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Host Range Susceptibility of *Enterococcus* sp. Strains Isolated from Diseased Turbot: Possible Routes of Infection

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Experiments were conducted to assess the pathogenicity of *Enterococcus* sp. strains isolated from diseased turbot for several fish species (turbot, salmon, trout, and seabream), as well as for mice. The intraperitoneal injection assays indicated that the tested strains showed host specificity for turbot, with a high degree of virulence (50% lethal dose of 10^4 cells per g of fish). The Spanish *Enterococcus* sp. isolates were nonpathogenic for the other fish species studied and for mice. The possible routes of infection were determined by bath exposure (with and without prior abrasion of the skin) and by intragastric inoculations with food and feces contaminated with the pathogen. The bath challenges indicated that the *Enterococcus* isolates were able to overcome the defense mechanisms present on the surface of the turbot only if the skin was abraded prior to the exposure. The antibacterial activities of components of a glycoprotein nature present in the turbot skin mucus are probably responsible in part for the resistance in noninjured fish to infection. On the other hand, we demonstrated the capacity of this pathogen to overcome adverse conditions in the stomachs of fish when associated with food or fecal material, since it is able to establish an infective state and to produce mortalities after 16 to 20 days postingestion. From all of these findings, we can conclude that horizontal transmissions through water and the fecal-oral route are the main avenues of infection of turbot streptococcosis.

Streptococcosis is a septicemic disease that affects freshwater and marine fish in both farmed and wild populations. Among commercially important fish species, this disease has been reported worldwide in yellowtail (*Seriola* spp.), eels (*Anguilla japonica*), menhaden (*Brevoortia patronus*), striped mullet (*Mugil cephalus*), and striped bass (*Morone saxatilis*) (for reviews, see references 1, 6, and 9). In addition, we have recently described the first epizootic outbreak of streptococcosis in turbot (*Scophthalmus maximus*) cultured in the northwest of Spain, which occurred in 1993 (21). Since that date, this disease has caused heavy economic losses to the turbot farming industry in Spain. The phenotypic and serological characterization of the *Enterococcus* sp. strains isolated from diseased turbot has been described elsewhere (20, 21).

There is a considerable degree of phenotypic heterogeneity among the gram-positive cocci associated with diseased fish, most of which show characteristics of the genus *Streptococcus* or *Enterococcus*. In fact, the taxonomic status of the fish-pathogenic streptococci is currently under revision, and new species, such as *Enterococcus seriolicida* (10), have already been established. In addition, some authors (3) have shown genetic similarities between *E. seriolicida* and *Lactococcus garvieae* by 16S rRNA sequence similarity.

To date, little is known about the epizootiology of these bacterial fish pathogens. Boomker et al. (2) reported some host specificity in freshwater isolates. In addition, it has been suggested that these bacteria can remain in seawater and mud around farms throughout the year, with seasonal changes in their distribution (7). Unfortunately, these authors investigated neither the reasons for the presence of streptococci in the aquatic environment nor whether these environmental isolates fit the phenotypic characteristics specific for the pathogenic strains. On the other hand, transmission is thought to be horizontal by direct contact or by contaminated fish food (1). The ingestion of gram-positive-coccus-laden fecal material from infected fish can be also a significant route for the horizontal transmission of these pathogens.

The aim of this work is to determine the host range of the *Enterococcus* species isolated from diseased turbot to know the potential danger for the culturing of other fish species. In addition, different putative routes of infection are evaluated to clarify the modes of transmission of this fish pathogen.

MATERIALS AND METHODS

Bacterial strains. Ten strains representative of a *Enterococcus* sp. previously described (20, 21), primarily isolated from moribund turbot and with different farm origins and years of isolation, were included in this study (Table 1). Two strains of *E. seriolicida* supplied by R. Kusuda (Fish Disease Laboratory, Faculty of Agriculture, Kochi University, Nankoku, Japan) and the *Enterococcus faecalis* reference strain ATCC 19433 were included for comparison. Strains were routinely grown on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) yeast extract. Stock cultures were maintained frozen at −80°C in tryptone soya broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 15% (vol/vol) glycerol.

