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Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*

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Abstract

The proportion of vibrio E, dominant in healthy turbot larvae, was artificially increased in the rotifer enrichment medium. The main effect of this vibrio enrichment was to improve the resistance of larval turbot challenged with a pathogenic strain of *Vibrio splendidus*, vibrio P. The improvement of the survival rates was significant with 1.5×10^6 CFU of vibrio E added per ml of enrichment medium, and it was still maximum at the highest dose tested (5×10^7 CFU.ml^{-1}; 80 ± 2% of survivors after 48 h of challenge, versus 50 ± 2 % for the control group).

Further *in vitro* characterisation showed that vibrio E was able to grow in the presence of the chelating agents EDDHA and EDTA, while producing siderophore, as revealed by chrome azurol S dyeing. The pathogenic vibrio P did not grow on iron-depleted medium, and it did not seem to produce siderophore. Consequently, a second experiment attempted to appraise the role of iron availability in the interaction between the pathogenic and probiotic vibrios. To this end, turbot larvae were fed with rotifers treated by the bacterial siderophore deferoxamine. This treatment significantly increased the resistance of larvae challenged with vibrio P in comparison with the control group, but to a lesser extent than the group enriched with vibrio E. There was also an increase of the growth rate due to either the siderophore treatment or the vibrio enrichment, as well. No further improvement was observed when the rotifers were simultaneously both treated and enriched. It was concluded that the probiotic effect of vibrio E may be at least partly due to competition for iron with the pathogen.

Keywords: bacteria, pathogenic vibrio, probiotic vibrio, experimental infection, siderophore, turbot, larva.

Production de sidérophore et effet probiotique de Vibrio *sp. associé aux larves de turbot,* Scophthalmus maximus.

Résumé

La proportion du vibrion E, dominant dans des larves de turbot en bonne santé, a été artificiellement augmentée dans le milieu d'enrichissement des rotifères. Cet enrichissement a eu pour principal effet d'améliorer la résistance des larves de turbot infectées expérimentalement par une souche pathogène de *Vibrio splendidus*, le vibrion P. L'amélioration des taux de survie était significative avec un ajout de 1.5×10^6 unités formant colonie (CFU) de vibrion E par ml de milieu d'enrichissement. L'effet était encore maximum avec la plus haute dose (5×10^7 CFU.ml^{-1}; 80 ± 2 % de survivants après 48 h d'infection expérimentale, contre 50 ± 2 % pour le groupe de contrôle). Le vibrion E peut être cultivé en présence des chélateurs EDDHA et EDTA, et il produit un sidérophore révélé par la coloration au chrome azurol S. Par contre, le vibrion P ne forme pas de colonies en milieu carencé en fer par chélation, et il ne semble pas produire de sidérophore. Une seconde expérience a donc été menée pour tester l'hypothèse d'un rôle de la disponibilité en fer dans l'interaction entre probiotique et pathogène. Des larves de turbot ont été nourries dans ce but avec des rotifères traités avec un sidérophore bactérien, la déferroxamine. Ce traitement a amélioré la résis-

tance des larves contre le vibrion P, mais moins que ne le permettait l'enrichissement en vibrion E. Le poids moyen des larves de turbot a augmenté aussi bien grâce au sidérophore qu'avec l'ajout du vibrion probiotique. Aucune amélioration supplémentaire n'a été observée avec l'emploi simultané des deux traitements. On peut en conclure que l'effet probiotique du vibrion E est dû au moins en partie à la compétition pour le fer avec le vibrion pathogène.

Mots-clés: bactéries, vibrion pathogène, vibrion probiotique, infection expérimentale, sidérophore, larve, turbot.

