Vibrio anguillarum感染に対するEnterococcus gallinarumのプロバイオティクス効果

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<tr>
<td>誌名</td>
<td>魚病研究</td>
</tr>
<tr>
<td>ISSN</td>
<td>0388788X</td>
</tr>
<tr>
<td>著者</td>
<td>Sorroza, L. Real, F. Acosta, F. Acosta, B. Deniz, S. Roman, L. Aamri, F.E. Padilla, D.</td>
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<tr>
<td>巻/号</td>
<td>48巻1号</td>
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<tr>
<td>掲載ページ</td>
<td>p. 9-12</td>
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<td>発行年月</td>
<td>2013年3月</td>
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A Probiotic Potential of *Enterococcus gallinarum* against *Vibrio anguillarum* Infection

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(Received August 31, 2012)

**ABSTRACT**—The aim of this study was to look for new potential probiotic bacterial strains for marine aquaculture. Only one of 195 isolates from the gut of cultured fish including sea bass *Dicentrarchus labrax*, gilthead sea bream *Sparus aurata*, meagre *Argyrosomus regius* and sole *Solea solea* showed a strong inhibitory effect against *Vibrio anguillarum*. This isolate, identified as *Enterococcus gallinarum*, also produced a moderated protective effect against *V. anguillarum* infection of sea bass. Intraperitoneal injection with the isolate showed no pathogenicity in sea bass. Thus, the isolate designated *gallinarum* L1 can have a potential of probiotic bacterium.

**Key words:** *Enterococcus gallinarum*, inhibitory activity, *Vibrio anguillarum*, probiotic, *Dicentrarchus labrax*

Infectious diseases are contributing to increase the losses in fish aquaculture, and also meaning serious problems due to the intensification of the production. *Vibrio anguillarum* has frequently showed to be one of the main known pathogens for cultured and wild marine fish.

The use of probiotics has been recognized like an excellent strategy for disease prevention, producing various beneficial effects for aquaculture as improved feed value, enzymatic contribution to digestion, inhibition or competition with location of pathogenic microorganisms, growth promoting factors and increase immune response. Most probiotic proposed as biological agents in aquaculture belong to the lactic acid bacteria group (*Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Pediococcus*, *Enterococcus* and *Streptococcus*) and the Genus *Vibrio*, *Bacillus*, *Pseudomonas*, *Roseobacter*, *Aeromonas*, *Alteromonas* and *Flavobacterium*. In this study we analyze different bacterial isolates from the intestine of four marine fish species in order to evaluate, by *in vitro* and *in vivo* tests, their potential inhibitory activity against *V. anguillarum*.

**Materials and Methods**

**Sampling**

We sampled different marine fish species: 60 sea bass *Dicentrarchus labrax*, 80 gilthead sea bream *Sparus aurata*, 30 meagre *Argyrosomus regius*, and 25 sole *Solea solea*. Fish (approximately 200 g B. W.) were anesthetized by overdose of MS-222 (Sigma Aldrich) and slaughtered on iced water. The intestinal content of each fish was aseptically removed and opened, and 1 g of the gut content was taken with sterile tips and homogenized in 9 mL of PBS. Serial dilutions were spread on Marine Agar, Brain Heart Infusion Agar, Sheep Blood Agar, Trypticase Soy Agar (TSA) and De Man Rogosa and Sharpe agar for 48 h at 25°C.

**Production of antagonistic effect and antibacterial substances against *V. anguillarum***

All isolates strains were tested for inhibitory effects against three strains of *V. anguillarum* (Table 1). Briefly, 100 µL of a culture of *V. anguillarum* in brain heart infusion broth (BHIB) was spread on TSA and each isolate was put over the plate with the loopful. Inoculated plates were incubated at 25°C for 24–48 h until to observe growth inhibitory halo. In order to determine the production of antibacterial substances in bacterial supernatants, isolates that showed antagonistic effect against *V. anguillarum* were analyzed following the well diffusion method with modifications.

In order to determine the production of organic acids as possible mechanism of action of the antagonistic effect against *V. anguillarum*, the supernatant of the isolate showing inhibitory activity was analyzed by liquid chromatography (Agilent 1200 RID, 300 mm*×*0.78 mm; Aminex HPX-87H column) by isocratic method.

**Table 1.** *V. anguillarum* strains used in the test of antagonistic effect

<table>
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<th>Origin</th>
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<tr>
<td>ATCC 14181</td>
<td>Brown trout (<em>Salmo trutta</em>)</td>
</tr>
<tr>
<td>CECT 4347</td>
<td>European eel (<em>Anguilla anguilla</em>)</td>
</tr>
<tr>
<td>IA-USC 975-1</td>
<td>Turbot (<em>Scophthalmus maximus</em>)</td>
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ATCC: American Type Culture Collection.
CECT: Spanish Type Culture Collection.
IA-USC: Institute of Aquaculture, Santiago de Compostela University.

**Identification of isolate**

Identification of the isolates that showed antagonistic effect against *V. anguillarum* was made by partial
sequencing of 16S rRNA gene PCR-amplified with universal primers and BLAST analysis.