Challenge experiments. Prior to any inoculation, the fish were analyzed by classical procedures (18) to determine if they were free of any bacterial pathogen that could interfere in the results of the experimental infections. Moreover, the fish were acclimated to the experimental conditions for 7 days between their arrival at the laboratory and the start of the inoculations.

i.p. inoculation. Four fish species that are important in Spanish aquaculture were chosen to determine the host range of the *Enterococcus* sp. isolates. Turbot (average weight, 10 g), rainbow trout (*Oncorhynchus mykiss* [12 g]), Atlantic salmon (*Salmo salar* [15 g]), and gilthead seabream (*Sparus aurata* [5 g]) were intraperitoneally (i.p.) inoculated according to the procedures described by Toranzo et al. (19). Briefly, a bacterial suspension was prepared in saline solution (SS [0.85% NaCl]) at a final concentration of 10^9 cells per ml. Tenfold dilutions were made, and doses ranging from 10^2 to 10^8 cells per ml were inoculated in groups of 10 fish of each species. Control fish were inoculated with sterile SS. Inoculated and control fish were distributed in 5-liter aquaria at a density of 10 individuals per aquarium. Each aquarium was continuously aerated with an airstone and maintained at 19°C. Daily, one-third of the water was changed, dead fish and feces were siphoned off, and fish behavior was observed. Mortalities were recorded daily over a 4-week period, and the degree of virulence (50%

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TABLE 1. Susceptibility of different fish species to the *Enterococcus* sp. strains isolated from diseased turbot by i.p. challenge[a]

Strain	Affected farm	Year	Virulence for fish				Pathogenicity for mice
			Turbot	Salmon	Trout	Seabream	
Enterococcus sp. strains							
RA-83.1	A	1992	+ (4.5×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
RA-99.1	A	1993	+ (5.0×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
RA-100.1	A	1994	+ (5.2×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
RA-146.2	A	1995	+ (1.0×10^4)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
AZ-12.1	B	1993	+ (7.0×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
AZ-71.1	B	1994	+ (6.3×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
AZ-90.2	B	1995	+ (3.7×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
RM-207.1	C	1993	+ (5.0×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
RM-212.1	C	1994	+ (4.0×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
RIM-17.1	D	1994	+ (8.4×10^3)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
E. seriolicida							
YT-3			− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)	− (>10^8)
SS-91014			− (>10^8)	− (>10^8)	− (>10^8)	ND	ND
E. faecalis							
ATCC 19433			− (>10^8)	ND	− (>10^8)	ND	− (>10^8)

[a] Virulence expressed as LD_{50}s (the numbers of bacteria needed to kill 50% of inoculated animals). +, virulent strains; −, avirulent strains; ND, not determined.

lethal dose [LD_{50}]) was calculated by the method described by Reed and Münch (14). In all cases, survivors were analyzed to determine the presence of carrier fish.

Since turbot was the unique fish species susceptible to the *Enterococcus* sp., subsequent challenge experiments employed it as the experimental fish. The influence of the fish size was assessed by challenging groups of turbot of different weights ranging from 10 to 400 g.

Bath challenge. Four groups of fish (10 turbot per group; 20 g per fish) were employed in the bath challenge. Group 1 was exposed to the bacterium suspended in seawater to a concentration of 2.5×10^7 CFU/ml. Small areas of the skin on the dorsal side of the body were abraded with a sterile scalpel. Group 2 was exposed to the same bacterial concentration, but the skins of the fish were not abraded. Groups 3 and 4 were handled in the same way as groups 1 and 2, respectively, but no bacteria were added to the water.

After the exposure, each group was housed in separate tanks. Maintenance was performed as for the i.p. infected fish. The experiment was terminated after 30 days. As before, after this period the survivors were analyzed to determine the presence of carrier fish.

Intragastric inoculations. To determine the importance of transmission through food and by the fecal-oral route, intragastric inoculations employing feed and feces contaminated with enterococcal strains were performed. Briefly, fecal materials collected from turbot and commercial feed were separately autoclaved (121°C/15 min) to avoid possible competition with any microorganism present in the sample, and then they were aseptically homogenized to produce an easily injectable slurry. These homogenates were mixed with an equal volume of bacterial suspensions to get a final concentration of 10^6 bacteria per ml. The mixtures were maintained at room temperature for 20 min to allow the absorption of the bacterial cells to the particulate material.

Groups of fish (10 turbot per group; 20 g per fish) were orally intubated with the feed-*Enterococcus* mixture, the feces-*Enterococcus* slurry, or the pathogen dissolved in SS. Intubation was performed with a 1-ml syringe equipped with a sterile combitip that was inserted into the esophagus of each fish. Each fish received 0.2 ml of the corresponding solution. Fish in the control group were similarly challenged, but with sterile fecal or feed slurry. Mortalities and establishment of the carrier state were monitored as described above.

