence of *Y.*

ruckeri, attempts weere made to develop methods for the detection of this factor, and to determine its nature, properties and location in the bacterial cell.

Unheated sonicated supernatants of HSF$^+$ *Y. ruckeri* strain 5 were resolved on 12% SDS-PAGE. Staining procedures used are listed in section 3.4.5. Sequential procedures are presented as a flow diagram in Fig. 7.11, examples of HSF band on SDS-PAGE are shown in Fig. 7.12 and a summary of the obtained results is given in Table 7.5. When similar samples to those resolved on SDS-PAGE, were resolved on a 12% native gel, only phosphine 3R gave positive results (very faint blue line in the HSF region). All of the other stains checked (sudan black B (1), neutral red and alcian blue) were negative. HSF could only be visualized in native-PAGE after immersion of the gels in a 10% SDS solution, and appeared as white bands, similar to those seen on SDS-PAGE. Interestingly, it was also observed that HSF in a native gel could also be stained with sudan black after exposure of the gels to 10% SDS solutions.

7.2.3.2 Differential staining of whole bacteria

The stains described in Section 3.2.7 were tested on bacterial smears of strains 5, 9 (HSF$^+$) and 19 (HSF$^-$), fixed with buffered formalin-acetone. None of the stains used could differentiate between HSF$^+$ and HSF$^-$ strains in simple smears.

7.2.3.3 Differentiation of HSF^+ and HSF^- strains using dye-supplemented media

Attempts were made to develop a bacteriological medium which could differentiate between HSF^+ and HSF^- strains. This was considered very useful from various viewpoints: **(1)** it would increase the screening capacity, when a large number of strains are tested **(2)** it could provide rapid valuable information in epidemiological studies **(3)** it could also be used in experiments conducted to find an isogenic pair of strains differing only in the HSF character.

Differential adsorption of sudan black, Coomassie blue, congo red, crystal violet and alcian blue by *Y. ruckeri* strains 2, 5, 9, 35 (HSF^+) and 19, 22, 39 (HSF^-) was investigated. Preliminary work was performed using dyes at a concentration of 100 μg/ml and incubating at 25°C. Sudan black and alcian blue presented solubility problems and had to be withdrawn from the test. Crystal violet was apparently adsorbed equally by HSF^+ and HSF^- *Y. ruckeri*, and since bacterial growth also decreased with the increase of dye concentration, no further work was undertaken with this dye. Coomassie blue gave promising preliminary results, showing a differential uptake of both dyes by the HSF^+ and HSF^- strains. Strains 2, 5, 9 and 35 (HSF^+) grew normally, and produced blue colonies with a light centre after 24-48 h, although large colonies, present in older plates, had dark blue centres. Strains 19, 22 and 39 (HSF^-) also grew normally on Coomassie plates, but they presented darker blue centred colonies. Further work, using Coomassie blue and congo red

was carried out, using a series of dye concentrations and studying the colony morphology over a range of temperatures. Bacterial strains used were 2, 5, (HSF^+), 19, 39 (HSF^-), which were grown at 10, 15, 20, 25 and 30°C on TSA plates containing 5, 25, 50, 75 and 100 μg/ml of either Coomassie blue or congo red. Growth was good and was not apparently affected by the addition of dyes to the base media.

Colonies of all strains absorbed the dyes in their periphery, but the centre of the colonies remained white in the HSF^+ strains.

The recorded results of the colour intensity (section 3.2.5.3) for all strains at the range of dye concentrations and temperature tested are presented in Tables 7.6 and 7.7. Both Coomassie blue and congo red were taken up in much greater amounts by HSF^- strains, and such uptake seemed to be temperature related. Therefore, with the exception of strain 19 growing at 30°C on Coomassie blue plates, it seemed that the higher the temperature, the more pronounced the differences in colouration between the HSF^+ and HSF^- strains. In further work, strains were grown at 25 and 30°C on TSA plates containing 100 $\mu g\ ml^{-1}$ of either Coomassie blue or congo red.

7.2.3.4 Use of ROD medium for differentiation of HSF^+ and HSF^- colonies

ROD had previously been used as a selective media to isolate *Y. ruckeri* from faecal samples (Chapters 5 and 6). It was noticed that not all strains of *Y.*

ruckeri that grew on ROD produced the characteristic yellow deposits surrounding the bacterial colony due to acid production (C. Rodgers, pers. comm.). When the possession of HSF (determined by SDS-PAGE), in a series of *Y. ruckeri* strains was related to their growth on ROD, it was shown that HSF^- strains did not produce the precipitation ring around the colonies, whereas all HSF^+ strains did. An illustration of such difference is presented in Fig. 7.13. In order to find out which one of the components of ROD was responsible for the differentiation, five modifications of the original composition of ROD media were performed as presented in Table 3.2. Five HSF^+ strains and three HSF^- strains were grown on each of these modifications. Results (Table 7.8) showed that the medium component responsible for precipitate formation was SDS. On the other hand, growth of HSF^- strains seemed to be reduced by SDS. Colonies of HSF^- strains growing in the presence of SDS became transparent after a few days of growth. Such results indicated that ROD media could be used as a selective and differential media for HSF^+ strains. Bacterial growth on the ROD formulation was quite slow, with 10 days at 25-30°C being required to ensure that all positive colonies had produced the precipitation ring. Therefore, a simpler medium which would allow quicker bacterial growth was desirable. The formation of such a medium is described in the following section.

7.2.3.5 Screening for HSF^+ colonies using TSA-SDS plates

The effect of SDS, added to TSA, was studied for its use in screening for HSF^+ colonies. SDS was added to TSA in increasing concentrations (1% to

5%), and 2 HSF^+ strains (2, 26) and 2 HSF^- strains (7, 19) were grown on the plates. SDS did not modify the amount of growth of HSF^+ strains, but it affected growth of HSF^- strains. HSF^+ strains formed white, creamy colonies which were surrounded by a cream deposit on media containing up to a concentration of 3% SDS. At higher concentrations, crystal formation occurred on the agar along with bacterial growth (Fig. 7.14). The amount of growth of HSF^- strains was not affected up to a concentration of 3% SDS, although colonies appeared transparent, probably due to cell lysis produced by SDS (Fig. 7.15). At SDS concentrations higher than 3%, growth was severely affected in comparison with HSF^+ strains and at 4% SDS, colonies were completely transparent (Fig. 7.16). Despite such dramatic effects on both growth and colony appearance, SDS, even at a concentration of 5%, was not completely lethal for HSF^- strains, since subcultures from such plates onto TSA or TSA + SDS, produced growth in all cases. However growth was reduced in comparison to that obtained with HSF^+ strains. Attempts to completely inhibit growth of HSF^- strains, by increasing the concentration of SDS in the media above 5% failed due to an incapacity of the agar plates to set.

Since TSA + SDS (1%) proved to be a very successful, quick (24-48 h, 25°C) and precise system to screen for the HSF character in *Y. ruckeri*, the entire available strain collection was tested on this medium. Results indicated that the majority of the serotype O1 strains could be designated HSF^+ when tested, and that only 15 were HSF^- namely strains 7, 19, 22, 39, 40, 45, 46, 49,

50, 51, 54, 55, 57, 59 and 71. However, some of these isolates were in fact, the same strain but from different donor (see Table 4.1). Consequently, because of duplication, this meant that there were actually only 9 O1 strains that were HSF$^-$. Other strains, representative of other serotypes, were HSF$^-$ with the exception of strain 47, a strain apparently classified as a serotype 06 representative (De Grandis *et al.*, 1988).

7.2.3.6 Screening for possession of HSF by combining Coomassie blue, congo red and SDS

An attempt to improve the screening for HSF was performed by combining the same media TSA plus 1% SDS with Coomassie blue or congo red (100 μg ml^{-1}). Congo red-SDS media proved unusable since the characteristic white-centred colonies of the HSF$^+$ strains did not occur and HSF$^+$ and HSF$^-$ strains could only be differentiated by the presence of a precipitation ring around the colonies (Fig. 7.17). Coomassie blue-SDS media improved the resolution obtained with Coomassie blue alone by producing a more pronounced white centre in the colonies and a precipitation ring around them (Fig. 7.18).

7.2.3.7 Study of the antibiotic sensitivity patterns of HSF$^+$ and HSF$^-$ *Y. ruckeri* strains

The possibility that the HSF character could be associated with antibiotic

resistance was investigated since a difference between the antibiotic sensitivity pattern between HSF^+ and HSF^- strains could be useful in the development of a more selective medium. A preliminary study of the susceptibility of two HSF^+ (2, 26) and two HSF^- (7, 19) strains to a range of 39 antibiotics (section 3.2.6.1) was performed. The four strains had a similar sensitivity to 37 of the antibiotics, but strains 7 and 19 were resistant to 10 μg fusidic acid (FC, Mastring 43), and 1.25 μg trimethoprim (TM, Mastring 41), whereas strains 2 and 26 were sensitive. Subsequently, a larger group of HSF^+ strains (4, 9, 11, 12, 13, 14, 15, 25, 27, 30, 38, 41) and two HSF^- strains (22, 39) as controls, were tested against TM (1.25 μg) and FC (10 μg). All strains tested, except strain 30, were sensitive to TM and, with the exception of 14 and 25, all strains were resistant to FC. Therefore, the HSF character did not seem to be related to sensitivity to either of those antibiotics tested and, consequently, no more work was undertaken in this area.

7.2.3.8 Attempts to obtain an isogenic pair (HSF^+/HSF^-) of mutants

Six different approaches were followed in an attempt to obtain an isogenic pair of mutants of *Y. ruckeri* differing only in the presence of HSF. Mutagenesis of strains, using acridine orange or ultraviolet radiation (UV) (Section 3.2.8) did not produce detectable mutants when treated cells were screened on ROD, TSA-CR, TSA-CB or TSA-SDS media. After UV treatment, a number of different colony morphologies appeared on the TSA-CR and TSA-CB plates. Such differences occurred both in colony size and

colour. Subculturing of abnormal colonies onto ROD showed that they all produced yellow deposits on the agar (i.e. they were HSF^+).

In the same way, attempts to activate possible lysogenic phages (Section 3.2.8.3) from either *Y. ruckeri* strains 5 (HSF^+) or 19 (HSF^-), when treated with UV irradiated supernatants of *Y. ruckeri* strain 5 were unsuccessful. No lytic phages for either HSF^+ or HSF^- *Y. ruckeri* were isolated from the water outflow of a rainbow trout farm, even after phage enrichment.

Attempts to transform an HSF^- strain (19) to an HSF^+ form was performed using both chromosomal and plasmid DNA of *Y. ruckeri* strain 48 (HSF^+). TSA - SDS was used as a selective media for transformants. The experiment was repeated four times, but none of the colonies growing on TSA-SDS media were white and able to produce a precipitate. Further work, to study if there was an incapacity of *Y. ruckeri* HSF^- strains to be transformed, was carried out with six HSF^- strains (7, 19, 39, 40, 47 and 57), which were treated for transformation with DNA from plasmid pUC18, which confers resistance to ampicillin (50 μg/ml). None of the strains acquired the capacity to grow in the presence of ampicillin (TSA-Amp). The same experiment was also performed using the HSF^+ strain 5 as a possible recipient but transformation was not achieved.

Finally, a series of strains, both HSF^+ and HSF^-, were grown on TSA-CB and TSA-CR plates, colour-modified colonies were pin-pointed, subcultured on

the same media and then transferred to TSA-SDS, to confirm if colour variations were due to a loss or acquisition of HSF. No HSF$^-$ spontaneous mutants were detected amongst the plates screened.

Of the 4 collection strains (7, 19, 38, 39), 3 were HSF$^-$ (7, 19, 39). These HSF$^-$ strains were obtained as type cultures from 3 different sources. One was a type culture collection (NCIMB) and the others were individual culture collections (C.J.R. and C.B.M.) (Table 3.1). Such duplications were included because it was thought that the strains from individual collections could have been subcultured on laboratory media more than the original strains and, therefore, could have suffered selection and lose of the HSF character. Cell extracts from them were run on SDS-PAGE to see if any of them had the HSF character. All the samples studied were consistently HSF$^-$.

7.2.3.9 Study of PK digests of sonicated supernatants containing HSF

When proteinase K digests (see Section 3.4.2.1) of supernatant sonicates of an HSF$^+$ strain (5) were resolved on 12% SDS-PAGE and observed unstained, HSF was present in all samples, in apparently the same amount (judged by the width of its band); and in the same position, independently of the length of incubation time with the enzyme (0 to 180 minutes). Gels were subsequently stained in Coomassie blue, and it could be seen (Fig. 7.19) that PK had digested all proteins present in the supernatant sample even at 'zero' (PK added to sample immediately prior to loading on the gel). Therefore, from this experiment, it was assumed that HSF had a non-proteinaceous

nature or that protein, if present, was not the only component.

7.2.3.10 Study of lipase digests of sonicated supernatants containing HSF

Three lipases from various origins, were used to digest cell extracts containing HSF from strain 5 (Section 3.4.3.1). Digestions were made for 2 h and overnight (approximately 18 h) at 30°C, and then digested cell extracts were compared on SDS-PAGE with a non-digested control. Results were similar for both digestion times, except that after overnight incubation it was evident that protein degradation had occurred (Fig 7.20). HSF was present in the non-digested control and in the Lipase I digest in apparently the same amounts (judged by width of the band), and at the same position on the gel. HSF on exposure to Lipase II migrated at a higher position on the gel (approximately 2 mm), and HSF digested with Lipase VII had a much thinner band than the non-digested control. The results indicated that some lipases were able to modify HSF structurally or degrade it.

7.2.3.11 HSF treatment with lipid solvents

Sonicated supernatants containing HSF (from strain 26) and without HSF (from strain 7) were treated with lipid solvents: chloroform/methanol (2:1) and diethyl ether (Section 3.4.3.2), and after 3 h extraction, resulting phases were screened on SDS-PAGE for the presence of HSF. HSF was visible only in the positive control (sonicated supernatant of strain 26) and in the aqueous phase and interphase of the ether extraction. HSF appeared as a very fine band in the aqueous phase, whereas it was very abundant in the interphase

sample. None of the solvent phases presented any visible HSF bands. Extractions with the same solvents mentioned above were performed in order to obtain lipid extracts of *Y. ruckeri* strains 7 and 26 (Section 3.4.7). Extracts were resolved in TLC plates and observed under UV light. No additional spots could be seen on the tracks corresponding to strain 26 (HSF^+) when compared with extract of strain 7 (HSF^-) (data not shown).

7.2.3.12 Effect of detergents and organic solvents on HSF

HSF present in cell extracts from strain 26 was treated with a number of detergents: SDS, Tween 20, Tween 80, Triton X-100 and 10% Zwittergent and 1.0% saponin or with organic solvents; chloroform, methanol and 1-butanol (section 3.4.3.2), and resolved on a 12% native acrylamide gel. The unstained gel presented the characteristic HSF band only in the sample treated with SDS. A transparent "mark" was present on the sample treated with 10% Zwittergent. Gel was soaked in 10% SDS solution, and after overnight incubation, a characteristic HSF band was present in samples treated with SDS or 1-Butanol. Samples treated with Zwittergent presented a transparent band at a slightly higher position (1mm) on the gel.

7.2.3.13 Study of the presence of HSF in membrane preparations

In order to obtain information about the location of HSF in the bacterial cell, boiled and non-boiled samples from an outer membrane protein extraction (Section 3.4.2.3) of the HSF^+ strain 5 and the HSF^- strain 19, were resolved

on SDS-PAGE and subsequently blotted. HSF could be seen on unstained gels in unboiled samples of sonicated cell extracts of strain 5, and in sarcosyl soluble (inner membrane) and insoluble (outer membrane) fractions. However, the relative amounts of the factor, as judged by the thickness of the band, were very different in the three samples with HSF present predominantly in the whole cell fraction and inner membrane fraction. In the outer membrane fractions, HSF was only observed after the gel was kept overnight at 4°C, whereas the HSF band in both the other samples was observed immediately after the electrophoresis was finished.

After blotting, HSF could only be noticed on the nitrocellulose sheet on the tracks of whole cell and inner membrane. Therefore, HSF did not seem to be particularly associated with the OMP fraction (Fig. 7.21).

7.2.3.14 Study of the presence of HSF in periplasmic protein extracts

Unboiled samples of a chloroform extraction of periplasmic proteins were studied on SDS-PAGE for the presence of HSF (Section 3.4.2.4). Chloroform extraction was performed on strain 5 suspensions with increasing amounts of cells. Non-treated whole cell extracts were used as a positive control. Periplasmic protein fractions and spheroplast fractions were run on SDS-PAGE. HSF appeared most clearly in whole cell extracts, forming a very strong band. In periplasmic protein fractions, the thickness of the HSF band increased accordingly with the volume of cells used for extraction of the periplasmic proteins. However, no HSF was detectable in spheroplast

samples.

Whole cell extracts and spheroplasts were resolved with and without chloroform. Chloroform had a deleterious effect on HSF with a consequent decrease of the apparent amount of the factor present on the gels. To avoid any inconsistency due to this effect, chloroform treated and untreated samples were always examined on the same gels. The apparent absence of HSF in spheroplast samples was independently of the chloroform treatment.

7.2.3.15 Study of the presence of HSF in extracellular products

Unboiled samples of cell extracts of strains 5 (HSF^+) and 19 (HSF^-), and their supernatants from both overnight cultures and cellophane overlays (Section 3.4.3.7) were resolved in SDS-PAGE and subsequently blotted. Both unstained gels and western blots showed HSF only in cell samples (Fig. 7.22).