INTRODUCTION

Considerable progress has been achieved in the production of turbot larvae, but the survival rates obtained in hatchery are still insufficiently reliable (Planas, 1994). The bacterial environment is clearly involved in this flaw, and several studies have attempted to control it. Recently, Munro *et al.* (1995) have shown in gnotobiotic conditions the detrimental effect of *Vibrio anguillarum*, while *Aeromonas* sp. and *V. alginolyticus* were harmless for turbot larvae. These authors have called for the selection of bacteria that could be established in the gut, while guarding the larvae from the invasion of opportunistic pathogens. This was the aim of Skjermo *et al.* (1997), who have proposed the so-called "microbial maturation of water" that delayed gut colonisation while increasing the survival rate of turbot larvae. An artificial enrichment of the flora with a lactic acid bacterium improved also the resistance of the larvae against a pathogenic vibrio (Gatesoupe, 1994). However, most attempts to find probiotic bacteria in fish have used vibrios, like *V. pelagius* which was able to colonise the gut of larval turbot soon after hatching (Ring *et al.*, 1996). Austin *et al.* (1995) succeeded in improving the resistance of Atlantic salmon against *Aeromonas salmonicida*, *V. anguillarum* and *V. ordalii* by previous immersion in a suspension of *V. alginolyticus*. The first experiment reported in the present paper was to test the probiotic effect of several doses of a new strain isolated from healthy larval turbot.

The mechanisms of action of probionts in fish have not been clearly understood so far, though there has been a rapid increase in the number of publications on this subject. Several kinds of substances produced by bacteria may account for antagonistic effects in the aqueous environment. Nair *et al.* (1985) showed that large proportions of marine bacteria produced bacteriolytic enzymes tested against *V. parahaemolyticus*. Imada *et al.* (1985) isolated a protease inhibitor from *Alteromonas* sp. that was effective against *Aeromonas hydrophila*. Several workers isolated bacterial strains with inhibitory effects against fish pathogens from seaweeds (Dopazo *et al.*, 1988), adult turbot (Westerdahl *et al.*, 1991) and halibut larvae (Bergh, 1995). Dopazo *et al.* (1988) tested the effect of culture filtrates on pathogenic bacteria and concluded that competition for nutrient could not account for this inhibition. However, Pybus *et al.* (1994) demonstrated that the inhibitory effect of *V. anguillarum* against *V. ordalii* was due to a siderophore. Moreover, Fouz *et al.* (1994) emphasized the role of iron availability in the virulence of *V. damsela* against turbot. The role of siderophore in the probiotic effect of bacteria has not so far been evidenced *in vivo*. With this in view, the second experiment presented here was an attempt to compare the effect of a purified bacterial siderophore to the effect of the candidate probiotic on larval turbot.

MATERIAL AND METHODS

Larval rearing

Two batches of one-day-old turbot larvae, *Scophthalmus maximus*, were reared till day 10 after hatching in eighteen 150 l cylindrical tanks with conical bottoms, according to the method previously described (Gatesoupe, 1995).

The first experiment dealt with the dose effect of bacterial culture added into the enrichment medium of rotifers. The six groups of three tanks each were designated by their dose of bacterial enrichment (in $ml.l^{-1}$, *i.e.* treatments 0, 0.1, 0.3, 1, 3.3 and 10). The second experiment investigated the interaction of bacterial enrichment with iron availability. The control group of four replicates (treatment C) was compared to three other groups: (1) enriched with the candidate probiotic (treatment B; 4 tanks), (2) treated with the bacterial siderophore deferoxamine (treatment D; 5 tanks), (3) both enriched and treated (treatment BD; 5 tanks).

The initial stocking density was 23 and 20 $larvae.l^{-1}$ in the two consecutive experiments, respectively. The rearing temperature was gradually increased from 14 °C to 18 °C and the salinity was 3.5‰. Seawater was renewed continuously, at the rate of 600 $ml.mn^{-1}$. The larvae were fed with rotifers that were transferred daily from their culture medium into clean seawater tanks (200 $rotifers.ml^{-1}$). The rotifers were enriched in experiment 1 with spray-dried fish autolysate, menhaden oil, soybean lecithin and DL-α-tocopherol, at the rates of 15, 7.7, 0.7 and 0.015 $mg.l^{-1}$, respectively, on a dry matter basis. The fish autolysate was omitted from the enrichment mix in experiment 2, in an attempt to reduce iron supply. The experimental addition of def-

eroxamine mesylate (chemical from Sigma; 5 $mg.l^{-1}$ of enrichment medium) was aimed at reducing the availability of iron in treatments D and BD (experiment 2). The rotifers were continuously offered to the larvae with a peristaltic pump, the flow rate of which was adjusted so that the whole volume was carried through in 24 h.