Virulence for mice. To assess the possible pathogenic potential of the *Enterococcus* sp. strains for mammals, a mouse pathogenicity assay was performed with 5 to 10 BALB/c mice (10 to 12 weeks old, 21 to 25 g), which were i.p. inoculated with doses ranging from 10^4 to 10^8 cells of each *Enterococcus* isolate. Control animals received phosphate-buffered saline (PBS; pH 7.4). The LD_{50} was calculated by the method described by Reed and Münch (14).

Bactericidal activity of surface mucus. Mucus was scraped from the skins of healthy turbot and Atlantic salmon with a glass slide which was passed along the animals from the caudal peduncle to the operculum. The mucosal material was dissolved in sterile SS at a final concentration of 5% (vol/vol), mixed thoroughly, filter sterilized (0.45-μm-pore-size filters), and stored at −30°C until used.

The antibacterial activities of the two skin mucus preparations were tested by two different procedures previously described (11): (i) the disc diffusion method on solid media and (ii) survival in skin mucus solution. The inocula employed were the respective isolates dissolved in sterile seawater at a concentration of 10^5 bacteria per ml. The assays were conducted in triplicate, and the means and their associated standard deviations were calculated. Two isolates of *Pasteurella piscicida* and *Vibrio anguillarum* with known sensitivity to turbot mucus and the *Staphylococcus aureus* reference strain ATCC 25923, which is resistant to lysozyme activity, were included as controls.

To preliminarily characterize the substances active in turbot skin mucus, temperature (47°C for 30 min; 56°C for 20 min; and 80°C for 10 min) and enzymatic (proteinase K, trypsin, β-galactosidase, achromopeptidase, lysozyme, β-amylase, and lypase) treatments of the mucus were carried out. For the enzymatic treatments, mucus preparations were mixed with the enzyme solutions (1 mg/ml in SS) in a ratio of 1:1, and the mixtures were incubated for 1 h at 37°C. In all cases, the residual activities of the mucus preparations were assayed by the disc diffusion agar plate method (11).

Survival experiments. Two different experiments were carried out to determine the possibility of transmission of this pathogen by food. Since food material used in the Galician farms are usually frozen fish, the viabilities of the isolates after different freezing periods were evaluated. The strains were dissolved in PBS at a concentration of 10^2 cells per ml, and the solutions were maintained at −30°C for 1 week and 1 and 3 months. After these periods, the bacterial solutions were defrosted and streaked onto BHA-1 plates. Representatives of other marine fish pathogens such as *P. piscicida* and *V. anguillarum* were included for comparison.

The second experiment was to evaluate the resistance of the *Enterococcus* sp. strains to acidic pH to determine the possibility of the strains surviving adverse fish stomach conditions. Aliquots of 5 ml of SS were adjusted to pH 3, 4, and 5. The *Enterococcus* strains were dissolved in SS or in a homogenate of sterilized dry feed in SS (1 g/ml) at a final concentration of 10^5 cells per ml. The bacterial solutions were mixed (proportion, 1:1) with the SS aliquots of various pHs. One hundred microliters of each mixture was plated at different times to determine the survival dynamic.

RESULTS

The *Enterococcus* sp. strains showed host specificity when the bacteria were inoculated i.p., turbot being the only fish species of those studied susceptible to streptococcosis. All dead or moribund turbot showed hemorrhages in the skin, mainly in the head and the base of the fins. Some of the fish presented a pronounced uni- or bilateral exophthalmia. Internally, the livers were pale and the peritoneal cavities were filled with ascitic fluid. Mortalities began by day 3, rapidly increased for approximately 2 days, and continued slowly for 3 to 4 days (Fig. 1). The degree of virulence of the *Enterococcus* sp. strains for turbot, expressed as LD_{50}, ranged between 10^3 and 10^4 bacteria for fish weighing approximately 10 g (Table 1). With increasing fish size, the LD_{50}s became higher, i.e., 5×10^4 bacteria for 50- to 75-g fish, 5×10^6 cells for 250-g fish, and 10^7 for turbot weighing 400 g. However, when the degree of viru-