7.2.3.16 Partial purification of HSF by column chromatography

Sonicated supernatants from an HSF^+ strain (26) and an HSF^- strain (19) were fractionated on an agarose-acrylamide column (Section 3.4.2.6) and 2 ml fractions were collected. Elution profile of the supernatant from strain 26 is presented in Fig. 7.23. Aliquots of the fractions obtained were incubated overnight at 4°C on a 96 well microtitre plate, with 10% SDS (2:1). After warming-up at room temperature (25°C) for 1-2 h, the SDS not associated to HSF went back into solution, whereas the SDS-HSF complex stayed as a white precipitate on the bottom of the well. Fraction numbers 53 to 58 of the

80 obtained from strain 26, gave a white precipitate (Figure 7.24) whereas none of the fractions from strain 19 presented any precipitate.

SDS-PAGE of fractions 51 to 60 was carried out. Other fractions, taken at random, which did not present a precipitate after incubation with 10% SDS, were also electrophoresed. The typical HSF band appeared for all fractions between 51 and 60 of strain 26, although the thickness of the band increased in a sigmoidal mode with a maximum between fraction 53 and 58 (the same samples positive in the microtitre system) (Fig. 7.25). Therefore, the SDS-PAGE was slightly more sensitive than the microtitre system but within the same order of magnitude. No HSF bands were observed in any of the other samples examined.

Coomassie blue stained gels showed that samples with HSF contained various proteins (Figure 7.26). A "distorting" effect caused by the presence of high amounts of HSF on the migration of the proteins was also noticeable. Partial purification of HSF by this system was successfully achieved in three consecutive attempts.

7.2.3.17 Study of partially purified HSF on native gels

Equal aliquots from fractions 54 to 57 from the column chromatography were pooled for further work and from now on this pooled sample is referred to as the HSF fraction. Boiled and non-boiled samples of whole cell extracts and the HSF fraction of strain 26 were resolved on 12% native gels. Samples

were arranged together in triplicate across the gel(s). After electrophoresis, each gel was cut and one part was stained with Coomassie blue, one was soaked in 10% SDS and another was immunoblotted. Results showed that:

(a) HSF presence in unstained gels seemed to diminish after overnight transfer to nitrocellulose.

(b) there were faint protein bands in the Coomassie blue stained gel and a recognition band on the western blot (using rabbit antisera anti-strain 26) in the same position as HSF.

(c) all bands on the western blot were very faint but samples from the HSF fraction produced much clearer results.

Therefore, this experiment indicated the possibility that HSF was transferred to nitrocellulose and recognised by the antisera. Thus, the possibility exists that HSF may have a proteinaceous component whereas previous experiments using cell extracts containing HSF on SDS-PAGE indicated that the factor was transferred to the nitrocellulose but there was an apparent lack of immunogenicity of the band. Cell extracts and HSF fractions of *Y. ruckeri* strain 26, were digested with proteinase K in order to determine if the bands recognised by the antiserum on the nitrocellulose were of protein nature. Digested and non-digested controls were resolved simultaneously, on a 12% native-PAGE in triplicate. The gel was cut after electrophoresis, as described above, and one third was developed with 10% SDS, one third stained with Coomassie blue and the other western blotted. The HSF band appearing in

unstained SDS-treated gels, migrates differently on the gel, depending on whether or not the sample had been digested with proteinase K. Digested samples of both cell extracts and HSF fractions appeared as a double band approximately 2 mm below the origin of the separating gel. Non digested samples migrated further (5 mm) and were visualized as single bands. Therefore, proteinase K had an effect on HSF migration on native gels, indicating the possible presence of protein in its structure. On western blots, very faint bands, corresponding to those seen on the SDS developed gel could be observed on the nitrocellulose sheet (Fig. 7.27).

Despite the evidence presented, visual correlation between bands appearing in gels and those recognised on nitrocellulose filters proved difficult due to the weak nature of such bands. This problem was solved by working with a gel of lower acrylamide content (7.5%). Proteinase K treated samples, as in the previous experiment, appeared in this case at 7 mm from the beginning of the gel, and non-proteinase-K treated samples appeared at approximately 12 mm. In both cases they were present as a multiple band. In both acrylamide gels and western blots, the HSF band appeared stronger than in 12% gels (Fig. 7.28) and correlation between both was evident. Fig. 7.29 shows the progressive formation of the HSF-SDS complex, when viewed under a low power microscope (100x).

7.2.4 *In vivo* Studies of Immunization of rainbow trout with HSF

A preliminary experiment was performed to study whether HSF was either

immunogenic or toxic for rainbow trout. Partially purified HSF, obtained by column chromatography, was kept at -70°C. It was noted that, after thawing, HSF^+ samples did not form the typical precipitate following incubation with 10% SDS in the 96 well microtitre system. One group of 14 fish was injected i.p. with one single dose, corresponding to approximately 10^6 c.f.u. per fish, of formalin killed *Y. ruckeri* strain 44 (HSF^+).

A second group of 17 fish was injected with one single dose of 0.1 ml of pooled partially purified HSF sample (thawed once). After 7 weeks both groups were challenged by i.p. injection (10^6 c.f.u. per fish) with live strain 44 following the standard protocol (Section 3.3.3.4). A non-immunized group of 14 fish was used as a control. After three weeks, the percentage of cumulative mortalities recorded were: Fish immunized with formalin-killed cells gave 7.1% mortality (equivalent to one fish dead), whereas those injected with HSF presented no mortalities. The control group showed 54.5% mortality.

7.3 DISCUSSION

The importance of the effect of growth and environmental conditions on the virulence of bacterial pathogens is a well-known phenomenon and it has been widely reported. Bacteria often lose virulence after growth *in vitro* but it may possibly be restored by animal passage. This may be the result of both

selection and phenotypic variation affecting cell envelope structures (Brown and Williams, 1985; Griffiths *et al.*, 1983; Lam *et al.*, 1984) and/or the activity of enzymes which facilitate *in vivo* growth and multiplication (Miller *et al.*, 1987). Of those characteristics compared here, in strain 5 and *in vivo* passaged derivative strain 26, none seemed obviously different, although the effect of environmental (culture) conditions was noticeable. Most such comparative work involved one or two steps of growth in laboratory media, which could have affected the expression by the passaged strain 26 of characteristics selected during sequential passage in fish. When an attempt to mimic *in vivo* conditions was made by growing *Y. ruckeri* in kidney extracts of an infected rainbow trout, no major new proteins seemed to be produced. Unfortunately, the bacterial growth obtained on kidney extracts was low and it did not allow production of sufficient samples to study other properties, such as LPS or periplasmic proteins, nor for use in western blotting. Future work in this area could probably answer some important questions, provided that improved culture media based on fish extracts were used, or work on completely *in vivo* conditions was possible. Immunoblotting studies could, simultaneously, provide fundamental information about differences in the antigenic bacterial make-up under *in vivo* or *in vitro* conditions. Neither the cells nor the extracellular products of strains 5 and 26 displayed cytotoxic effects in any of the poikilotherm and homeotherm cell lines used. This observation supports the lack of toxicity obtained (section 5.2.3), in fish injected i.p. with supernatant of *Y. ruckeri* strain 26. Also, no protein bands were detected on SDS-PAGE and western blots of the culture supernatants.

Romalde *et al.* (1988), studied the effects of ECP on 20 serotype O1 strains of *Y. ruckeri* on 4 cell lines and found variable results depending on the strain tested. Only ECP from strain NCMB 1316 was reported to be cytotoxic for the four cell lines. From work carried out in this study (section 7.2.2.1) it was found that strain NCMB 1316 was non-virulent, although NCMB 1316 (strain 7) was not used in cytotoxicity experiments in this study, it seems unlikely that the cytotoxic effects observed by Romalde *et al.*, (1988) are related to virulence.

The results obtained from the i.p. infections performed with a series of serotype O1 strains indicated the existence of different degrees of virulence amongst them. *Y. ruckeri* strains 7 and 19 proved to be avirulent, whereas strain 30 seemed to have an intermediate degree of virulence when compared with strain 26, 5 or 48. Differences in virulence amongst serotype O1 strains have been reported by Cipriano *et al.*, (1987); Flett (1989) and Davies (1990). Although challenge protocol, bacterial dose used for infection and fish species varied, the overall conclusion from their work points to the fact that there are important interstrain variations in virulence, which is expressed both in mortality rates and in the level of infection established in the fish (Flett, 1989; Davies, 1990).

A direct correlation between mortalities and infectivity rates, for the tested strains, was also found in this work. Although only four strains were tested, it is of interest to note that antiserum titres produced by rainbow trout were

higher in fish immunized with HSF⁻ avirulent strains 7 and 19 than in those immunized against strain 26(HSF⁺). Davies (1990) studied the serum killing capacity against a series of serotype O1 strains and showed that in general, although not always, avirulent strains presented a higher killing index than virulent strains.

In the present work a heat-sensitive factor (HSF) in extracts of *Y. ruckeri* was found. Possession of the HSF was closely correlated with virulence (shown by i.p. challenge), and all HSF⁺ strains caused 100% mortality, whereas HSF⁻ strains failed to produce any. However, it should be noted that i.p. challenge might circumvent the natural routes of entry that the pathogen uses in order to establish infection and disease. When the effect of possession of HSF on mortalities following exposure to the bacteria by immersion challenge was studied, all HSF⁺ strains produced mortalities while HSF⁻ strains failed to cause death. There were, however, apparent differences in the degree of virulence amongst the HSF⁺ strains. This suggests that HSF is one important virulence determinant, but that other bacterial components might also be necessary. The capacity of the different strains to establish infection was also different, although markedly reduced or even absent in the case of the HSF⁻ strains.

The study of other cell components of the virulent and avirulent strains, showed that HSF was the only consistent variation in SDS-PAGE of whole cell profiles or sonicated supernatants which could be correlated with

virulence. Such results do not rule out, however, the possible co-operation of other factors in the virulence of *Y. ruckeri* serotype O1 strains. Following this line of thought, it is relevant to compare the findings of this work with those of Davies (1990). Davies established an OMP-typing system, to differentiate strains of *Y. ruckeri* and performed a series of experimental infections in order to study if there was a correlation between the OMP-type and virulence. The OMP-typing scheme of Davies (1990) recognized five OMP-types (1-5) of which OMP-types 1, 2 and 3 represented the majority o the strains (95%). This author also grouped the serotype O1 strains of *Y. ruckeri* into six clones (1-6). All challenge work performed by this author was done by bath immersion (5×10^7 c.f.u. ml^{-1} for 1 h), and strains were considered virulent if they were capable of both producing mortalities and raising a persistent infection of fish after three weeks. Therefore, although the challenge method used by Davies was similar to the immersion challenge used in this work, he did not use parenteral infection. As indicated by the results of the i.p. challenge, this gives more clear cut results, especially when the strains under study are of intermediate virulence level. Davies' findings indicated that virulent isolates consisted of serotype O1 strain of the OMP-type 1 (clone 2) and the OMP-type 3 (clone 5). There were, however, strains which did not fit into this scheme. Some of the strains used in this work had been donated by Davies and, therefore, a direct comparison was possible. All strains tested by Davies of the OMP-type 3 (clone 5) were virulent, except strains RD126 (71), RD138 (72) and RD140 (73). The results obtained here show that when these strains were grown on ROD media, strain RD126 (71)

was HSF⁻ whereas RD138 (72) and RD140 (73) were HSF⁺. However, the reaction on ROD was extremely weak (very little precipitate was formed around the colonies), which could explain why those strains were avirulent (0% mortality) when Davies tested them. On the other hand, Davies considered strains of the OMP-type 2 (clone 3) avirulent, although strains RD20 (68) and RD62 (70) produced 11% and 7% mortality respectively in his infection trials. The reason stated for this was that neither strain was recovered from infected fish at 3 weeks post-challenge. One further strain of this clone was tested, RD38 (30), which produced neither mortality nor infected survivors. This strain had been used in the present work for i.p. infection (Table 7.1), and was shown to have an intermediate virulence level. When all three strains (30, 68 and 70) were tested on ROD, they were HSF⁺ but produced a weak reaction . It seems, therefore, that strains of OMP-type 2 (clone 3) would be considered of intermediate virulence under the criteria used in this work.

Studies on serum resistance, also carried out by Davies (1990), showed that all serotype O1 virulent isolates were serum resistant, with the exception of RD38 (strain 30), which as shown above, is considered here to be of intermediate virulence. Serotype O7 strains RD36 (29), RD60 and RD150 (76) were also serum resistant, although at a much lower level than the serotype O1 strains. In this study strains 29 and 76, were found to be HSF⁻. Despite these results, it could be unwise to conclude that the HSF character is not associated with serum resistance, since the origin of such resistance

could be different depending on serotype. On the other hand, HSF seems to be mainly restricted to serotype O1 strains (only strain RS80 (47), a member of serotype O6 has also been found to be HSF^+). In general terms, the HSF character seems to be compatible with the OMP-type scheme proposed by Davies, with the OMP-types 1 (clone 2) and 3 (clone 5) being considered virulent. However, in this work Davies' OMP-type 2 (clone 3) was found to be of intermediate virulence. More work is required in order to test whether such a correlation is universal or if exceptions occur.

Since more than one OMP-type represents the virulent strains, a comparative study of LPS patterns could also produce very valuable information and perhaps clarify the correlation of virulence with serum resistance.

Plasmid-associated virulence has been described widely (Portnoy and Falkow, 1981). The occurrence of different plasmid profiles amongst *Y. ruckeri* serotypes has been reported (De Grandis and Stevenson, 1982; Toranzo *et al.*, 1983; Stave *et al.*, 1987), and workers have speculated that a correlation between presence of the 70mDa plasmid in serotype O1 strains and virulence may exist (Stave *et al.*, 1987). Plasmid analysis carried out in this work comparing virulent and avirulent strains did not show any obvious differences, although the possible existence of small mutations in the plasmid could not have been detected by studying only plasmid profiles on agarose gels.

Work carried out by Cipriano *et al.* (1987), apparently contradicted the

general assumption that only serotype O1 strains are able to cause disease and concluded that serotype O2 strains were as virulent as serotype O1 strains. This work was performed on brook trout (*S. fontmalis*), and suggests that different hosts may be more susceptible to different *Y. ruckeri* serotypes. Epidemiological reports support this point, since most virulent serotype O1 strains have been isolated from rainbow trout, whereas serotype O2 strains have caused problems in both atlantic salmon (*S. salar*) in Norway (Davies, 1990). Also, O2 strains have been isolated pacific salmon in North America (McCarthy and Johnson, 1982). Therefore, virulence mechanisms selected amongst the different serotypes could differ, and their expression could depend on finding the ideal *in vivo* conditions displayed by different hosts.

Only a systematic study of the relationship between all the above mentioned factors, supported with a consistent *in vivo* testing model, could clarify further the operational virulence mechanisms of *Y. ruckeri*.

The virulence-associated HSF was found as a major unreactive band in western blots, and at first it was thought to be unrecognised by the rabbit antisera used, presenting a white area on the blot, completely free of background. Subsequently it was found to correspond with a white opaque band appearing in the same area on SDS-PAGE. HSF could only be visualized if no overheating occurred during the electrophoresis procedure. To see the HSF band, it was usually necessary, although not always, to cool down the gel (4°C) for a period of time (2 h to overnight) and then leave it

to warm up at room temperature. The HSF band was then visible and stable for at least 72 h (the longer time examined). Therefore, cooling of the gel was found to accelerate or improve visualization. Possible explanations for this phenomenon are given below.

When native gels were used, HSF could only be visualized by immersing the gel in SDS solution at room temperature without previous fixation. These results clearly indicated that SDS was responsible for the appearance of the band, although not for the band's formation. Similar conclusions could be drawn from the results obtained from the addition of SDS to the bacteriological media (TSA). When unstained SDS-PAGE gels were observed under the light microscope, a well organized structure, formed by bright spherical structures of variable sizes, could be seen along the band. One possible explanation is that very specific conditions occurred in the zone of the 12% SDS-PAGE, occupied by proteins of 120 kDa, which corresponded to those of the critical micelle concentration (CMC) for SDS, in which micelles are formed above certain concentrations. The behaviour of SDS as a surface active agent in solutions has been studied by Shinoda *et al.* (1963). The study of the physical-chemical characteristics of SDS as an ionic surface active agent is a complex field outside the scope of the present study, but nevertheless, some brief discussion is needed to try to understand the nature of HSF. Shinoda *et al.* (1963), studying the behaviour of SDS in water solutions, described a series of phenomena which could be correlated with some of the findings of this work. The work reported that a saturation

concentration effect which corresponds with the CMC of a singly dispersed ionic agent (i.e., micelle formation), occurs only at a certain concentration. At very high solute concentrations, a change in the solute:solvent ratio makes the formation of micelles less favourable. This could explain why in some cases, the band appearing on SDS-PAGE had a "flat" doughnut shape, since conditions for CMC could only be achieved on the periphery of the zone but not in the centre, where the bulk of the substance initiating the process occurs. This could also explain the appearance of the ring formation observed on TSA-SDS plates. Rings were formed around large colonies but not in direct contact with them. Although, presumably, most of the HSF is cell-associated, since it was never observed in ECP. Therefore, it does not appear to be exported, at least in the same form as that occurring in the cells.

Another property which seems relevant to comment on applies to the solubility properties of the micelle depending on whether it is in a liquid or solid state. When in a liquid state the solute can mix with various solvents of markedly different molecular sizes, but in the solid state it can only form crystals with molecules of identical size. This fact, together with the capacity of some surface active agents to form dimers, could explain the "ladder" structures observed on 7.5% native gels.