Characterisation of bacteria

The pathogen used in these experiments was the "vibrio P" described in Gatesoupe (1995). This strain was further studied by Nicolas *et al.* (1996), who found its phenotypic characteristics and 16S rRNA sequence identical to those of the strain A 515, a scallop pathogen identified as *Vibrio splendidus*. The bacterium tested as candidate probiont was designated as strain E. It was isolated on Petrifilm (Aerobic Count Plates, 3M Health Care), as the dominant bacterium in eight-day-old turbot that looked especially healthy, with good growth and survival rates. These two strains were characterised, together with another strain S that was found dominant with the siderophore treatment D in experiment 2. Api 20 E and Api 50 CH strips were used for strain characterisation according to the manufacturer's instructions (bioMérieux). The 20 E suspension medium was replaced by sterile half-salinity water (1.8%; equal volumes of distilled and sea water), and the 50 CHE medium was supplemented with 50 $\mu l.ml^{-1}$ of a sterile saturated NaCl solution. The 20 E and 50 CH strips were incubated for 48 and 96 h, respectively, at 20 °C. Additional tests employed for strain characterisation were sensitivity to ampicillin, cefalotin and O/129 discs (10, 30 and 500 µg per disc, respectively; Sanofi Diagnostics Pasteur) and the Oxidation-Fermentation test of Hugh and Leifson (OF basal medium, AES Laboratoire). Plate Count Agar (PCA, Biokar Diagnostics) was dissolved in half-salinity water and the pH was adjusted to 7.8 for assessing the temperature requirement. Five temperatures were tested (4, 20, 30, 35 and 40 °C). In order to estimate the Na^+ requirement for growth, different NaCl concentrations (0, 3, 6, 8 or 10 %) were added to PCA dissolved in distilled water, plus 0.4 % $MgSO_4.7H_2O$ and 0.1% KCl; pH was adjusted to 7.8. The antagonism of strains E and S towards vibrio P was tested according to the double-layer method modified by Westerdahl *et al.* (1991). The hard and soft layers were prepared by adding 14 and 1.5 $g.l^{-1}$ of agar, respectively (Pastagar B, Sanofi Diagnostics Pasteur) to Tryptone Soy Broth (TSB, Biokar Diagnostics) dissolved in half-salinity water.

Assessment of iron availability for the bacteria

The ability of the three bacterial strains to obtain iron from their environment was tested in two ways. First, the chelators Ethylene-Diamine-Tetra-acetic Acid (EDTA disodium salt, dihydrate, Rhône-Poulenc Prolabo) and Ethylene-Diamine-Di-o-Hydroxy-phenyl-acetic Acid (EDDHA, Sigma) were added to PCA (half-salinity, pH 7.8). Three concentrations of both chemicals were tested (100, 200 and 400 $\mu g.ml^{-1}$), plus three other doses of EDDHA (20, 50 and 75 $\mu g.ml^{-1}$). Secondly, CPCA, a special medium for detecting siderophore production, was prepared by modifying the method of Schwyn and Neilands (1987). Three solutions were prepared separately for dyeing 1 l of CPCA: (1) 60.5 mg of chrome azurol S in 50 ml distilled water, (2) 2.7 mg of $FeCl_3.6H_2O$ in 10 ml HCl 0.01 N and (3) 72.9 mg of hexadecyltrimethylammonium bromide in 40 ml distilled water, under slight heating (chemicals from Sigma). Solutions 1 and 2 were mixed and gently added to solution 3 while stirring. The chrome azurol dye solution was autoclaved separately from PCA that was dissolved in half-salinity water. The pH of this agar solution was naturally close to 6.8, which was designated as the convenient value by Schwyn and Neilands (1987). After cooling at 60 °C, PCA and dye were slowly mixed on a hot plate-stirrer in a laminar flow hood. The CPCA plates were blue, and production of siderophore was deduced from the appearance of orange halos around the colonies.