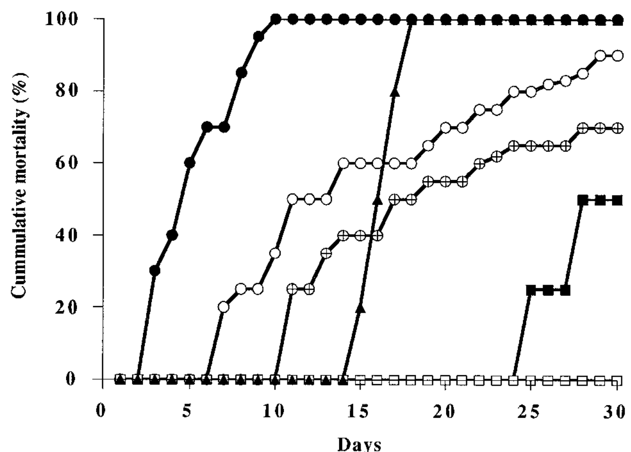

FIG. 1. Cumulative mortality of turbot (20-g fish) caused by the *Enterococcus* sp. strain RA-99.1 in the i.p. bath and intragastric challenges. ●, i.p. inoculation (10^7 bacteria per ml); ○, i.p. inoculation (10^6 bacteria per ml); ⊕, i.p. inoculation (10^5 cells per ml); ■, bath exposure with skin abrasion (10^7 cells per ml); □, bath exposure without skin abrasion (10^7 cells per ml); ▲, intragastric inoculation of *Enterococcus*-laden food (10^7 bacteria per ml).

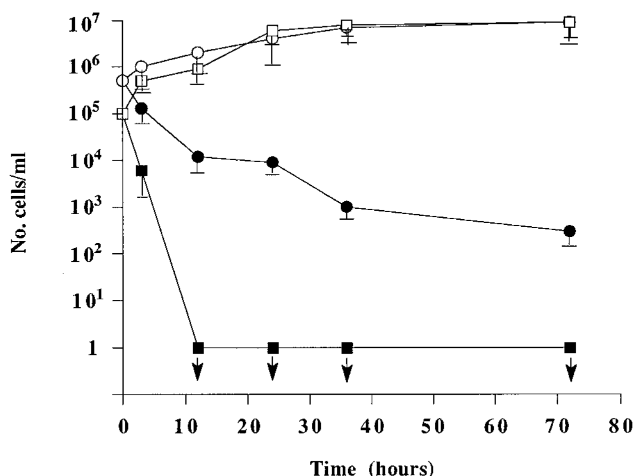

FIG. 2. Comparison of the survival rates in turbot skin mucus of an *Enterococcus* sp. strain and *P. piscicida*. ●, *Enterococcus* sp. strain AZ-12.1 in turbot mucus; ○, *Enterococcus* sp. strain AZ-12.1 in seawater; ■, *P. piscicida* DI 21 in turbot mucus; □, *P. piscicida* DI 21 in seawater. Arrowheads indicate values less than the detection limit. Vertical bars represent standard deviations.

lence was calculated in relation to body weight, in all cases the results were similar, with values of approximately 10^4 bacteria per g of fish. The challenge organism was reisolated from the internal organs of all dead and moribund fish. In addition, turbot survivors carried the inoculated *Enterococcus* strains, mainly in kidney and brain tissues. On the other hand, no mortalities or clinical signs of disease were observed in any of the other fish species assayed. However, it is interesting to point out that a carrier status was demonstrated in salmon, since these fish harbor the pathogen in their visceral organs (mainly kidneys and brains) after a period of 30 days postinfection. No carrier fish were detected among the inoculated trout or seabream after the same period.

The results obtained in the mouse pathogenicity assay indicated that under laboratory conditions, no strains were pathogenic for mice (LD_{50} of $>10^8$ cells).

Regarding the bath exposure experiments performed with turbot, mortalities were recorded for the group of abraded fish after 25 to 30 days postinoculation but not for the turbot with uninjured skin or for the control groups (Table 2) (Fig. 1). The *Enterococcus* strains were recovered from dead fish which showed soft hemorrhagic livers, intestines filled with pus, and ascitic fluid in their peritoneal cavities. Interestingly, the inoculated abraded turbot did not develop ulcers at the sites of skin abrasions. After the experimental period, the skin was normal in abraded areas.