Temperature is a very important factor for the CMC, and there is a very narrow range at which the micelle is in the thermodynamically preferred form. This could explain why the best way to visualize HSF was to allow the

temperature to rise gradually from low (4°C) to ambient (25°C). In this process, the temperature would pass the Krafft point, (Shinoda *et al.*, 1963) above which micelles will be the more stable form. It is, however, important to bear in mind that this explanation of the phenomena observed on SDS-PAGE, when HSF is present, is based on the interpretation of behaviour of surfactants in liquid systems, where all components are defined. Direct correlation with phenomena in gel systems, where complex samples (cell extracts) are being resolved by application of an electric current, has to be interpreted with caution.

The opaque aspect of the band could be explained as the consequence of the "clouding effect" (Shinoda *et al.*, 1963). When the temperature of a solution of a non-ionic surface active agent is elevated, it suddenly becomes turbid in a narrow temperature range. This temperature is called the "clouding point". The "clouding point" of dilute aqueous solutions of non-ionic surface active agents is affected by the addition of co-existing substances. When an ionic active agent like SDS is present in the mixture, the "clouding point" becomes broadened, and the mixture generally presents clouding over a wider range of temperatures. Therefore, the opacity of the band observed in SDS-PAGE, and the presence of micelle-like structures, could be due to the effect of a mixture of an ionic and non-ionic agents. If this is so, HSF might be a substance of such chemical structure that it can bind an ionic surface agent such as SDS and form both micelles and clouding.

The opaque aspect of the HSF band could be also explained as a purely physical phenomenon. A beam of light would suffer "scattering" through a micellar suspension if the size of the micelles were not homogeneous. When the HSF band was examined under the light microscope, a variation in the size of the micelles was evident. Therefore, the opacity of the band could be due to variation in the sizes of the formed micelles causing light scattering, which would result in visualization of the band.

Substances of different chemical nature are able to form micelles in the presence of surface agents, providing precise conditions prevail. Lipid and lipoproteins form micelle structures in water solutions (Gurr & James, 1980) and specific examples have been reported for micellar complexes of apolipoprotein A-I and phosphatidylcholine/cholesterol (Jonas and McHugh, 1983), or complexes involving only proteins and a surfactant agent (Rapoza and Horbett, 1988; Lopata *et al.*, 1988). As a number of widely different chemical compounds can interact to form micelles, the nature of HSF cannot be ascertained from the evidence presented in the above discussion.

At this stage in the work, SDS-PAGE of unheated cell extracts was the only technique available to study the putative virulence factor, and initially histochemical strains were used on these gels to investigate the nature of the HSF band. When SDS-PAGE gels were stained with a range of dyes, the HSF-SDS complex was found to bind a number of stains, except those for proteins (Coomassie blue and silver nitrate). Whether the band was stained

as a result of chemical affinity or due to the presence of electrostatic forces in the HSF-SDS region, which bound the dyes, remains unclear. Dye absorption is one of the methods for measuring the CMC, since the fluorescence of some dyestuffs changes considerably at the CMC (Shinoda *et al.*, 1963). Under the conditions normally used in staining, Alcian blue carries at least 2 and possibly 4 positive charges. It has been postulated that it would combine by salt linkages with polyanionic substances and was observed to combine with and to precipitate polyanions in the same way as cationic detergents (Pearse, 1968). Congo red staining has been proposed for amyloids, but when used in conjunction with simple light microscopy, the specificity of the Congo red method is low (Pearse, 1968). Sudan black B may behave like a cationic dye and binds to the hydrophilic phosphate ester groups of phospholipids (Kernan, 1981). When a bromination step was used before Sudan black staining in protocols 2, 3 and 4, no colouration of the band was achieved. Bromine combines with unsaturated lipids at the site of double bonds (Pearse, 1968). Therefore, it seems that if lipid is present, it would probably have saturated bonds. In any case, when native gels were stained with the same dyes, only phosphine produced a faint band in the HSF region, and after visualization of HSF with 10% SDS, Sudan black stained the band, but also quite faintly.

It is, therefore, difficult to present any hypothesis about the nature of HSF, based on the use of histochemical stains, since results obtained using SDS-PAGE are only indicative of the staining profile of the HSF-SDS complex.

The results obtained using native gels seem to point to a possible presence of a lipid component in the HSF band. This should be confirmed by using delipidized control samples, simultaneously with purified or semipurified HSF. When the same histological dyes were tested on bacterial smears no differentiation between HSF^+ and HSF^- strains could be made, probably due to the fact that SDS was not present in the samples, as occurred in the case of the native gel. Also, HSF is probably not exposed on the outside of the cells for the dye binding to take place.

Differentiation between pathogenic and non-pathogenic strains of *Yersinia entercolitica* is possible, using bacteriological media containing Congo red (CR) (Robins-Browne and Prpic, 1985; Riley and Toma, 1989) or crystal violet (Bhaduri *et al.*, 1987; Robins-Browne *et al.*, 1989). Virulent plasmid-bearing clones acquired a darker colour, due to a differential binding capacity for the dye compared to the plasmid-less nonvirulent clones. In the case of the crystal violet, temperature influences the binding capacity of the bacteria. *Y. ruckeri* HSF^+ and HSF^- clones were not differentiated by the addition of crystal violet to the media. However, CR did permit differentiation, although in this case, HSF^- avirulent colonies appeared a darker colour than HSF^+ virulent ones. Coomassie blue (CB) added to TSA, produced similar results, and in both cases, there was a temperature effect in the uptake binding of the dye. The reason why a difference could be established in this case, in contrast to stained bacterial smears, is probably due to active uptake of the dye into the cell. This would, obviously, require viable cells. It appears

contradictory that the two dyes used (CB and CR) produced different results when studied on SDS-PAGE gels, but similar results in the dye-TSA system. A possible explanation for this could be that none of the dyes are actually bound by HSF. Therefore, HSF^+ colonies have a lighter colour, since HSF is a possible major component of their periplasm and consequently remains colourless in the presence of either stain. This would produce a less intense overall colouration. The apparent contradiction with the results obtained staining SDS-PAGE is clarified by the results obtained when HSF^+ and HSF^- *Y. ruckeri* strains were grown on TSA-SDS-CB or TSA-SDS-CR. The difference in colour due to CB binding was emphasized by the SDS since, as shown before, CB does not stain the SDS-HSF complex. On the other hand, when CR was used together with SDS, colonies of either HSF^+ or HSF^- strains appeared similar. CR, as discussed above, can stain the HSF-SDS complex, and therefore, when SDS is present, colonies of HSF^+ strains are no longer of lighter colour than those of HSF^- strains. The combination of CB and SDS in TSA plates offered a good bacteriological medium to screen for virulent serotype I (HSF^+) strains of *Y. ruckeri*. Until now, the use of ROD medium was the only medium available to both distinguish *Y. ruckeri* from other bacteria and to differentiate the HSF^+ clones. TSA-CB-SDS, is a simplified medium and can be used to screen for HSF^+ *Y. ruckeri*. The use of TSA-CB-SDS plates in epidemiological trials or in studies of bacterial survival in fish, should be complemented with TSA-CB plates in order to detect the HSF^- strains. Otherwise, due to the transparent appearance of the HSF^- colonies, they could be overlooked. This could explain why HSF^-

strains were never isolated from faeces, plated onto ROD, of fish surviving infection. However, the low levels of kidney infection, obtained from samples on TSA, in the same fish does not rule out the possibility of a total clearance of the pathogen from the gut.

Despite all attempts made to obtain an isogenic pair (HSF^+/HSF^-), no mutant clones were detected. For example, attempts to transform an HSF^- strain of *Y. ruckeri* to HSF^+ using the $CaCl_2$ technique of Mandel and Higa (1970) did not work. However, transformation has been recently achieved in various *Yersinia* species by means of electroporation techniques (Conchas *et al.*, 1990), and a similar approach could be attempted using *Y. ruckeri*. Transposon mutagenesis is another logical option to try, presenting the advantage that the transposable elements of DNA segments can move to new locations in DNA molecules by processes which require neither extensive DNA sequence homology between the element and the site of insertion, nor the *rec* genes needed for classical homologous cross over (Berg and Berg, 1983). Transposons can encode for resistance to antibiotics, which makes them an ideal genetic engineering tool. The use of transposon mutagenesis to obtain an isogenic pair of strains involving loss of virulence has proved successful in *Listeria monocytogenes* (Gaillard *et al.*, 1986).

Later work to elucidate the nature of HSF employed both cell extracts and HSF fractions, obtained through gel filtration, and resolved on 12% and 7.5% native PAGE. These indicated that a protein component was probably part

of HSF in addition to the lipid component previously recognized. This work also indicated the possibility of a polymeric nature of HSF since the factor appeared as a multiband array in the lower percentage gel (7.5%). Further characterization of HSF might be possible by the use of a range of enzymes such as phospholipases, amylases, other lipases and proteinases. Western blotting studies of the different digests could also provide information about which components of the HSF structure are immunogenic. The use of immobilized enzymes to perform the digestion would allow elution of HSF without the contaminating enzyme. This would allow the use of preparative gels from which the digest could be obtained in a pure form and enable the resultant compound to be studied *in vivo*.

However, further study of conditions effecting the stability of HSF is required, to make sure that optimal conditions for enzyme digestion are used. Attempts to obtain HSF by lipid extraction procedures showed that it was apparently not extractable by either of the solvent systems used. HSF only appeared in the interphase and aqueous phase after lipid extraction with ether. This finding suggests that, if any lipid component of HSF is extracted, it could suffer degradation or modification in such a way that, when resolved in SDS-PAGE, it could not be visualized. The chloroform-methanol extraction supports this argument since, although HSF was not apparently present in the solvent phase; it was also absent from the other fractions. The term lipid applies to a very wide and heterogeneous group of chemical substances, which have in common their insolubility in water and their

solubility in organic solvents (Kates, 1986). When in the cells, lipids can form part of complexes or exist in association with other components. Depending on the nature of the lipid and the kind of association in which it participates (Van der Waals, hydrophobic association, hydrogen binding, electrostatic, hydrophobic association or covalent link) different extraction procedures are required (Kates, 1986). HSF seems to be a complex structure in which lipid is present. If the lipid component is highly polar and/or the link with any protein is very strong, then the solvent extraction will be unsuccessful, (Kates, 1986). This could happen when cell extracts of HSF^+ strains are treated with ether and could explain why it remains in the interphase. The lack of additional spots in the tracks of a HSF^+ strain, after TLC, also indicates that the factor was either degraded or not extracted.

Work carried out with a series of solvents and detergents indicated that SDS was the only substance able to produce the appearance of the HSF band on the gels. It was also apparent that care should be taken in the future when HSF^+ *Y. ruckeri* cells are submitted to such treatments since all compounds used except 1-butanol and perhaps Zwittergent, modified or destroyed HSF in such a way to make it non-visible on the native gels after developing with 10% SDS.

HSF seems to be located in the periplasmic space of the cells. To avoid misleading results, adequate controls need to be used when chloroform is used to release periplasmic proteins in order to visualize HSF. Such controls

should involve the use of a series of periplasmic protein extracts obtained using increasing amounts of cell suspensions but identical amounts of chloroform. Other protocols for periplasmic preparations could be used if more quantitative results are desired. Methods involving cold osmotic shock or slow and rapid freeze/thaw (Lall *et al.*, 1989) could be tried. However, HSF was not detectable in culture supernatants, either from overnight liquid cultures or from cells grown using the cellophane overlay technique. This does not rule out the possibility of HSF being exported to the outside of the cell, but suggests that, if exportation occurs, HSF would be in a modified, non-detectable configuration when treatment with SDS is used for visualization. Also the unusual white band of the HSF-SDS complex did not appear on Western blots of ECP. Indication of a possible polymeric structure for HSF was obtained when ladder-like bands appeared in 7.5% native-PAGE of HSF^+ samples. It would be difficult to offer an explanation of how HSF interacts with the host if it is restricted only to the periplasmic space although it has been reported that cells of other members of the *Enterobacteriaceae*, growing in liquid media, shed fragments of their outer membranes. Such fragments contain proteins, phospholipidis and LPS, (Munford *et al.*, 1980).

Significantly, HSF was found to be immunogenic when fractions were resolved in native gels (i.e. lacking SDS). HSF was originally thought to be non-immunogenic, at least in rabbits, due to the peculiar band formed on western blots using HSF^+ cell extracts from SDS-PAGE. A possible hypothesis was that HSF could play a role in "camouflage" during bacterial

invasion, helping to avoid recognition by the host and therefore, improving the chances of the establishment of infection. By contrast, if HSF is immunogenic, other explanations are required. So far, HSF has only been detected as a component of the cellular periplasmic space, and it is in this form in which its immunogenicity has been proved. If HSF undergoes structural modifications in order to be functionally active, it is not yet known whether such a product can be is recognized by the host. Unfortunately, attempts to perform western blots using rainbow trout antiserum immunized against HSF^+ or HSF^- strains of *Y. ruckeri* were unsuccessful and therefore it is not possible at this stage to confirm whether or not HSF is immunogenic in fish.

HSF fractionated by chromatography, and frozen, was not detectable by SDS treatment after thawing. This could indicate breakdown into units unable to react with SDS. Therefore, an alternative method to store it should be found. A preliminary experiment injecting such preparations into fish produced a certain degree of protection with subsequent challenge using a *Y. ruckeri* HSF^+ strain. These initial results are encouraging, but more work needs to be done to assess whether or not purified HSF can protect rainbow trout (*O. mykiss*) against ERM infection.

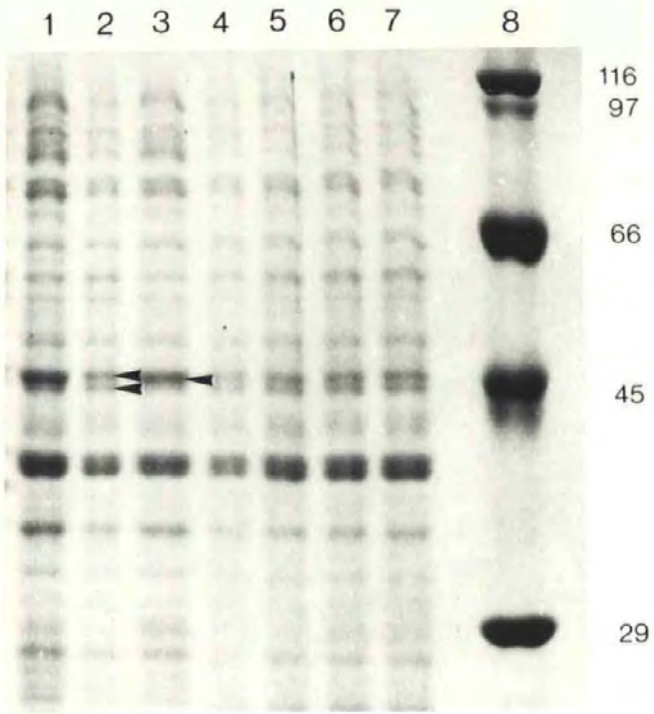
In conclusion, this work has produced evidence for the first time of a virulence factor found in serotype O1 strains of the fish pathogen *Y. ruckeri*. HSF does not seem to be like any of the previously described virulence

factors. Its nature and characteristics appear to be quite unique, and therefore offers a interesting challenge for further investigation. Future work on the study of HSF could be approached from many different angles but a more detailed chemical study would be advisable as a first step. Genetic studies attempting the mutagenesis and cloning of the genes encoding the factor could certainly answer fundamental questions which are unknown at present. Functional studies of involvement of HSF in the pathogenic process by examining whether or not its presence is related with serum resistance or evasion of phagocytosis, would also be an obvious area of work. Finally, it would be interesting to carry out a comparative study covering a wide range of Gram negative bacteria in order to determine whether HSF is restricted to *Y. ruckeri* or not.

Fig. 7.1: **Comparison of whole cell protein profiles of *Y. nickeri* strains 5 and 26 grown under different culture conditions.**

(1): strain 26 grown on BHIB; (2): strain 26 grown on BHIA; (3): strain 5 grown on BHIB; (4-7): strain 5 derived from different dilutions (1:4 to neat) of cell suspension (A_{625}nm = 1.5); (8): molecular weight standards (KDa).