Enrichment of the rotifer medium with the candidate probiotic

Strain E was cultivated for 24 h at 20 °C on TSB enriched with 1.3% sodium chloride. With 10% of inoculum rate (volume/volume), the final concentration in the TSB culture medium averaged $5 \pm 1 \times 10^9$ Colony Forming Units $(CFU).ml^{-1}$, counted daily on Thiosulphate-Citrate-Bile Salt agar (TCBS, AES Laboratoire) dissolved in half-salinity water (24 h incubation at 20 °C).

The strain E culture was prepared daily, and introduced the following day into the medium of rotifer enrichment for experimental treatments. The effect of initial probiont concentration was investigated in experiment 1, where the control group of three tanks was compared to five other triplicates treated with 0.1, 0.3, 1, 3.3 and 10 $ml.l^{-1}$ of bacterial culture (5×10^9 $CFU.ml^{-1}$), respectively. The bacterium was added at the rate of 4 $ml.l^{-1}$ into the enrichment media of two treatments in experiment 2, either alone or in combination with deferoxamine (treatments B and BD, respectively).

Counts of bacteria associated with the larvae

Twenty larvae per tank were sampled at day 8 for bacterial counts. They were handled in sterile conditions, while using half-salinity water for all the operations. The samples were first rinsed over 180 µm net, then they were suspended and crushed in 4.5 ml water in glass homogeniser. The suspension was diluted and counted on Petrifilm (10^{-4} dilution) and TCBS plates (10^{-4} and 10^{-5} dilutions), after incubation at 20 °C for 5 days and 24 h, respectively. Colonies were sampled in each Petrifilm plate at the rate of 9 and 25 CFU per

treatment in the two consecutive experiments, respectively. They were cultivated for 24 h at 20 °C on PCA (half-salinity water, pH 7.8), then harvested bacteria were suspended in half-salinity water for Api 20 E characterisation.

Challenge against the pathogen

The larvae were challenged with the pathogen at day 9 following Gatesoupe (1995). One day before inoculation, vibrio P was incubated at 20 °C on half-salinity TCBS agar. Then it was suspended in sea water, and the inoculum concentration was adjusted by measuring the transmission of 570 nm light through the suspension. The doses of infection were checked by counting the vibrios on TCBS agar plates. The concentration of the pathogen was thus estimated at 1.2 and 6×10^6 CFU.ml^{-1}, in the challenge medium of the two consecutive experiments, respectively. This challenge was performed in 500 ml polycarbonate square bottles (Nalgene), where 50 larvae were gently introduced per bottle (100 larvae.l^{-1}). The suspension of vibrio P was added to five bottles per replicate. A sixth bottle was used as a non-infected control for each replicate. In total, 108 bottles were used per experiment to assess the survival rates after 48 h of incubation at 18 °C.

The data obtained in each experiment were compared by analysis of variance (ANOVA), and when there was a significant difference, the effects of the individual treatments were assessed by an a priori test (Sokal and Rohlf, 1969).

RESULTS

Characterisation of the candidate probiotic strain E

Although it was non-motile, strain E showed many characteristics of vibrio, and its profile resembled that of *Vibrio alginolyticus*, but it was lysine decarboxylase negative and citrate negative (Table 1). Its variable ornithine decarboxylase activity confounded further identification according to Alsina and Blanch's keys (1994). No inhibition zone was observed in the plates testing the antagonism of strain E against vibrio P. It is important to note that it differed from vibrio P in its resistance to iron chelation by EDDHA, which inhibited its growth only at the highest concentration tested (400 µg.ml^{-1}), and EDTA, which was not inhibiting even at this dose. Moreover, the production of siderophore by strain E was observed on CPCA. The main other biochemical differences from vibrio P were the lack of β-galactosidase and arginine dihydrolase in strain E. The carbohydrate metabolism of the two strains seemed similar, except for sucrose, amygdalin and melibiose.