The intragastric inoculations of turbot with *Enterococcus*-laden feces or food resulted in the death of 100% of the orally intubated fish after a period of 16 to 20 days, while no mortalities were observed in the control fish inoculated with sterile feces or food (Table 2) (Fig. 1). Dead fish had no external signs of illness. Internally, organ adhesions and pus-filled intestines were observed. It is noteworthy that in fish orally intubated with the *Enterococcus* sp. in sterile saline, no symptoms and/or deaths were recorded (Table 2).

All of the *Enterococcus* sp. strains tested were sensitive to the antimicrobial action of turbot skin mucus by the disc diffusion method. *S. aureus* ATCC 25923, which was employed as an indicator for the presence of lysozyme, was also sensitive to turbot mucus, indicating that substances other than lysozyme were responsible for this activity. Figure 2 lists the survival rates for *Enterococcus* sp. strains from diseased turbot in seawater containing mucus. The numbers of viable cells decreased slightly during the first 24 h and then rapidly decreased, reaching values of approximately 10^2 cells per ml. All of the isolates survived well in seawater (control), maintaining viable cell populations that were the same as or higher than those present at the start of the experiment. The preliminary characterization experiments showed that the antibacterial activity of the mucus was completely lost after 10 min at 80°C and also after proteinase K and the β-galactosidase treatments. In addition, trypsin and achromopeptidase showed a partial inhibitory effect. These results suggested that the antibacterial substance(s) is of a glycoprotein nature.

TABLE 2. Percentages of mortality achieved in the bath challenge and intragastric inoculation of *Enterococcus* sp. strains in turbot

Strain	% Mortality with bath challenge[a]				% Mortality with intragastric inoculation[b]		
	Abraded fish		Nonabraded fish		Bacteria + food	Bacteria + feces	Bacteria
	Bacteria	No bacteria	Bacteria	No bacteria			
RA-99.1	50	0	0	0	100	100	0
AZ-12.1	100	0	0	0	100	100	0
RM-207.1	75	0	0	0	90	100	0
RIM-17.1	90	0	0	0	100	100	0

[a] The bacterial concentration in the water bath was 2.5×10^7 bacteria per ml. Mean time to death was 18 days.
[b] Inoculated doses were of 2×10^5 bacteria per ml of food or feces. Mean time to death was 28 days.

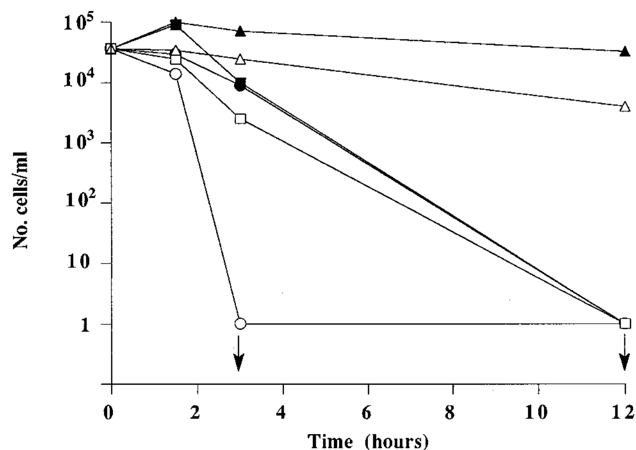

FIG. 3. Survival rates of the representative *Enterococcus* sp. strain RA-99.1 at pH 3 (●), 4 (■), and 5 (▲). Open symbols, survival of the *Enterococcus* strain alone; closed symbols, survival of the *Enterococcus* strain in association with particulated food. Arrowheads indicate values less than the detection limit.

The low-pH and low-temperature-survival assays showed that the *Enterococcus* sp. isolates were able to maintain their viability after freezing periods at −30°C for up to 3 months, 100% of the initial bacterial cells being recovered. In contrast, *P. piscicida* and *V. anguillarum*, the other fish pathogens included in the experiment for comparison, did not survive this low temperature. After 1 week at −30°C, their bacterial cells were lysated, as demonstrated by light microscopy observations (data not shown).

The rate of survival at low pH was greater for the bacteria associated with food material than for the bacterial cells alone (Fig. 3) for all pH values assayed (pH 3, 4, and 5). The *Enterococcus* isolates did not survive longer than 3 h when resuspended directly in SS at pH 3 but persisted until 12 h at that pH value when they were associated with feed homogenate. At pH 4 and 5, while the enterococci in saline experienced a strong and rapid antibacterial effect (more pronounced at pH 4), they were capable of multiplication and growth in the first 3 h prior to a slow and relentless decrease (Fig. 3) when they were associated with feed.