Fig. 7.2: **Comparison of whole cell protein profiles of *Y. nickeri* strains 5 and 26 grown under different culture conditions. (1): strain 26 grown on BHIA; (2): strain 26 grown unshaken on BHIB; (3): strain 26 grown shaken on BHIB; (4): strain 5 grown on BHIA; (5): strain 5 grown unshaken on BHIB; (6): strain 5 grown shaken on BHIB.**


1 2 3 4 5 6 7 8
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97
66
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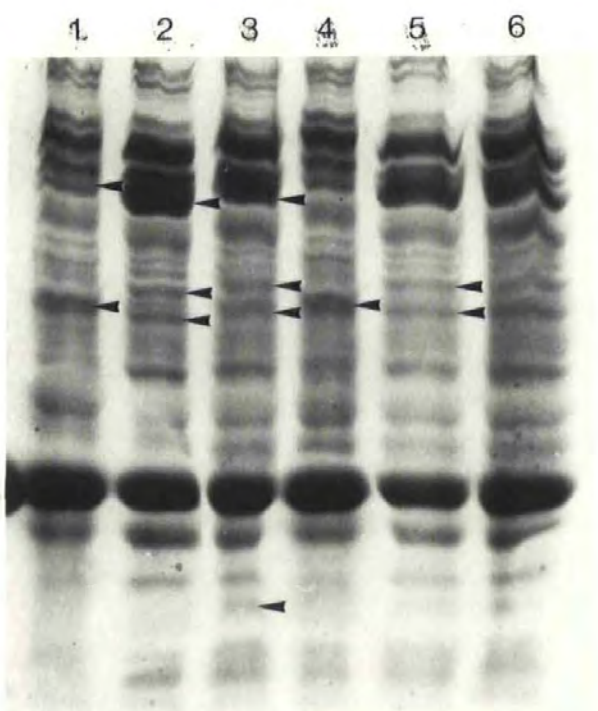
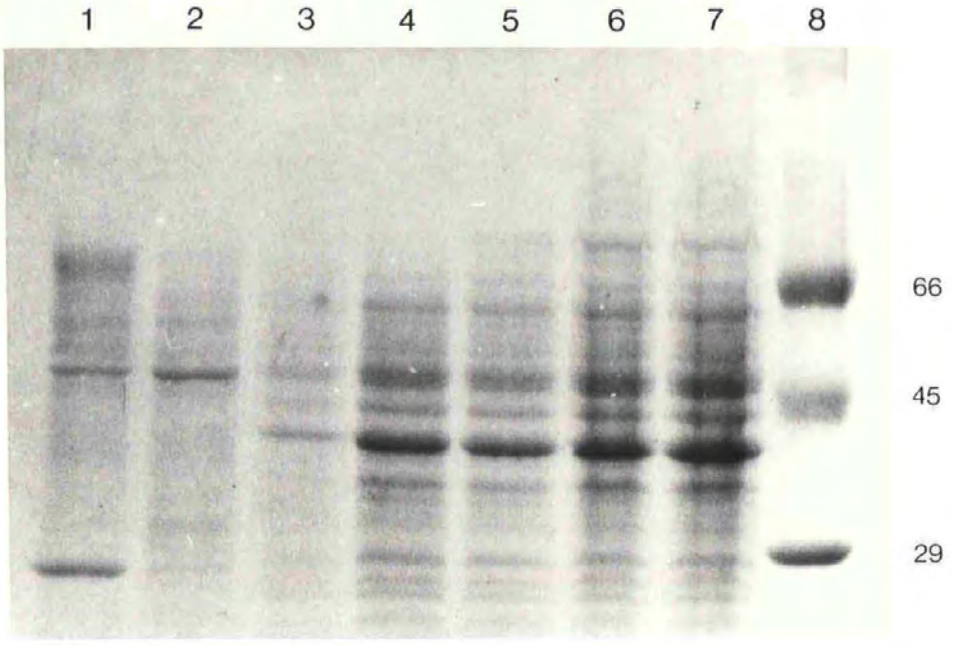

1 2 3 4 5 6

Fig. 7.3: **Effect of growth temperature and media on the whole cell protein profiles of *Y. nickeri*.**

Lanes 1 and 2: protein profiles of kidney extracts from non-infected rainbow trout; (3): strain 26-F1 grown at 15°C; (4): strain 26 grown at 15°C; (5): strain 5 grown at 15°C; (6): strain 26 grown at 23°C; (7): strain 5 grown at 23°C; (8): molecular weight standards (KDa).

Fig. 7.4: **Periplasmic protein profiles of *Y. nickeri* strains: 48, 19, 7, 5 and 26 are presented in lanes 1, 2, 3, 4, 6 and 8 respectively. Lane 10: molecular weight standards (KDa).**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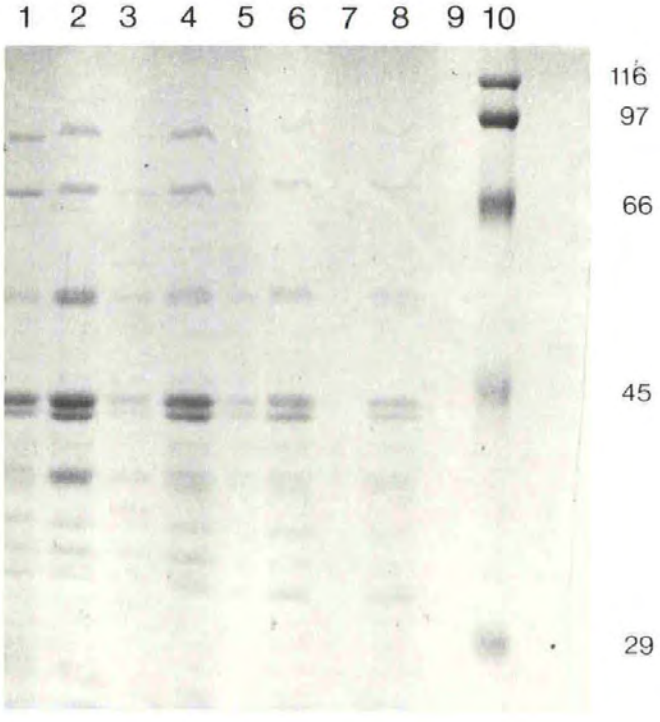
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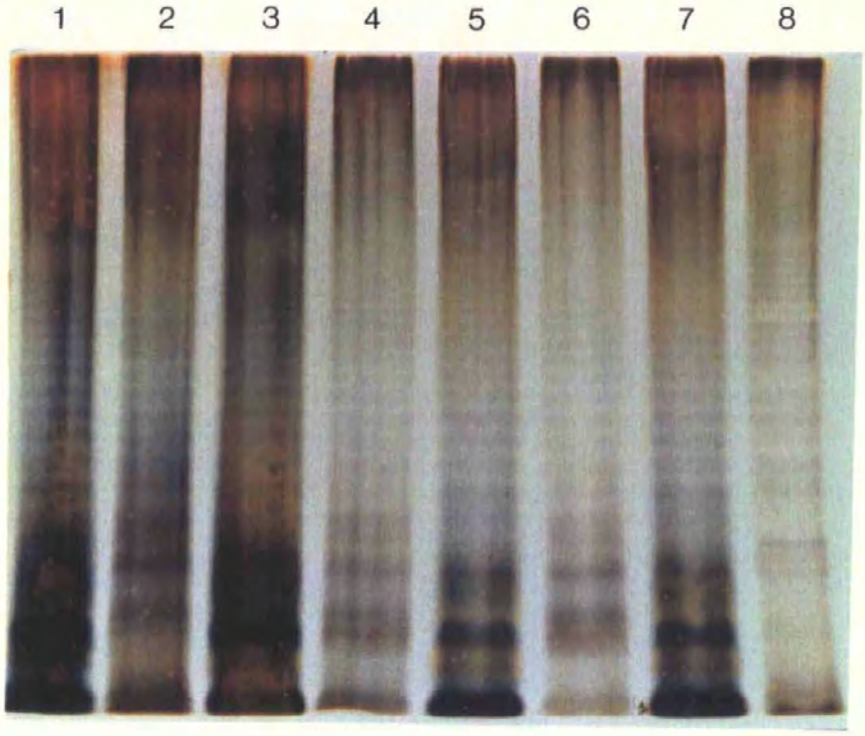
Fig. 7.5: **Comparison of LPS profiles of *Y. ruckeri* strains 5 and 26 grown at different temperatures. (1): PK digested cells of strain 5 grown at 23°C; (2): non-digested control; (3): PK digested cells of strain 26 grown at 23°C; (4): non-digested control; (5): PK digested cells of strain 5 grown at 15°C; (6): non-digested control; (7): PK digested cells of strain 26 grown at 15°C and (8): non-digested control.**

Fig. 7.6: **Comparison of LPS profiles of *Y. ruckeri* strains 19 and 26. (1): PK digested cells of strain 19; (2): non-digested control; (3): PK digested cells of strain 26; (4) non-digested control.**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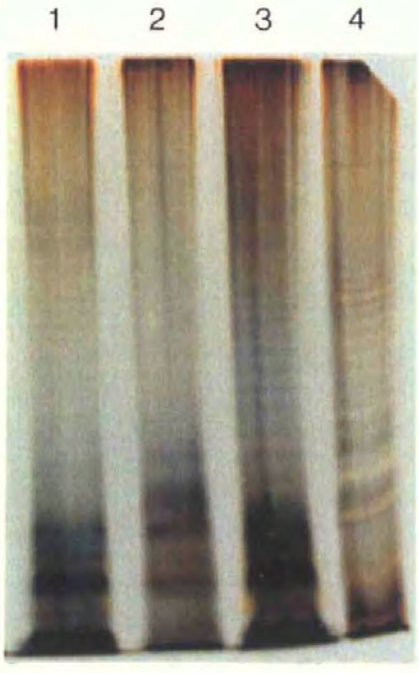
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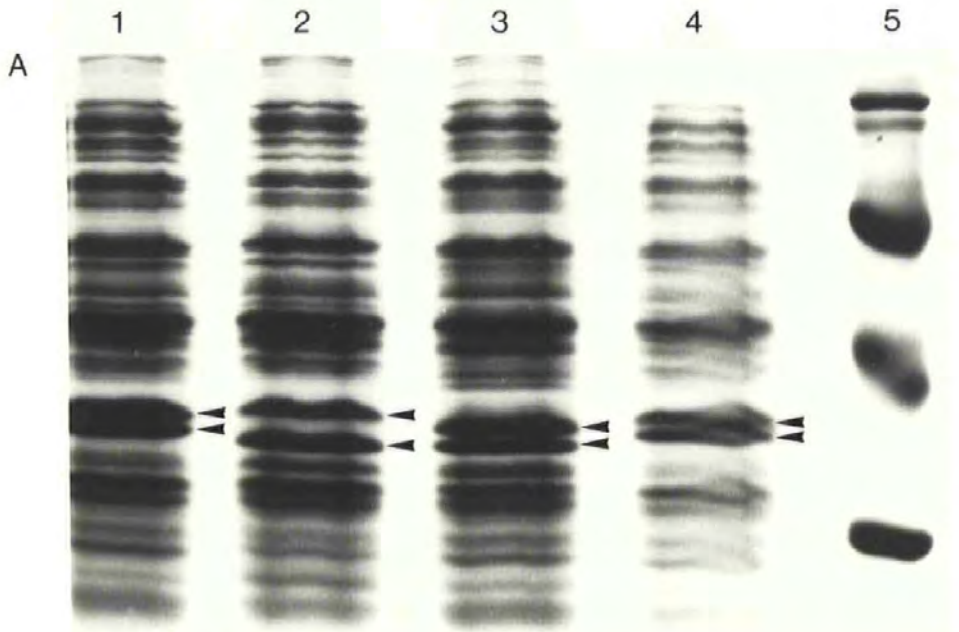
TABLE 7.1: MORTALITIES OCCURRING AFTER I.P. INJECTION OF RAINBOW TROUT WITH FIVE SEROTYPE (I) STRAINS OF *Y. ruckeri*.

Strains	Inoculum c.f.u	Mortality	% Mortality
5	10^4	2/5	40
26	10^4	4/5	80
30	10^7	3/5	60
7	10^3	0/5	0
57	10^7	0/5	0
48	10^7	4/5	80

Fig. 7.7: **Comparison of whole cell protein profiles of *Y. ruckeri* strains 5, 7, 19, 26, 30 and 48.**

A: Lanes 1 to 4: strains 48, 7, 30 and 26 respectively; line 5: molecular weight standards (KDa).

B: Lanes 1 to 3: strains 7, 19 and 5 respectively.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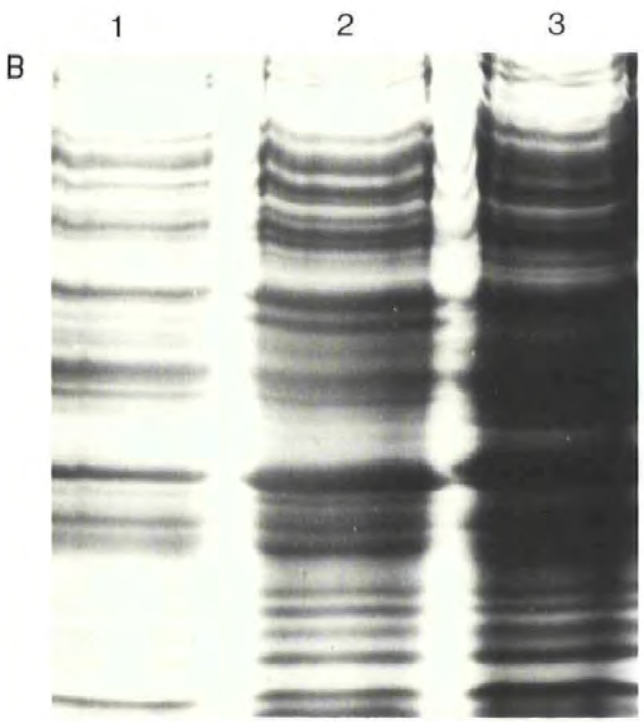
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Fig. 7.8: **Western blot of 3 strains of *Y. nickeri*. (1): strain 5; (2): strain 7 and (3): strain 48.**

A: top side.

B: reverse side of the nitrocellulose (mirror image).

Fig. 7.9: **Unstained gel after SDS-PAGE of unheated cell extracts of 3 strains of *Y. nickeri* (1): strain 5; (2): strain 48 and (3): strain 7.**

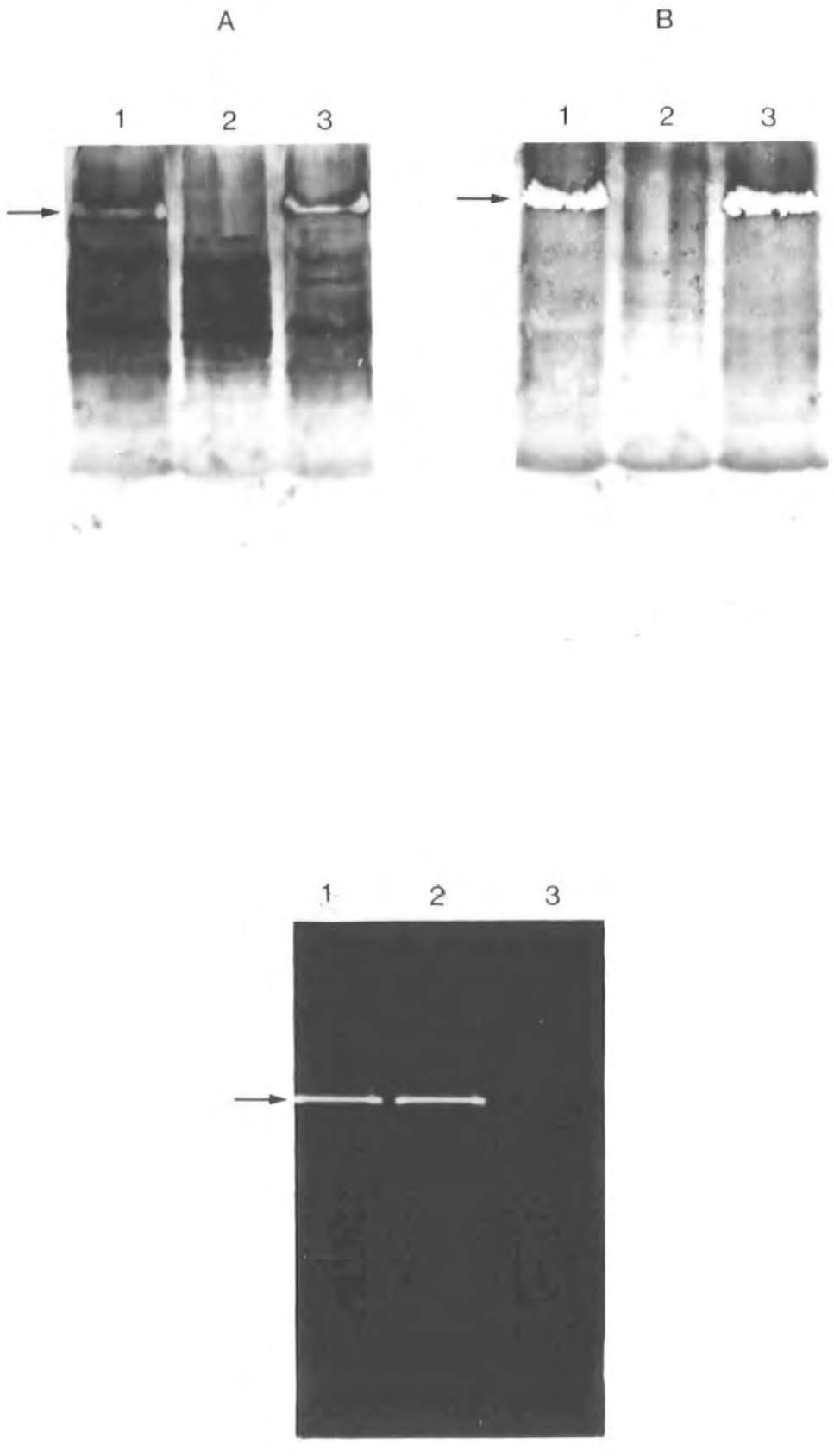
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TABLE 7.2: STRAINS OF *Y.nickeri* SCREENED FOR THE POSSESSION OF THE HSF FOR PATHOGENICITY STUDIES			
Strain number	**Geographic origin**	**Fish source**	**Presence of HSF**
2	Canada	*O.mykiss*	+
4	USA	*not known*	+
5	England	*O.mykiss*	+
7	USA	*O.mykiss*	-
9	Denmark	*O.mykiss*	+
11	Spain	*O.mykiss*	+
12	Spain	*O.mykiss*	+
13	Italy	*O.mykiss*	+
14	Australia	*O.mykiss*	+
15	Ireland	*C.auratus*	+
19	USA	*O.mykiss*	-
20	England	*R.rutilus*	+
22	Norway	*S.salar*	-
25	Spain	*O.mykiss*	+
27	England	*O.mykiss*	+
28	England	*O.mykiss*	+
30	USA	*M.salmonoides*	+
34	USA	*O.mykiss*	+
35	USA	*O.mykiss*	+
36	USA	*not known*	+
38	USA	*O.mykiss*	+
39	USA	*O.mykiss*	-
41	USA	*O.mykiss*	+
42	USA	*not known*	+
44	England	*O.mykiss*	+
57	Norway	*S.salar*	-

HSF presence determined by SDS-PAGE of unheated cell extracts.