Effects of the treatments on the flora associated with turbot

In experiment 1, all colonies isolated from the larvae treated with the highest dose of bacterial enrichment (10 ml.l^{-1}) were identified as strain E by their Api 20 E profiles, while the proportion of this strain was only 11% of the total CFU with the dose of 0.1 ml.l^{-1} ($p < 0.05$; Fig. 1). The highest dose of enrichment significantly increased the total count of bacteria on Petrifilm (28 *versus* 8 to 11 $\times 10^4$ CFU.larva^{-1} with the other doses; $p < 0.001$). A similar effect was observed in TCBS counts (14 *versus* 3 to 4×10^4 CFU.larva^{-1} in the other groups; $p < 0.001$). Vibrio P was isolated in small proportion in the intermediate treatments (11, 25,14 and 11% of the isolates in treatments 0.1, 0.3, 1 and 3.3, respectively).

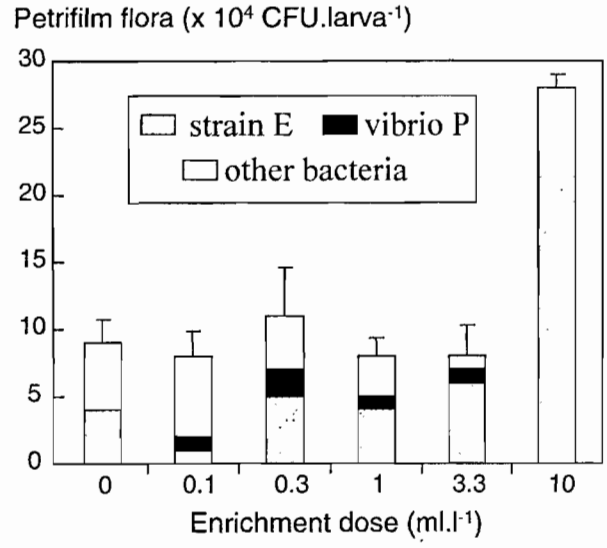

Figure 1. – The effect of enrichment dose on the proportion of strain E and total Petrifilm count in eight-day-old turbot in experiment 1 (symbols T: standard errors for total counts).

In experiment 2, The colonies identified as strain E represented 67% of the isolates from treatment B, versus 20% with treatment D ($p < 0.01$; Fig. 2). The total counts on Petrifilm were significantly higher in treatments B and BD (9 and 11 $\times 10^4$ CFU.larva^{-1}, respectively) than in treatments C and D (5×10^4 CFU.larva^{-1}; $p < 0.01$). A similar difference was observed by counting the TCBS plates (4×10^4 CFU.larva^{-1} with treatments B and BD, versus 13 and 5×10^3 CFU.larva^{-1} with treatments C and D, respectively; $p < 0.01$). Another strain, designated as strain S, was found dominant in treatment D, where it represented 80% of the isolates. The proportion of this strain was significantly lower in treatment C (46%), and minimal in treatment B (4%; $p < 0.001$). Its characteristics resembled those of a pseudomonad (positive reactions for motility, catalase, gelatinase, aesculin,

Table 1. - *Characteristics of the pathogenic vibrio P, the candidate probiotic E and the strain S, enhanced by the deferoxamine treatment D in experiment 2.*