DISCUSSION

Transmission of streptococcosis in fish is thought to be horizontal, with infection occurring from direct contact with infected fish or contaminated fish food (6). However, the results obtained in transmission experiments vary depending on the fish species employed. Thus, Robinson and Meyer (15) showed that the disease could be transmitted experimentally by introducing infected golden shiners into healthy aquaria and by placing healthy fish in a suspension of the pathogen. In contrast, Rasheed and Plumb (13) failed to reproduce the disease in Gulf killifish dipped in bacterial suspensions unless they injured the fish prior to dipping. On the other hand, Taniguchi (17) reported that oral infection of yellowtail with contaminated food was successful. These different results can also be due to the fact of great heterogeneity within the streptococcal group, in which are included a variety of *Streptococcus-Enterococcus* species such as *S. iniae*, *E. seriolicida*, beta-hemolytic *Streptococcus* species, nonhemolytic *Streptococcus* species, and *Enterococcus* species (6, 9). All of these facts make it impossible to extrapolate from one case to another. Therefore, epizootiological study of the new strains isolated is necessary to establish adequate preventive measures.

The results obtained here from the i.p. injection experiment indicated that the tested strains were highly pathogenic for turbot and were nonpathogenic for the other fish species (salmon, trout, and seabream) and for mice. However, the detection of carrier salmon is important in terms of epidemiology, suggesting that under farming conditions, even salmon free of disease could act as a reservoir of the bacteria and could be the origin of infections in turbot facilities located in the same area. On the other hand, we cannot at present rule out the existence of a carrier status for trout and seabream, with the bacteria existing in a viable but nonculturable status or harbored within the fish macrophages.

The bath challenge experiment showed that the invasive capacity of the *Enterococcus* sp. strains isolated from turbot was limited and that prior abrasion of the skin was needed to establish infection. These results agree with those of Rasheed and Plumb (13) for Gulf killifish and suggest that proper handling procedures are important as a first step in the prevention of disease. In addition, and as indicated by Ferguson et al. (4), the presence of other pathogens producing mucosal damage may promote the initiation or progression of streptococcal disease. This finding was supported by the fact that the *Enterococcus* sp. strains were sensitive to the antimicrobial activity of turbot skin mucus and indicated that, in contrast to other pathogens such as *Flexibacter maritimus* or *Cytophaga*-like bacteria (for which the body surface is the primary infection site [5, 11]), normal skin is not a usual portal of entry for *Enterococcus* cells into the fish body. Similar results have been reported for other fish-pathogenic bacteria such as *P. piscicida* (11).

It has been demonstrated (16) that few bacteria are able to survive the adverse conditions of the stomach (pH values of lower than 3 and digestive enzymes) but that they can rapidly grow once they are in the intestine. The results from the intragastric inoculations indicated that enough bacterial cells survived such conditions to establish systemic infections when the bacteria were associated with fecal or food material. However, when administered alone, the pathogen was unable to overcome these adverse conditions. Moreover, the same results were obtained in the in vitro survival experiments which showed that the *Enterococcus* strains were able to survive the low pH values only when they were associated with particulated food material but not when they were dissolved only in SS. This is probably the reason why several authors (8, 13) failed to reproduce the infection by this route, concluding that the digestive tract is not a route of entry for streptococci in healthy fish. In contrast, our results clearly demonstrated that contaminated food and feces from diseased or carrier fish play an important role in the transmission of streptococcosis. It has been suggested that contaminated diets could be the principal source of streptococcal infections in farmed fish (6). Several authors (12, 22) found streptococci with similarities to the fish pathogens in fresh and frozen wild fish used for farmed-fish diets. Our results showing that the *Enterococcus* sp. strains isolated from turbot survived longer than 3 months at −30°C supported that suggestion and indicated that adequate controls of fish diets should be established in order to prevent foodborne infections.

In summary, the *Enterococcus* sp. strains isolated from turbot show host specificity for this marine fish. The routes for horizontal transmission are mainly the fecal-oral route and through water if body injuries exist. In addition, contaminated diets can serve as an important source of infection. From all of these findings, it seems clear that proper management proce-

dures (including the reduction of overcrowding, overfeeding, unnecessary handling or transportation, and the prompt removal of feces and moribund fish) are the first preventive measures needed to avoid outbreaks or, at least, to reduce their severity.

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