TABLE 7.3 EFFECT OF POSSESSION OF HSF ON VIRULENCE OF *Y. ruckeri*

Strain	HSF	IP Injection		Bath Inoculation	
		Inoculum[1]	Specific Mortality (%)	Inoculum[2]	Specific Mortality (%)
2	+	5.0×10^6	5/5 (100%)	4.5×10^7	14/20 (70%)
5	+	6.0×10^6	5/5 (100%)	5.5×10^7	11/20 (55%)
9	+	9.0×10^6	5/5 (100%)	4.0×10^7	13/20 (65%)
35	+	1.0×10^6	5/5 (100%)	3.0×10^7	2/20 (10%)
19	-	1.0×10^6	0/5 (0%)	2.5×10^7	0/20 (0%)
22	-	9.0×10^6	0/5 (0%)	5.0×10^7	0/20 (0%)
39	-	1.0×10^6	0/5 (0%)	3.0×10^7	0/20 (0%)
Control		0	0/5 (0%)	0	0/20 (0%)

[1] i.p. inoculum = number of colony-forming units injected.

[2] Bath inoculum = number of colony-forming units ml^{-1} of tank water, 1 hour exposure.

TABLE 7.4: RECOVERY OF *Y. ruckeri* FROM FISH SURVIVING INFECTION BY IMMERSION AFTER 4 WEEKS

Strains	Kidney (TSA)		Faeces (ROD)	
	N° positive fish	% positive fish	N° positive fish	% positive fish
2	2/6	33	2/6	33
5	8/9	89	0/9	0
9	3/6	50	5/6	83
35	8/10	80	2/8	25
19	1/10	10	0/10	0
22	0/10	0	0/10	0
39	0/10	0	0/10	0

Fig. 7.10: **Comparison of cell sonicated supernatants of *Y. ruckeri* strains 2, 5, 19 and 39. Lanes 1 to 4: heated samples of strains 39, 19, 5 and 2 respectively. Lanes 5 to 8: non-heated samples of strains 39, 19, 5 and 2.**

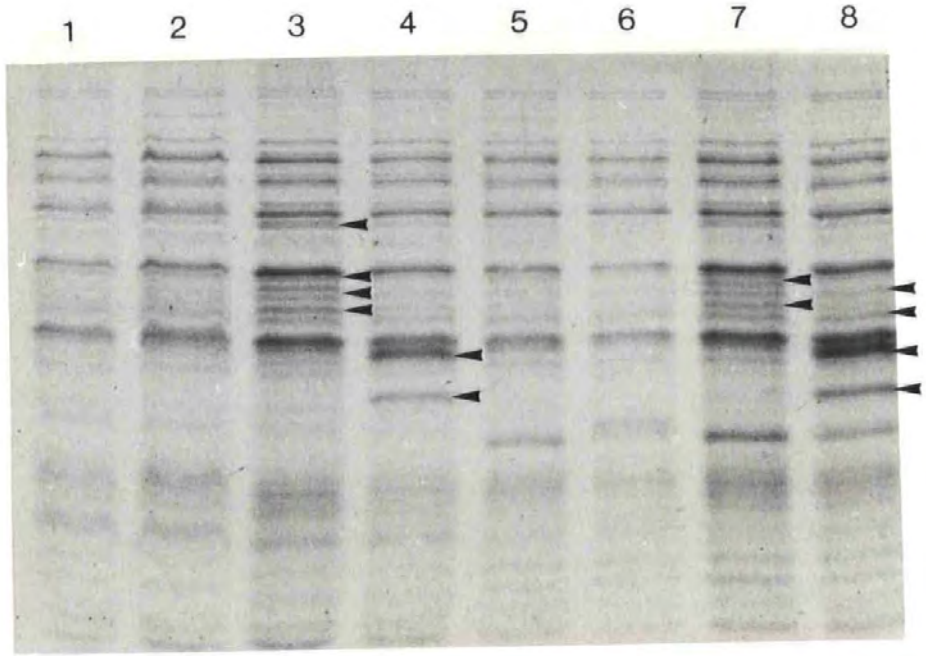
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FIGURE 7.11: FLOW DIAGRAM INDICATING SEQUENTIAL SCHEME FOLLOWED FOR THE IDENTIFICATION OF THE HSF NATURE ON SDS-PAGE GELS

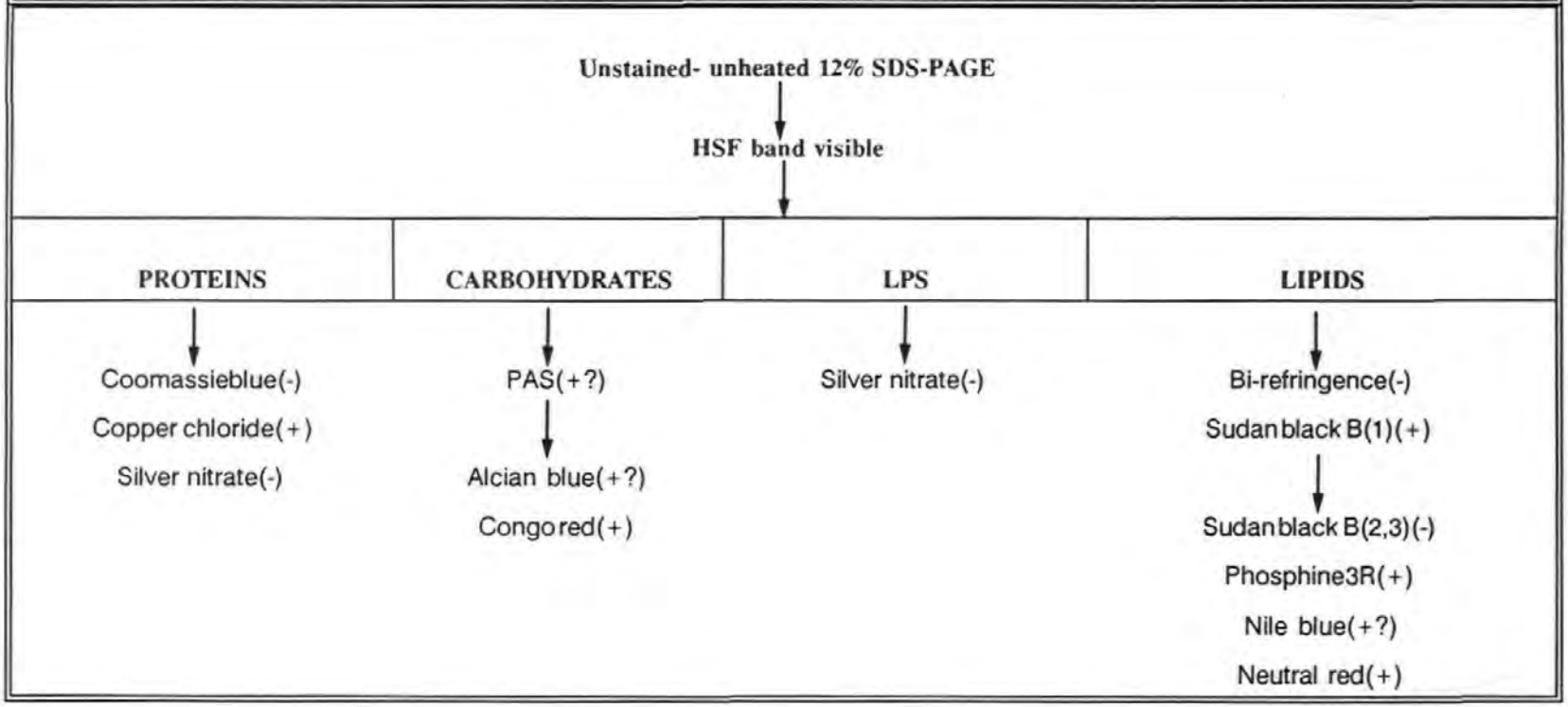
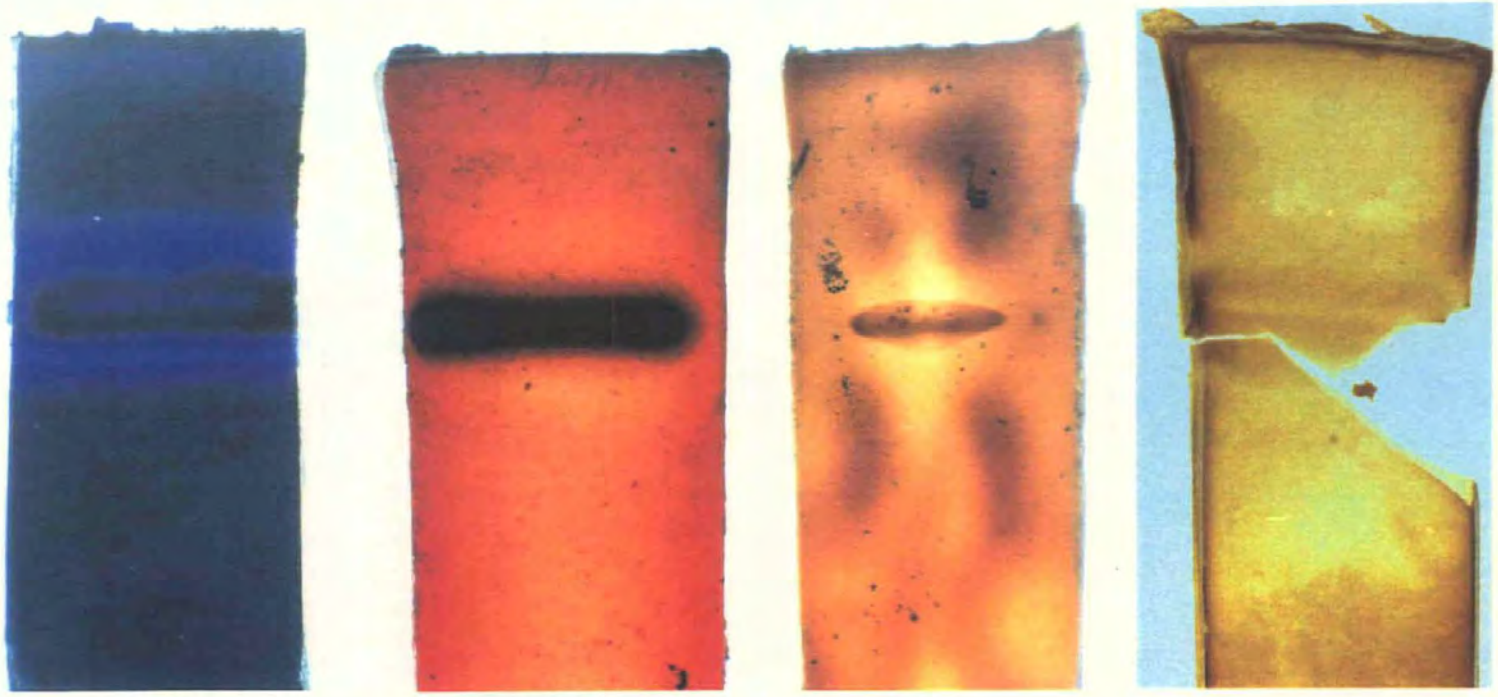


Fig. 7.12: **Appearance of the HSF band on 12% SDS-PAGE after staining with (1): Alcian blue; (2): Congo red; (3): Sudan black B(1) and (4) Phosphine 3R.**



1 2 3 4

TABLE 7.5 : SUMMARY OF RESULTS OBTAINED FROM STAINING HSF PRESENT ON 12 % SDS-PAGE.

METHOD	RESULT (HSF visible)	COMMENTS
Unstained- unheated	White opaque (+)	-Micelle like spheres visible under light microscope.
Coomassie Blue (proteins)	(-)	
Silver nitrate (proteins)	(-)	
Copper chloride (negative stain for proteins)	White opaque (+)	
PAS (glycoproteins)	Dark red-purple (+?)	-HSF band darker than other bands on the gel. -Stained or coated?
Alcian blue (sialo mucins)	Blue (+?)	-Centre of HSF band dark blue surrounded by light blue. -Background colour blue.
Congo red (amyloid)	Deep red (+)	
Silver stain (LPS)	(-)	
Bi-refringence (lipid spherocrystals)	(-)	
Sudan black B (SBB) (1) (general lipids)	(+)	-Micelle-like spheres visible under light microscope.
Bromine-SBB (2) (lipids)	(-)	
Bromine-acetone-SBB (3) (phospholipids)	(-)	-Gel damaged by the acetone.
Nile blue (acidic lipids-neutral fats)	Deep purple (+)	
Acetone-Nile blue (phospholipids)	Deep blue (+?)	-Gel damaged by acetone. -All gel stained blue. -HSF band darker than background.
Phosphine 3R (neutral fats)	Yellow-orange (+)	-Fluorescent under u.v.light and also visible under white light.
Neutral red (lipids)	Yellow-pink (+)	-Stain bound electrostatically to the gel. -Band also visible under white light.

TABLE 7.6: NUMERICAL ASSESSMENT OF CONGO RED UPTAKE BY *Y. ruckeri*

STRAIN 2 (HSF+)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	1	1	NG	1	2
75	1	1	1	NG	NG
50	1	1	1	1	2
25	1	1	1	1	1
5	1	1	1	1	1

STRAIN 5 (HSF+)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	1	1	2	1	1
75	1	1	1	1	1
50	1	1	1	1	1
25	1	1	1	1	1
5	1	1	1	1	1

STRAIN 39 (HSF-)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	2	3	4	5	5
75	2	3	4	5	5
50	1	3	4	4	5
25	1	3	3	3	4
5	1	1	1	1	1

STRAIN 19 (HSF-)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	3	3	4	3	5
75	3	3	3	NG	NG
50	1	3	4	3	5
25	1	2	4	2	3
5	1	1	1	1	1

NG : No Growth.
Intensity of colour see (section 3.2.5.3)

TABLE 7.7. NUMERICAL ASSESSMENT OF COOMASSIE BLUE UPTAKE BY *Y. nickeri*

STRAIN 2 (HSF+)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	1	1	1	1	1
75	1	1	1	1	1
50	1	1	1	1	1
25	1	1	1	1	1
5	1	NG	1	1	1

STRAIN 5 (HSF+)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	1	1	1	1	1
75	1	NG	1	1	1
50	1	1	1	1	1
25	1	1	1	1	1
5	1	1	1	1	1

STRAIN 39 (HSF-)

Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	4	5	5	5	5
75	4	4	5	5	5
50	4	5	5	5	4
25	2	3	3	4	4
5	1	2	1	1	1

STRAIN 19 (HSF-)

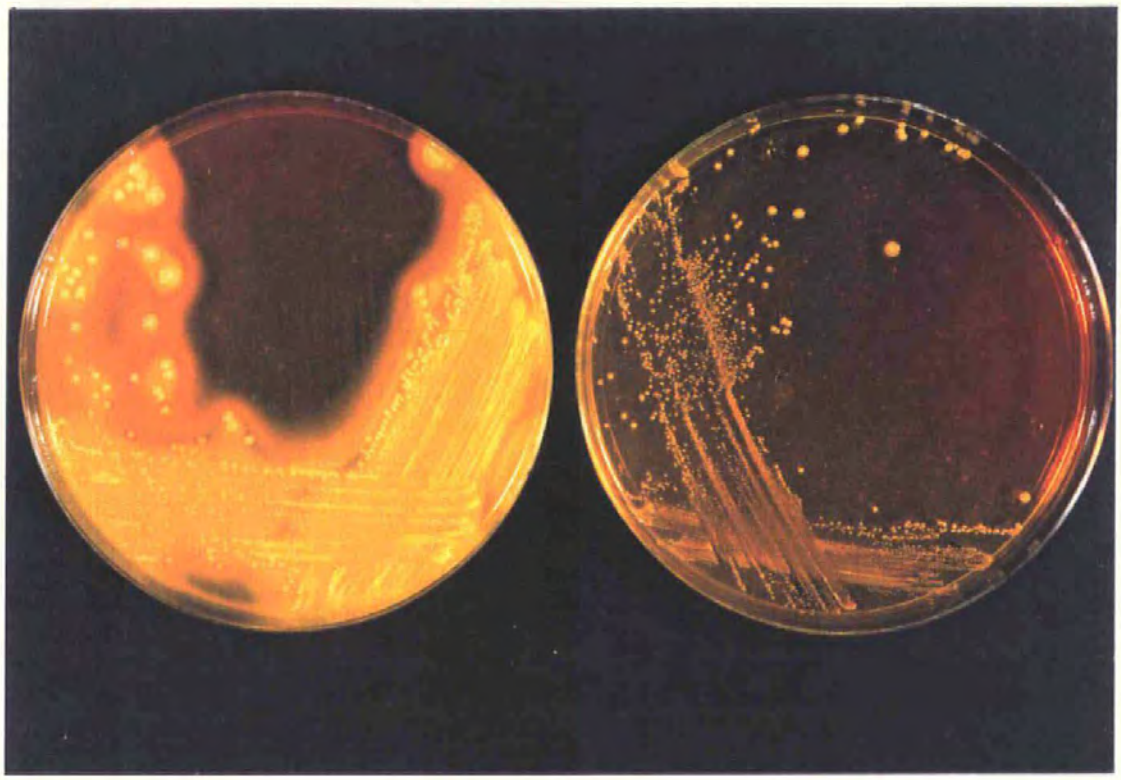
Conc µg/ml	Temperature (°c)				
	10	15	20	25	30
	Intensity of colour				
100	4	4	4	4	1
75	4	5	4	4	1
50	4	4	3	4	1
25	2	3	2	2	1
5	1	2	1	NG	1

NG : No Growth.
Intensity of colouration (see section 3.2.5.3)

Fig. 7.13: *Y. ruckeri* grown on ROD media. (1): strain 5 (HSF^+), note the yellow deposits surrounding the bacterial growth in contrast with (2): strain 19 (HSF^-).