Bacterium	Vibrio P	Strain E	Strain S
EDDHA (dose for complete inhibition in $\mu g.ml^{-1}$)	< 20	400	50
EDTA (dose for complete inhibition in $\mu g.ml^{-1}$)	200	> 400	> 400
siderophore production on CPCA	-	+	+
motility	+	-	+
TCBS colonies	green	yellow	yellow
% NaCl for growth	3-6	3-8	3
temperature for growth (°C)	4-20	20-40	4-35
ampicillin 10 µg	r	r	s
cefalotin 30 µg	s	r	s
β-galactosidase	+	-	-
arginine dihydrolase	+	-	-
ornithine decarboxylase	-	v	-
indole	+	+	-
acetoin production (Voges Proskauer)	+	+	-
reduction of nitrates to nitrites	+	+	-
oxidation of glucose (Hugh and Leifson)	+	+	-
fermentation of glucose (Hugh and Leifson)	+	+	-
Acid production from:			
arabinose	-	-	+
sucrose	-	+	+
amygdalin	+	-	-
mannitol	+	+	-
glycerol	+	+	-
ribose	+	+	-
galactose	+	+	-
melibiose	+	-	-
starch	+	+	-
gluconate	+	+	-
D-fructose	+	+	-
D-mannose	+	+	-
N-acetyl-glucosamine	+	+	-
cellobiose	+	+	-
maltose	+	+	-
trehalose	+	+	-
glycogen	+	+	-

v = variable reaction, r = resistant, s = susceptible. Common characteristics (1) positive tests: catalase, gelatinase, aesculin, oxidase; (2) negative tests: Gram, lysine decarboxylase, citrate, H_2S, urease, tryptophan desaminase; (3) no acid production from: inositol, sorbitol, rhamnose, erythritol, D- and L-xylose, adonitol, β-methyl-xyloside, L-sorbose, dulcitol, (-methyl-D-mannoside, α-methyl-D-glucoside, arbutin, salicin, lactose, inulin, melezitose, D-raffinose, xylitol, α-gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, 2- and 5-keto-gluconate; (4) resistance to O/129 (0.5 mg). Strains E and S were not antagonistic to vibrio P in the *in vitro* test.

oxidase, and acid from arabinose and sucrose; Table 1). It might belong to the genus *Alteromonas* because of its Na^+ requirement for growth. This strain was inhibited by 50 $\mu g.ml^{-1}$ of EDDHA, which indicated a sensitivity intermediary between those of vibrio P and strain E. It was also resistant to iron chelation by EDTA, and it produced siderophore on CPCA plates. No inhibition zone was observed in the plates testing its antagonism against vibrio P.

Challenge experiments

The larvae kept for 48 h in the challenge bottles had 94% survival rate in both experiments, when they were not infected with vibrio P.

The survival rates of the infected larvae in experiments 1 and 2 (Tables 2, 3, respectively) were significantly different among treatments ($p < 0.001$). In experiment 1, the best survival rates of infected larvae (78% on average) were observed with the highest enrichment doses (3.3 and 10 $ml.l^{-1}$). Intermediate results were obtained with the other doses, and the lowest survival rates were observed with the lowest dose (55%; 0.1 $ml.l^{-1}$) and the control treatment (50%, not significantly different from treatment 0.1). In the second experiment, 46% of the infected larvae in treatments B and BD survived after 48 h, versus 36% survival rate in the control group. An intermediate survival rate (41%) was obtained with the siderophore treatment D.

Larval rearing performances

The mean survival rate was higher in experiment 1 than in experiment 2 (82 ± 5% and 35 ± 3% at day 10, respectively; Tables 2, 3), but the means of treatments were not significantly different.

There were generally higher growth rates in the experimental treatments than in the control group in both experiments. However, the mean weight observed with the enrichment dose of 3.3 $ml.l^{-1}$ in experiment 1

Table 2. - Effects of bacterial enrichment doses on larval performances in experiment 1 (mean ± standard error; superscripts indicate significant differences among survival rates of the challenge test, or among mean weights, according to the a priori test of Sokal and Rohlf, 1969).

Enrichment dose ($ml.l^{-1}$)	Challenge survival rate after 48 h (%)		Survival rate at day 10 (%)	Mean weight at day 10 (mg)
	Not infected larvae	Infected larvae		
0	$93^{a} \pm 4$	$50^{e} \pm 2$	59 ± 18	$0.35^{b} \pm 0.01$
0.1	$96^{a} \pm 2$	$55^{ce} \pm 2$	85 ± 15	$0.32^{b} \pm 0.01$
0.3	$94^{a} \pm 3$	$65^{c} \pm 2$	79 ± 8	$0.34^{b} \pm 0.01$
1	$92^{a} \pm 1$	$59^{c} \pm 3$	92 ± 5	$0.41^{a} \pm 0.02$
3.3	$96^{a} \pm 1$	$76^{b} \pm 4$	89 ± 15	$0.34^{b} \pm 0.01$
10	$95^{a} \pm 1$	$80^{b} \pm 2$	87 ± 1	$0.38^{a} \pm 0.01$
ANOVA	$p < 0.001$		not significant	$p < 0.001$

Table 3. - Effects of bacterial enrichment and deferoxamine treatment on larval performances in experiment 2 (mean ± standard error; superscripts indicate significant differences among survival rates of the challenge test, or among mean weights, according to the *a priori* test of Sokal and Rohlf, 1969).

Bacterial enrichment	Treatment		Challenge survival after 48 h (%)		Survival rate at day 10 (%)	Mean weight at day 10 (mg)
	Deferoxamine	Code	Not infected larvae	Infected larvae		
-	-	C	$93^{a} \pm 3$	$36^{d} \pm 2$	32 ± 11	$0.41^{b} \pm 0.01$
+	-	B	$93^{a} \pm 3$	$45^{b} \pm 2$	36 ± 3	$0.46^{a} \pm 0.01$
-	+	D	$95^{a} \pm 1$	$41^{c} \pm 2$	34 ± 5	$0.48^{a} \pm 0.01$
+	+	BD	$95^{a} \pm 2$	$47^{b} \pm 2$	37 ± 6	$0.46^{a} \pm 0.01$
		ANOVA	$p < 0.001$		not significant	$p < 0.001$

(0.34 mg) did not differ from that of the control (0.35 mg), whereas those obtained with the doses of 1 and 10 $ml.l^{-1}$ were significantly higher (0.41 and 0.38 mg, respectively; $p < 0.001$). The mean weights of the three experimental treatments of experiment 2 were significantly higher than that of the control group (0.47 *versus* 0.41 mg; $p < 0.001$).

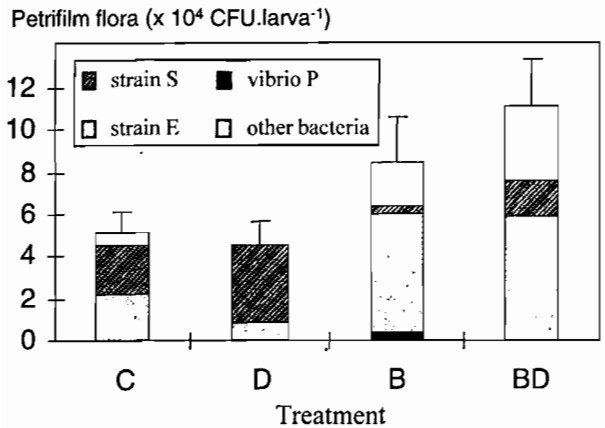

Figure 2. – The effect of the siderophore and/or bacterial treatment on the proportion of the dominant strains E and S, and total Petrifilm count in eight-day-old turbot in experiment 2 (symbols T: standard errors for total counts).

DISCUSSION

Most vibrios are motile in standard media, but some exceptions were reported, like a *Vibrio* sp. pathogenic to clam and oyster larvae (Brown and Tettelbach, 1988), and the probiotic strain of *V. alginolyticus* studied by Austin *et al.* (1995). Strain E is immotile but probably belongs to this genus considering its other characteristics, and it will be referred to in the following as "vibrio E".