Fig. 7.14: *Y. ruckeri* strain 26 grown on TSA with increasing amounts of SDS: (1) to (4) represent SDS concentration of 1%, 2%, 3% and 4% respectively. Plates were grown for 72 h.

1 2



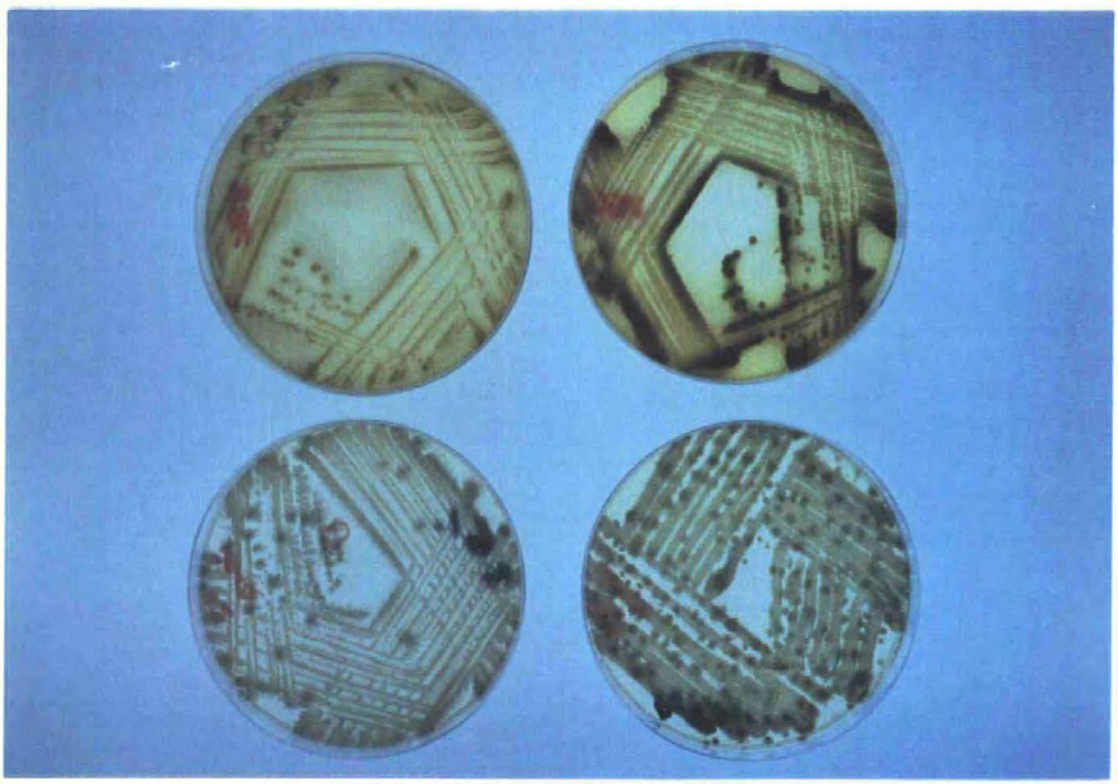


TABLE 7.8: MODIFICATIONS OF ROD MEDIA.

Growth on media for 10 days at 25°c

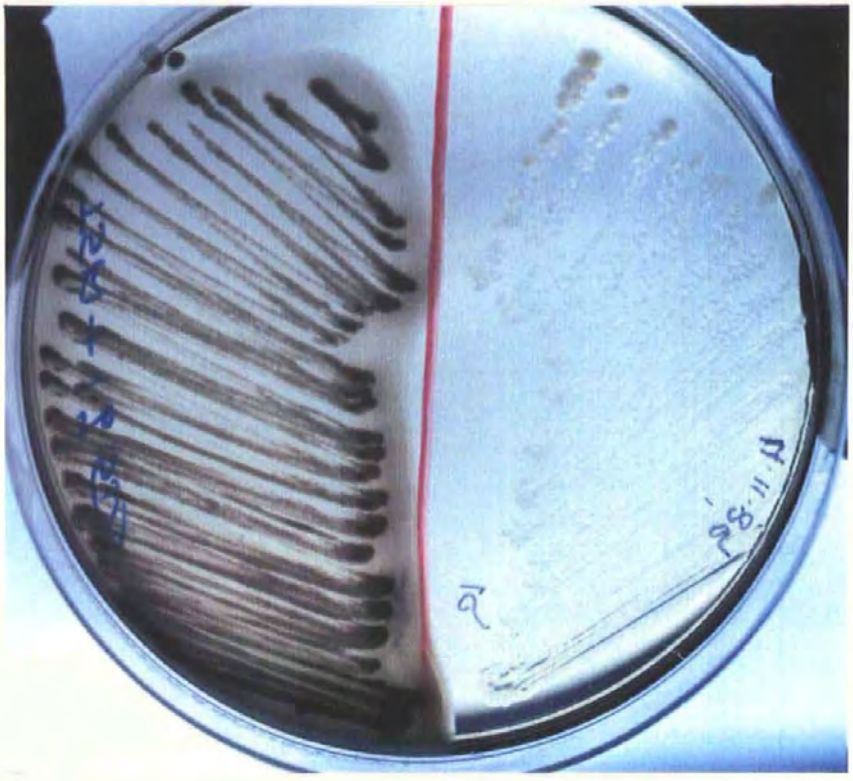
Strains	HSF^{+}					HSF^{-}		
Media	**2**	**5**	**9**	**30**	**35**	**19**	**22**	**39**
BM + Tryptone +1% SDS	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	N^{+}	N^{+}	N^{+}
BM + Ornithine + 1% SDS	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	N^{+}	N^{+}	N^{+}
BM + Tryptone and Ornithine + 1% SDS	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	N^{+}	N^{+}	N^{+}
BM + Tryptone and Ornithine. No SDS	N^{+++}	N^{+++}	N^{+++}	N^{+++}	N^{+++}	N^{+++}	N^{+++}	N^{+++}
BM + Ornithine, Maltose and Phenol Red	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	Y^{+++}	N^{++}	N^{++}	N^{+}

BM : Basal Media.
Y : Indicates presenceof deposit around bacterial growth.
N : Indicates absenceof deposit.
Superscriptsindicate extent of bacterial growth: $^{+++}$ heavy growth, $^{++}$ moderate growth, $^{+}$ poor growth.

Fig. 7.15: *Y. nuckeri* grown on TSA + 1% SDS. (1): strain 26 (HSF^+); (2): strain 19 (HSF^-). Plates were grown for 72 h.

Fig. 7.16: *Y. nuckeri* grown on TSA + 4% SDS. (1): strain 26 (HSF^+); (2): strain 19 (HSF^-). Plates were grown for 72 h.

1 2



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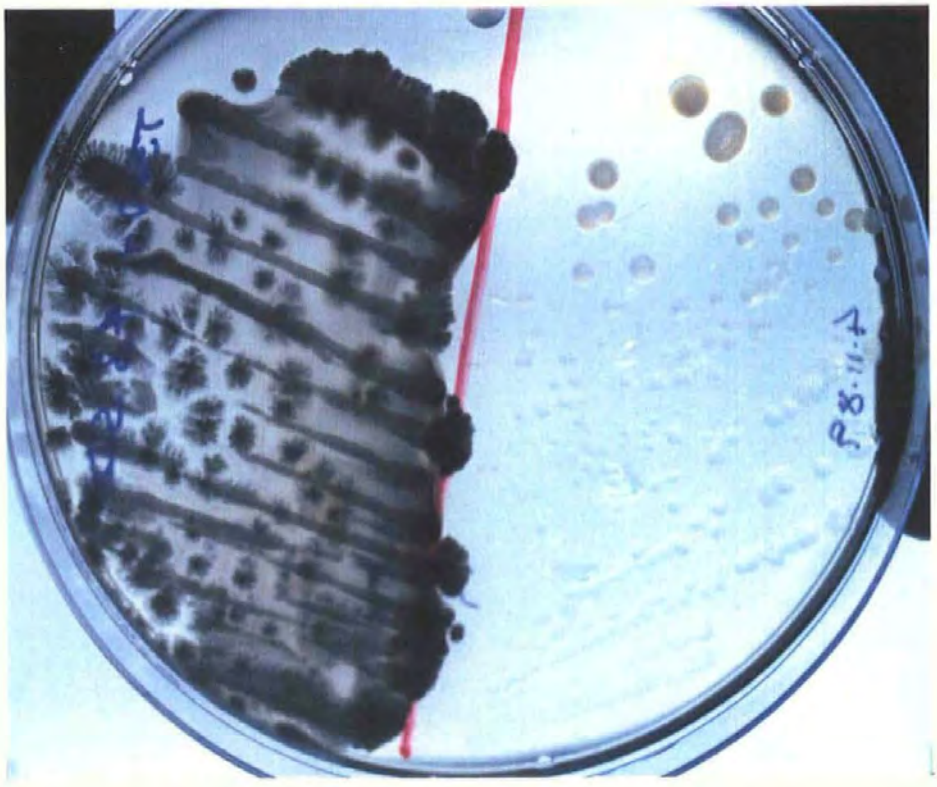


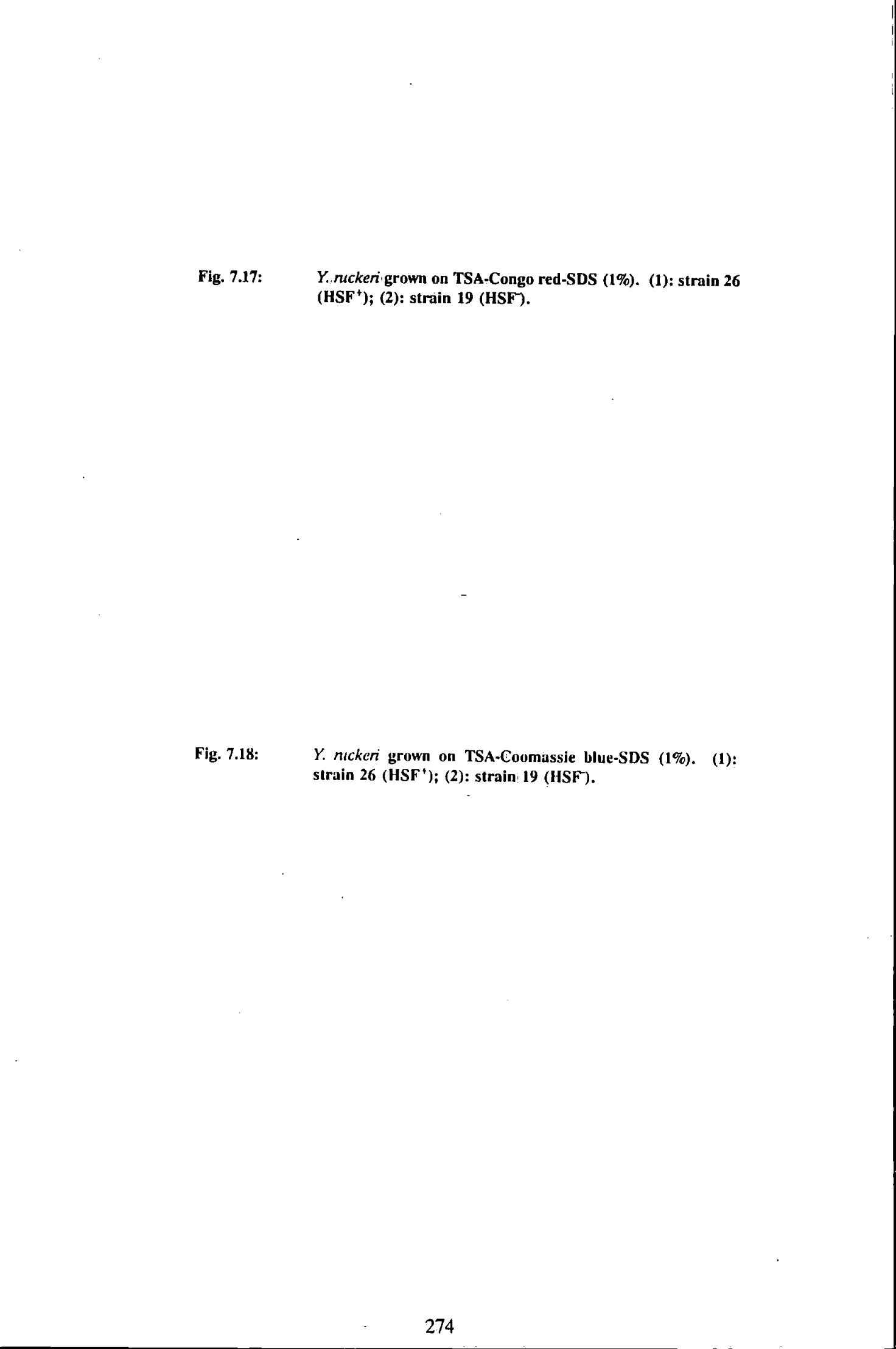
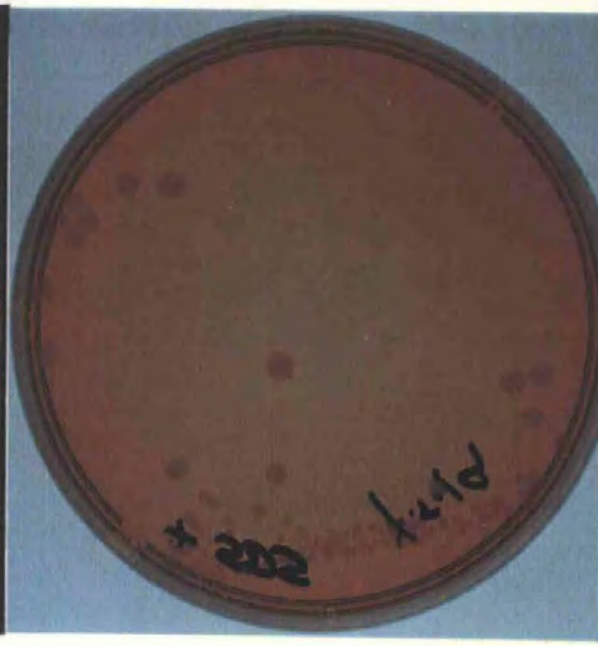


Fig. 7.17: *Y. nuckeri* grown on TSA-Congo red-SDS (1%). (1): strain 26 (HSF^+); (2): strain 19 (HSF^-).

Fig. 7.18: *Y. nuckeri* grown on TSA-Coomassie blue-SDS (1%). (1): strain 26 (HSF^+); (2): strain 19 (HSF^-).