Though the proportion of vibrio E was naturally high in the flora associated with the control larvae, it was possible to significantly increase this proportion by enrichment with bacterial broth culture. This effect was less dramatic than that obtained with Lactic Acid Bacteria (LAB) in previous experiments (Gatesoupe, 1994), but LAB were naturally present at a low level, or even not detected in larval turbot. However, the vibrio enrichment significantly affected the Petrifilm-TCBS flora, at variance with the previous results obtained with LAB enrichment. At its highest dose, which corresponded to 5×10^{7} $CFU.ml^{-1}$ in the enrichment medium, vibrio E was fully dominant in Petrifilm flora (3×10^{5} $CFU.larva^{-1}$) in the present experiments, whereas LAB cohabited with Petrifilm flora at the same level ($7\text{-}9 \times 10^{4}$ $CFU.larva^{-1}$) for a similar enrichment dose ($3\text{-}5 \times 10^{7}$ $CFU.ml^{-1}$) in the previous experiments.

The main probiotic effect of the vibrio enrichment was to increase the survival rate of the larvae after 48 h of challenge, as previously observed with LAB. The

optimal range of enrichment dose was 10^7 CFU.ml^{-1} for both LAB and vibrio E. An excessive dose of vibrio enrichment was not reached in the present experiments. The highest experimental dose accounted for 1% of the volume of enrichment medium, and the initial amount of TSB dehydrated medium corresponded to 300 mg per litre of enrichment medium. It does not seem reasonable to further increase this load of organic matter, because it could be detrimental for the rotifers.

The growth rate of turbot larvae might be increased when the rotifers were enriched with vibrio E, but it remains questionable. The dose response is indeed unclear, because this effect was not observed with an intermediary dose in the first experiment (1.7×10^7 CFU.ml^{-1}).

Vibrio E did not show any inhibitory effect against vibrio P *in vitro*, when there was no iron depletion. The potential pathogen P was still observed at a low level in larvae treated with a dose as high as 2×10^7 CFU of vibrio E per ml of enrichment medium. Vibrio P seems normally present in the larvae, but it is dominated by other strains. It is likely that this opportunistic pathogen becomes dominant only after the larvae have been exposed to unfavourable conditions (Gatesoupe, 1990).

Among pathogenic vibrios, Borrego *et al.* (1996) distinguished those obtaining iron with siderophores, like *V. anguillarum* and *V. vulnificus*, from those able to grow under iron limitation without producing siderophores, like several strains of *V. harveyi* and *V. pelagius*. Vibrio P is sensitive to iron chelation and it did not seem to produce siderophore. That may account, at least partly, for its relatively low virulence. The lack of siderophore production should be interpreted carefully, and it seems that there is not ideal medium to assess the intrinsic ability of bacteria to produce siderophore. On the one hand, the original medium of Schwyn and Neilands (1987) is too purified to allow this production by some bacteria (Gram, 1996). However, a less purified medium proposed for *Vibrio harveyi* required the addition of a chelator which could depress bacterial growth (Owens *et al.*, 1996). The present CPCA medium was a compromise, as it contained a low concentration of iron other than that complexed with the indicator, while allowing good growth rates of the strains tested.

In contrast to vibrio P, vibrio E produces siderophore and it can grow in iron-chelated medium. The probiotic effects of vibrio E were partly reproduced by the purified bacterial siderophore deferoxamine, that increased both the resistance against vibrio P and the growth rate of turbot. There was no further improvement of the results when the rotifers were treated both with vibrio E and deferoxamine, the effects of which seem redundant rather than additive. The hypothesis that these effects were due to competition for iron is therefore tenable.

From a nutritional point of view, the use of fish autolysate in the enrichment mixture of rotifers as food for larval turbot cannot be recommended any longer. On the one hand, the rearing performances of turbot and sea bass larvae have not been significantly affected by omitting this additive (Gatesoupe, 1986 and unpublished data). On the other hand, this additive may endanger the larvae by increasing iron availability, thereby favouring pathogenic vibrios.

From a prophylactic point of view, non-pathogenic bacteria which produce siderophores are promising candidate probiotics against pathogens with low iron-uptake capability, *e.g. Vibrio* P. Their potential defence against more virulent pathogens like *V. anguillarum* is however unlikely.

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