1

2



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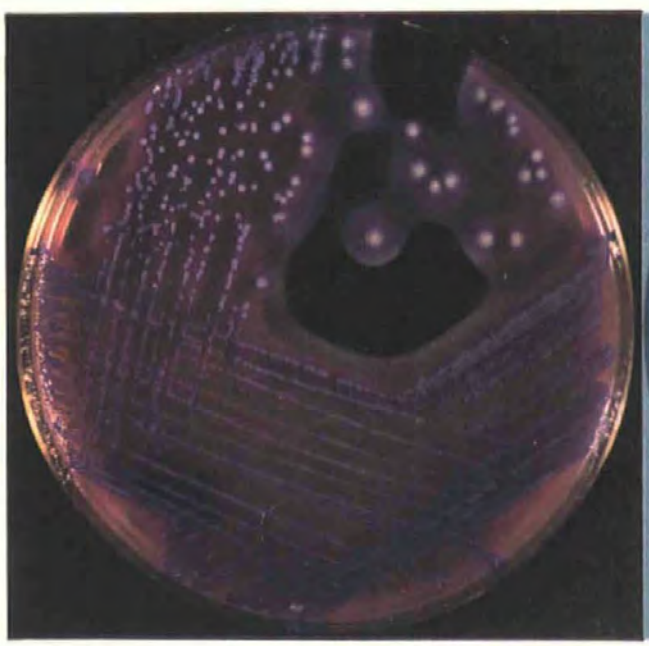

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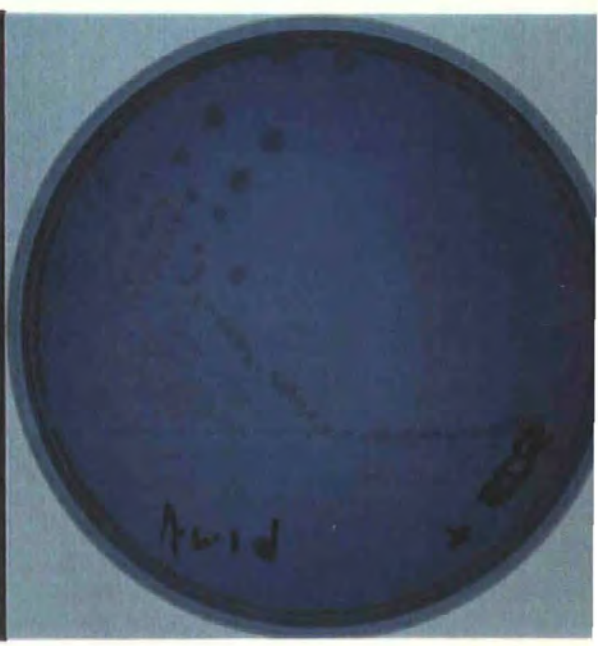
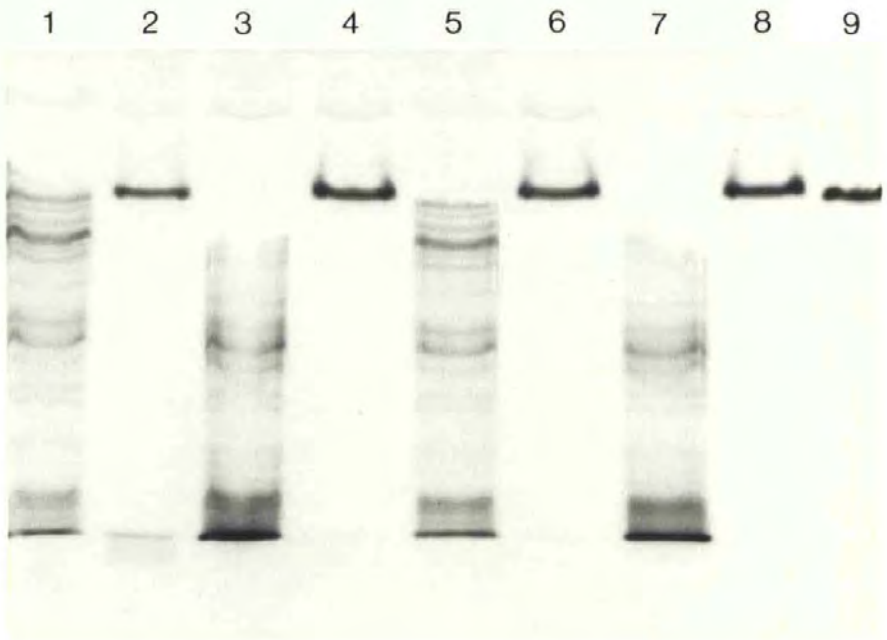


Fig. 7.19: **Protein profiles of PK digests of sonicated supernatants of *Y. ruckeri* strain 5, containing HSF. 12% SDS-PAGE stained with Coomassie blue. Lanes 2, 4, 6 and 8: sonicated supernatants digested for "zero", 60, 120 and 180 minutes respectively. Lanes 1, 3, 5 and 7 show the correspondent non-digested controls. Lane 9 showing PK only.**

Fig. 7.20: **Protein profiles of Lipase digests of sonicated supernatants of *Y. ruckeri* strain 5, containing HSF. 12% SDS-PAGE stained with Coomassie blue.**

Lanes 1 to 3: sonicated supernatants digested for 2 h with Lipase VII, II and I respectively. Lanes 4 to 6: Lipases VII, II and I respectively. Lane 7: non-digested sonicated supernatant. Lane 8: molecular weight standards (KDa).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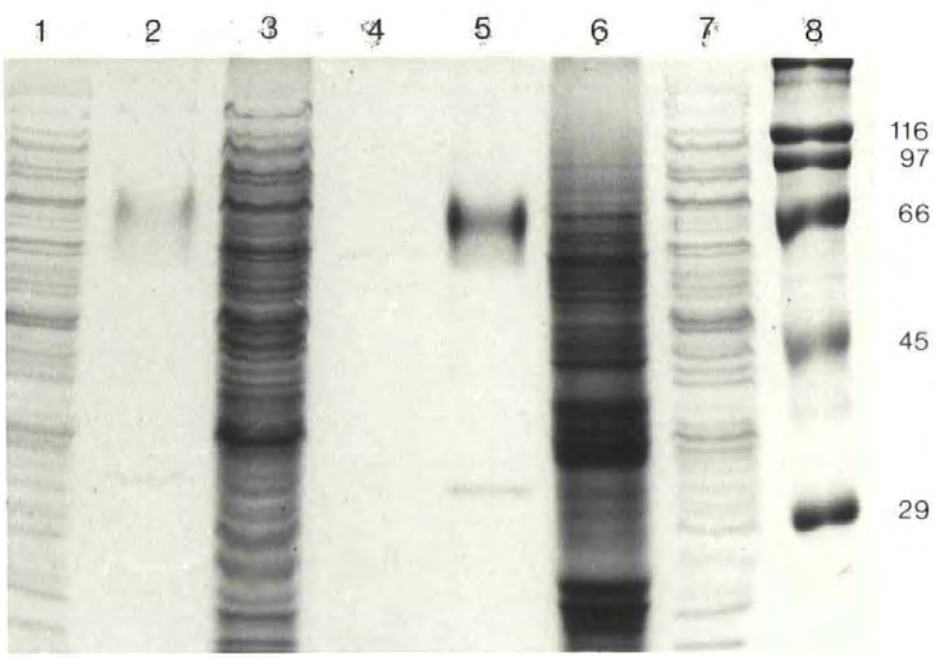
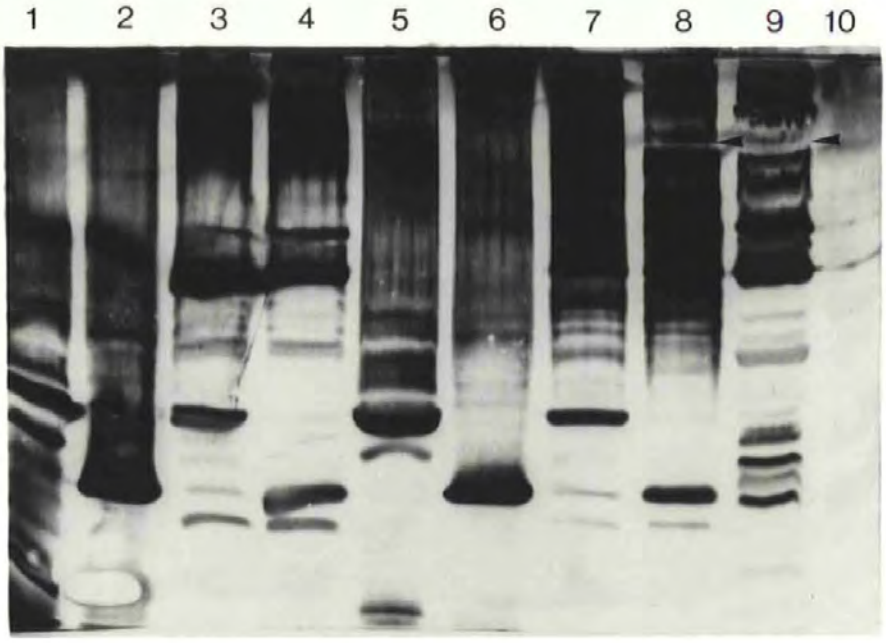

1
2
3
4
5
6
7
8
116
97
66
45
29

Fig. 7.21: Western blot of membrane preparations of *Y. ruckeri* strains 5 (HSF^+) and 19 (HSF^-). (1): heated outer membrane fraction of strain 19; (2): non-heated outer membrane of strain 19; (3): heated inner membrane of strain 19; (4): non-heated inner membrane of strain 19; (5): heated outer membrane of strain 5; (6) non-heated outer membrane of strain 5; (7): heated inner membrane of strain 5; (8): non-heated inner membrane of strain 5; (9): non-heated cell extract of strain 5 and (10): molecular weight standards. HSF band arrowed.

Fig. 7.22: Western blots of cell extracts, culture supernatants and ECP of *Y. ruckeri* strains 5 and 19.

A: samples obtained from cellophane overlays. Lanes 1 and 2: ECP of strains 19 and 5 respectively. Lanes 3 and 4: cell extracts of strains 19 and 5 respectively.

B: samples obtained from overnight cultures. Lanes 1 and 2: culture supernatants of strains 19 and 5 respectively. Lanes 3 and 4: cell extracts of strains 19 and 5 respectively. HSF band arrowed.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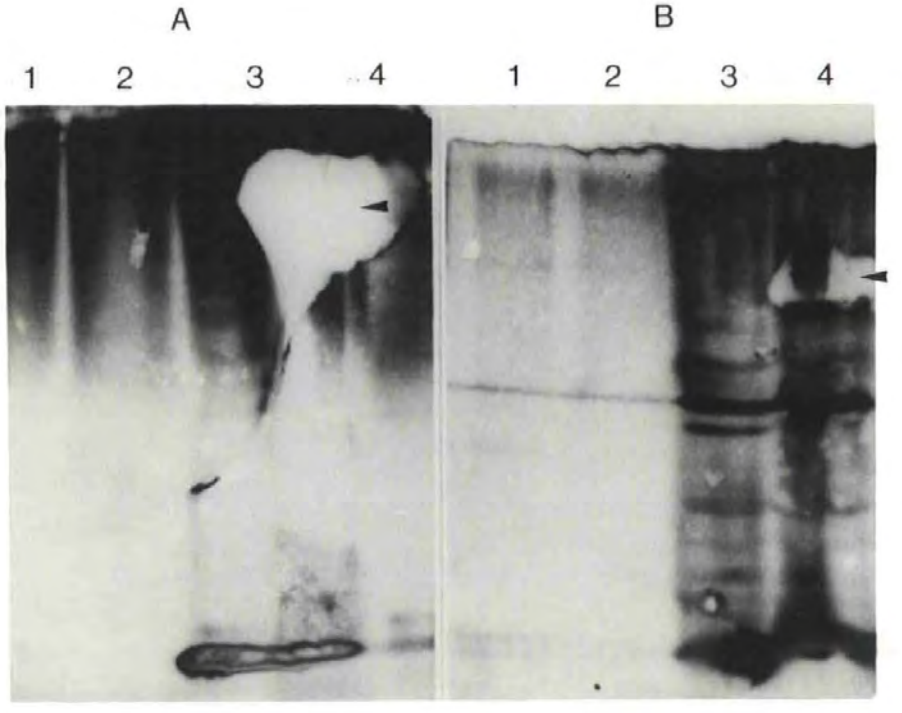
A
B
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4

Fig. 7.2 3: Elution profile of *Y. ruckeri* (strain 26) sonicated supernatant on ultragel AcA22

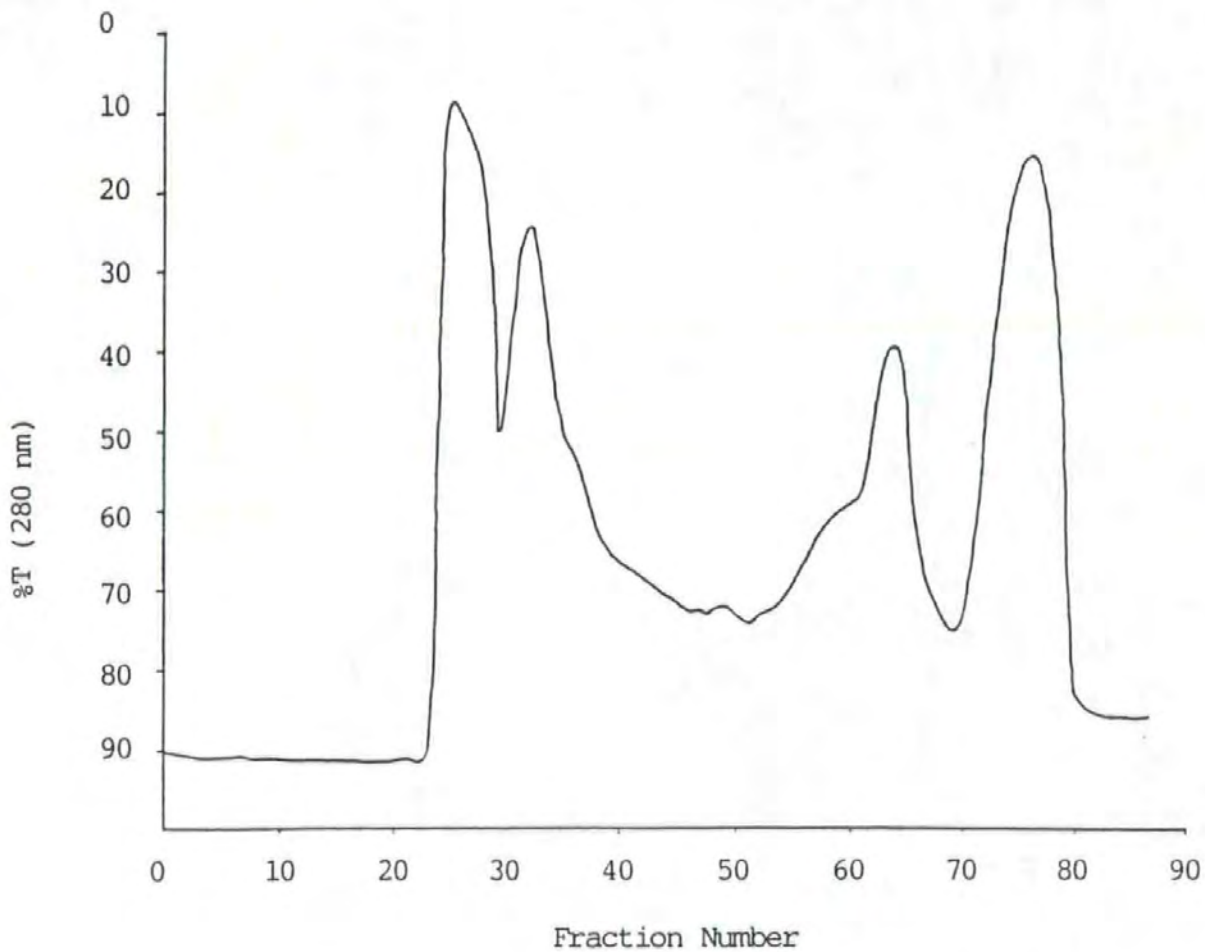
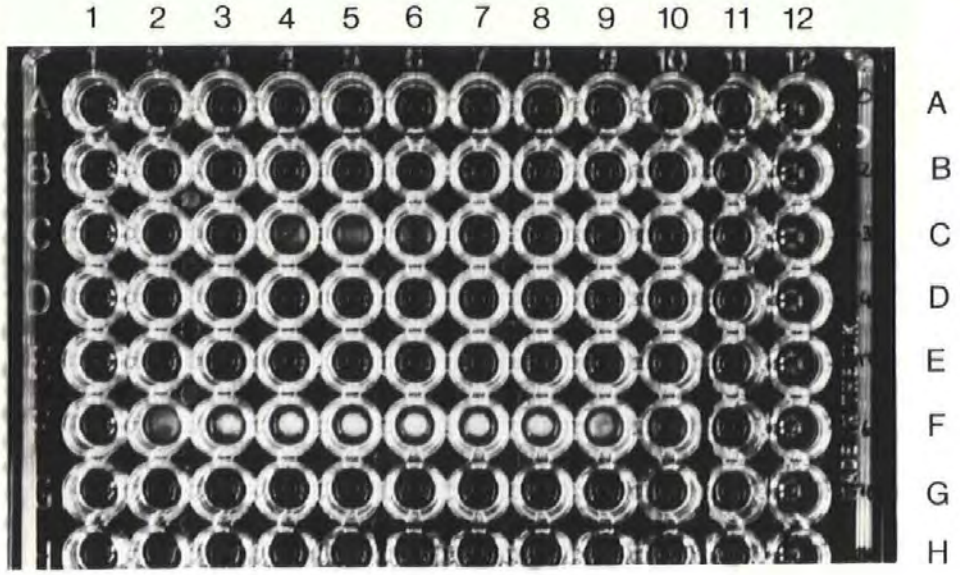


Fig. 7.24: **Microtitre plate containing aliquots of fractions from sonicated supernatants of *Y. ruckeri* strain 26, obtained by column chromatography. Observe the presence of a white precipitate (HSF) in wells 3F to 8F.**

Fig. 7.25: **Unstained 12% SDS-PAGE of aliquots of fractions 52 to 59 (Lanes 1 to 8), from *Y. ruckeri* strain 26. HSF band in white.**

Fig. 7.26: **Coomassie blue stained 12% SDS-PAGE of aliquots of fractions 52 to 60 (Lanes 1 to 9) from *Y. ruckeri* strains. Observe the "distorting" effect (arrowed) caused by the presence of HSF.**


1 2 3 4 5 6 7 8 9 10 11 12
A
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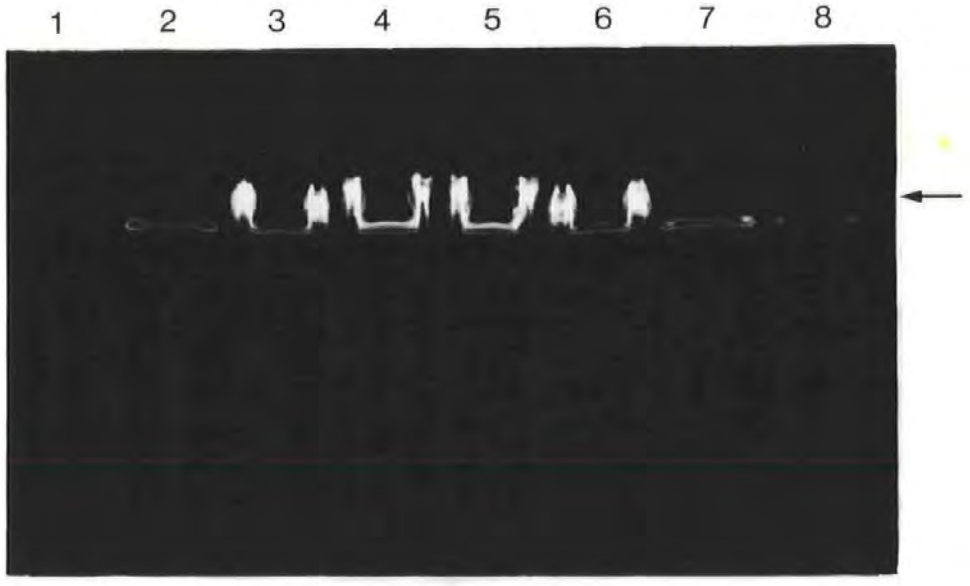
C
D
E
F
G
H


1 2 3 4 5 6 7 8

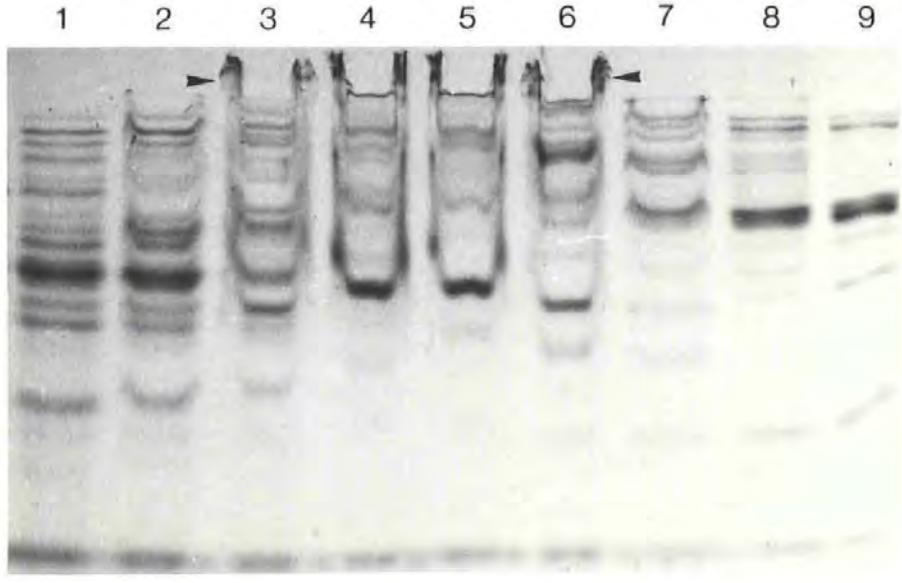
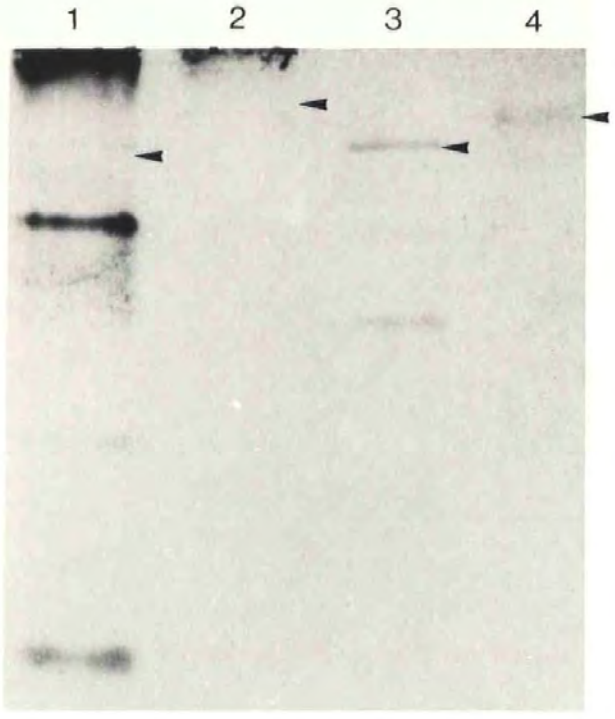

1 2 3 4 5 6 7 8 9

Fig. 7.27: **Western blot from a 12%-PAGE. (1): unheated cell extracts of *Y. ruckeri* 26; (2): PK-digested cell extracts; (3): unheated HSF fraction of *Y. ruckeri* strain 26; (4): PK-digested HSF fraction. Arrows indicate HSF bands.**

Fig. 7.28: **Western blot from a 7.5%-PAGE. (1): unheated cell extracts of *Y. ruckeri* strain 26; (2): PK-digested cell extracts; (3): unheated HSF fraction of *Y. ruckeri* strain 26; (4): PK-digested HSF fraction. Arrows indicate HSF bands.**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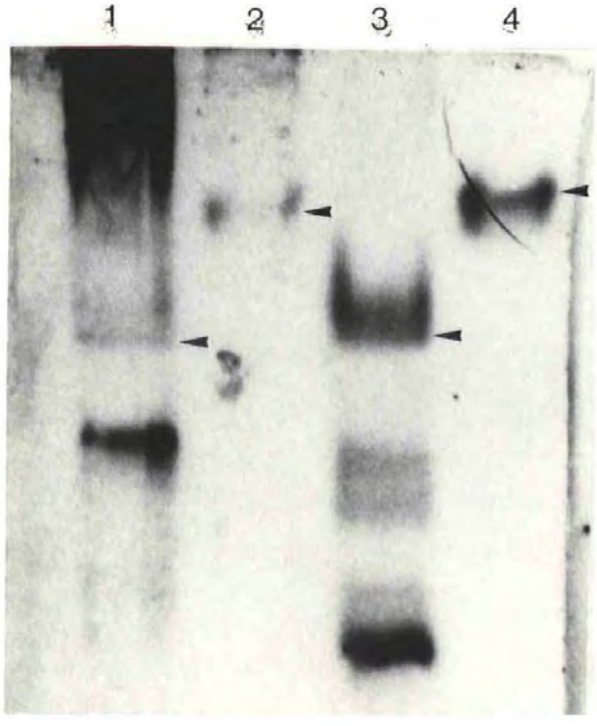
1
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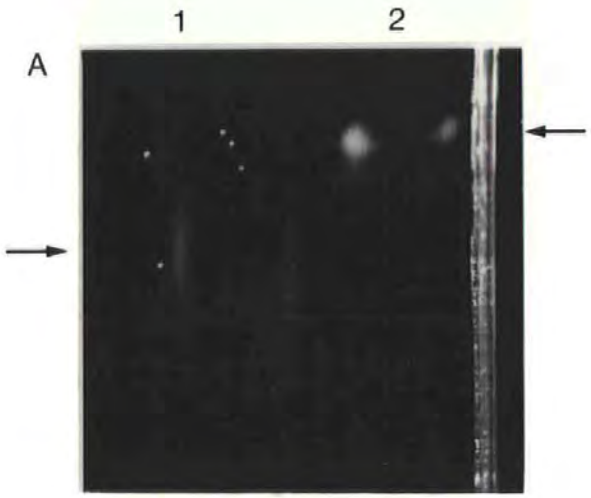
4

Fig. 7.29: **Visualization of the HSF-SDS complex on unstained 7.5%-PAGE treated with 10% SDS.**

A: HSF-SDS complex appearing on gel after 5 min of treatment with 10% SDS. (1): unheated HSF fraction of *Y. nickeri* strain 26; (2): PK-digested HSF fraction. Arrows indicate the HSF-SDS complex.

B: Progressive formation of the HSF-SDS complex. (1): Granular appearance of the band inside the gel, approximately 10 min after treatment with 10% SDS; (2): crystal-like formations on the gel, progressively covering the "granular" band inside the gel. Approximately 15 min after treatment with SDS; (3): granular and crystal like formations 30 min after treatment with SDS.

C: Aspect of the gel 3 h after treatment with SDS, showing the HSF-SDS complex of (1): HSF fraction and (2): PK-digested HSF fraction.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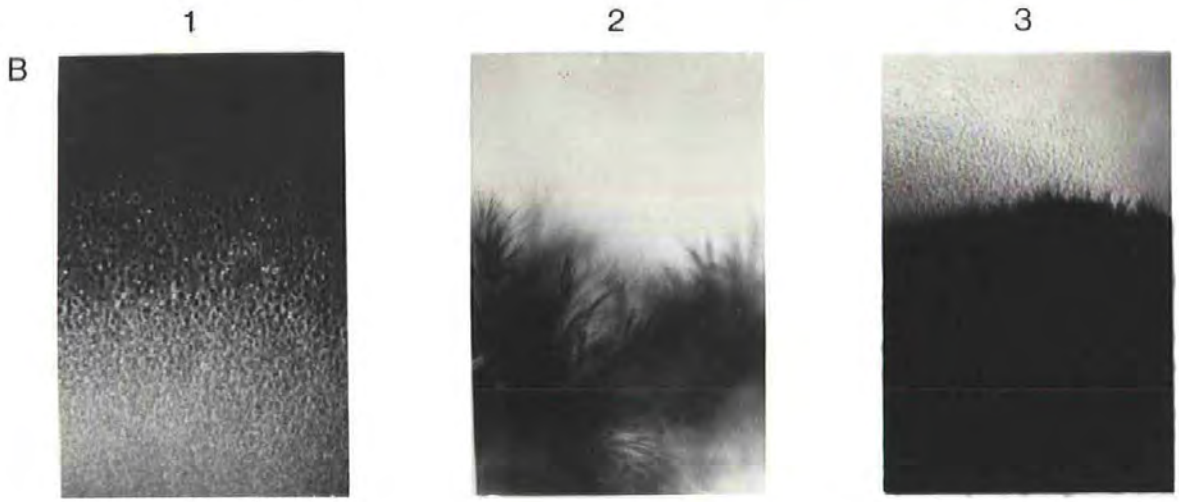
A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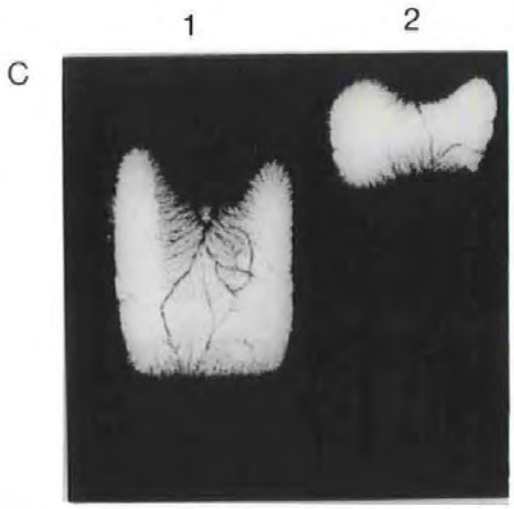
B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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The biochemical characterization of the large number of strains used in this study indicated that *Y. ruckeri* is a very homogeneous species. With the exception of the motility test, results were in close agreement with those of other authors. As a result of this characterization work, representative serotype O1 strains were selected for further work.

Because of the lack of consistency in published work referring to the virulence of *Y. ruckeri* a major aim of this study was to standardize a model for reliable artificial infection.

A series of preliminary studies established the optimal conditions for *in vitro* culture of *Y. ruckeri* (strain 5), which were grown on full strength BHI for 5 hours at 30°C (static conditions). This was followed by further investigations *in vivo*, in order to monitor any potential variations in the pathogen's virulence which could be related to these laboratory conditions.

An increase in the virulence of the original strain 5 was accomplished by a series of 7 serial passages of the pathogen in fish. The virulence of the resulting strain (26) was stable when stored in glycerol (-20°C or -70°C) for at least one year. Results also showed that virulence levels could be

maintained after 6 consecutive subcultures of strain 26 on laboratory media.

Despite the precautions taken to keep a homogeneous fish supply, significant differences in response to *Y. ruckeri* infection were found in stocks of fish which had been spawned in the winter. This stressed the necessity of performing a preliminary LD_{50} test for all lots of fish received.

Following intraperitoneal injection, environmental factors such as fish stocking densities or water temperature seemed to have a more marked effect on mortality than factors related exclusively with the fish status (i.e. size). Conditions for inoculum preparations (time of incubation and concentration of media) were important in the infection process when both mortality results and data of bacterial recovery from surviving fish were considered together. As a result, inocula of *Y. ruckeri* grown on full strength of BHI for 5 h at 30°C in static conditions were chosen to perform infections. Interestingly, such conditions were the same as those of optimal *in vitro* growth, except for the incubation in static conditions. When infection by immersion was performed, no obvious effect of bacterial dose on the infection process was observed within the range of bacterial concentration studied. Exposure time to the pathogen, however, seemed to affect the establishment of *Y. ruckeri* in the lower intestine of the fish, suggesting that longer exposure to the pathogen led to development of a higher number of carrier fish. Therefore, it could be interesting to study new models of infection based on a slow delivery of bacteria over a long period of time. Such a model could possibly

mimic the natural conditions of infections in a more accurate way than one single high inoculum. Use of a continuous culture (chemostat) system would offer considerable advantages and further work could be undertaken to endeavour to overcome the difficulties of standardization of a homogeneous inoculum.

Subsequently, infection by immersion using bacterial cultures suspended in their own media, delivered to the fish directly in tanks was preferred to options involving lengthy inoculum preparation (e.g. addition of latex particles) and unnecessary handling (e.g. abrasion or HI) of the fish, since experimental evidence indicated that there were no significant differences in the results produced providing that any area-effect due to the position of the tanks in the aquarium was eliminated.

In order to test the usefulness of the infection model, a series of experiments were undertaken in which dietary vitamin E was the variable under study. Vitamin E depletion in fish fed low vitamin E diets (7 mg kg^{-1}) was evident from the haematological, biochemical and histological results obtained. However, unexplained mortality patterns were observed in the groups of fish fed different dietary vitamin E levels. Fish fed diets with an intermediate level of vitamin E (86 mg kg^{-1}) gave the highest mortalities. There was no obvious explanation for such a result. A complex interaction between the requirements needed by *Y. ruckeri* to display full pathogenicity and the conditions occurring in the fish as a response to a depleted dietary vitamin

E level could account for the observed phenomena.

Serum antibody levels did not correlate with the mortality patterns obtained, suggesting that additional immunological tests should be included in this kind of study in order to obtain a more accurate picture of the response of the immune system to infection under the conditions studied.

The different dynamics observed in the recovery of *Y. ruckeri* from fish following infections by i.p. injection and immersion, emphasised the importance of the infection model used in the results obtained. It was also obvious that more information about the pathogenicity mechanisms of *Y. ruckeri* was needed, in order to be able to interpret the kind of results obtained in the dietary study of vitamin E. In this respect, a comparison between *Y. ruckeri* strain 5 and its homologous passaged strain 26 of higher virulence was carried out, using SDS-PAGE. No obvious differences were found among the various characteristics compared. The effect of environmental (culture) conditions was, however, noticeable when intrastrain variations were studied. Additionally, neither cells nor ECP of strains 5 and 26 displayed cytotoxic effects in the cell lines tested.

Since the comparative work performed with this pair of strains did not give any evidence about the virulence factors of *Y. ruckeri*, a comparison between a series of serotype O1 strains was undertaken.

Preliminary *in vivo* studies showed that there were important interstrain variations in virulence. Such results agreed with observations made by Flett (1989) and Davies (1990). It was also noticed that virulent strains produced lower antiserum titres in rainbow trout than avirulent ones. Western blotting and SDS-PAGE studies of cell extracts revealed the presence of a previously undescribed heat sensitive factor (HSF) occurring only in the virulent strains. Although HSF was the only factor which could be clearly associated with virulence in the present study, further work involving other cell components (OMP, LPS, plasmids) would produce very valuable information which should help to explain the virulence mechanisms of *Y. ruckeri* serotype O1 strains.

From work carried out using SDS-PAGE, it was thought initially, that HSF was non-immunogenic (at least in rabbits). However, Western blots performed from native gels showed later that HSF was in fact immunogenic. There were also indications that partially purified HSF injected into rainbow trout could protect them from challenge with a virulent strain of *Y. ruckeri*.

Initial work with HSF was based on its study using SDS-PAGE, since the complex formed by SDS with HSF was the only means of vizualizing the putative virulence factor on the gels. Such a complex is probably the result of micelle formations.

To date, the nature of HSF has not been determined. Histochemical studies using the complex indicated that HSF reacted with all differential staining

except those for proteins, whereas when native gels were used, only Phosphine 3R (a lipid stain) produced a reaction. Digestion of cell extracts with lipases also pointed to the possibility of a lipid component in the factor. The combination of Coomassie brilliant blue and SDS in TSA plates proved to be a good bacteriological medium to screen for virulent HSF^+ strains of *Y. ruckeri*. From these results, the importance of using HSF as a diagnostic characteristic in epidemiological studies can be realised.

Despite the attempts made to obtain an isogenic pair (HSF^+/HSF^-), no mutant clones were detected in any of the techniques used. Future work using electroparation techniques or transposon mutagenesis could probably prove useful in achieving such a pair of strains. Successful implementation of such genetic techniques is essential to be able to assess the significance of HSF in the pathogenic process.

HSF appears to be located in the periplasmic space of the cells and it does not seem to be exported, at least in a detectable form, to the outside of the cells. This makes it difficult to hypothesize about its role in pathogenicity. Western blotting studies of proteinase K partially purified HSF indicated the involvement of a proteinaceous component in the make-up of the factor. This fact could explain the reasons why HSF is not detectable in organic phases after solvent extractions. The nature of HSF appears to be unique and interesting; and only detailed physico-chemical analysis is likely to reveal sufficient information to assess its properties. Further studies are also needed

to elucidate the role played by the HSF of *Y. ruckeri* in the various stages of the host-pathogen interactions.

In summary, this work has demonstrated the value of a reliable and reproducible *in vivo* testing model. This has allowed the investigation of factors affecting interactions between *Y. ruckeri* and its fish (rainbow trout) host. Significantly, this has led to the discovery of a previously undescribed factor which could be of value in an understanding of pathogenic mechanisms and epidemiological studies. Further avenues for such investigations are now possible.

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