**Fish bile and pH resistance**

In the analysis of fish bile resistance, 100 µL of fresh bile from sea bass was added to 900 µL of bacterial culture at 10<sup>7</sup> CFU/mL. In the analysis of resistance to acid pH, 100 µL of bacterial culture at 10<sup>7</sup> CFU/mL was added to 900 µL of PBS with a pH range 3–7. Both test samples were incubated for 1.5 h at 22°C. The results were obtained by counting viable cells on TSA plate.

**Adhesion and exclusion mucus assays**

Ten sea bass (average 400 g B. W.) were starved for 48 h, and the gut were removed and homogenized in PBS. Mucus was centrifuged twice at 12,000 xg for 5 min at 4°C to remove particulate and cellular material. Then, the solution was adjusted to 0.5 to 1 mg/mL protein in PBS by Bradford Protein Assay Kit (Sigma), and was sterilized by UV light exposure for 30 min. BSA coated and non-coated polystyrene plates were also used to determine if the adhesion is due to nonspecific factors.

The adhesion to the intestinal mucus was evaluated following previously described method using green fluorescent nucleic acid (SYTO 9) (Invitrogen) and the adhesion of the tested isolates to the intestinal mucus was expressed as the percentage of fluorescence of the bound bacteria in relation to the fluorescence of the bacterial suspension added initially.

A competitive exclusion assay was also performed in order to analyze if the selected isolates are able to compete with V. anguillarum for binding sites in the intestine. The competitive exclusion rate was expressed as the ratio between the percentage of adherence of V. anguillarum 975–1 with and without the tested isolate.

**Feeding with selected isolate for fish protection**

In order to determine possible harmful effects, we have used sea bass and mice (strain BABL/c). In fish, 100 µL at 10<sup>8</sup> CFU/mL of selected isolate was intraperitoneally injected by triplicate at two separate groups of 20 sea bass (average 10 g B. W.). Twenty mice were intraperitoneally inoculated with 0.1 mL containing 10<sup>8</sup> or 10<sup>10</sup> CFU/mL. Both control groups were injected with the same volume of sterile PBS. Fish were monitored daily for any adverse signs for 30 d after inoculation, and mice for 20 d. Fish and mice were slaughtered and necropsied to evaluate any possible lesions by histopathological study, and analyzed by microbiological methods on TSA to detect the presence or absence of the inoculated bacterium.

For preparation of the experimental diet containing the tested isolate, the isolate was cultured in BHIB for 24 h at 25°C. Culture was centrifuged at 2,500 xg for 20 min at 4°C, and the cell pellet was twice washed and resuspended in PBS to 10<sup>10</sup> CFU/mL. Twenty-five milliliters of the suspension was spread on 120 g of commercial feed (BioMar YM 558; Dueñas), mixed and dried for 24 h at 37°C to obtain a final concentration of 2 x 10<sup>6</sup> CFU/g of the feed.

For the protection assay, fish challenge was performed by triplicate with 25 sea bass per tank. Sea bass (average 18 g B. W.) were fed daily at 2% body weight with the experimental diet for 20 d before challenge, and then were immersed in a suspension of the virulent strain V. anguillarum 975–1 at 10<sup>8</sup> CFU/mL for 8 h (probiotic test group), with the water temperature increased from 21°C to 24°C to magnify the effect of infection in all the experimental groups. For positive control group, fish were infected and fed with the commercial diet without probiotic, and for negative control group, non-infected fish were fed with the commercial diet without probiotic, were employed. Fish were observed daily for 20 d after infection, and mortality was attributed to V. anguillarum if the bacterium was recovered in pure culture from the internal organs of dead fish.

All data were statistically analyzed with SPSS statistics program for Windows, version 18.0 (SPSS, Inc). Fish protection assay was analyzed using a nonparametric Mann-Whitney test.

**Results and Discussion**

A total of 195 strains were obtained from the cultured gut, but only one, isolate L1 from sea bass, showed a strong antagonistic effect against V. anguillarum (Fig. 1a). Supernatant obtained from this strain also inhibited V. anguillarum, demonstrating the production of extracellular substances with antagonistic effect (Fig. 1b). This isolate was identified as Enterococcus gallinarum by 16S rRNA gene partial sequence, showing 99.9% similarity (1042/1043 bp). This strain, designated E. gallinarum L1, belongs to the lactic acid bacteria group, one of the most important groups of bacteria with probiotic activity described. Lactic acid bacteria constitute a part of the gut microbiota of several fish species. Genus Enterococcus is frequently isolated from marine environments as much as intestine from cultured fish in integrated farms, and also from food, like minced meat and spoiling boiled shrimps, being considered as probiotic by the production of different antimicrobial substances.

One of the main mechanisms of inhibitory action of E. gallinarum L1 against V. anguillarum, is of production of antibacterial substances, including lactic and acetic acids, and small quantities of ethanol, with predominance of lactic acid with regard to the other substances. Analysis of supernatant by HPLC yielded that the iso-
The presence of survival rate was obtained at pH 4 (40%). The susceptibility of the isolate in acid pH does not mean that *E. gallinarum* L1 is unable to survive and colonize the intestine because this does not occur *in vivo* where bacteria will mix with food and the action of the acid pH will not be direct\(^6\). The isolate showed a satisfactory survival rate in the fish bile assay (75.7%).

The isolate significantly adheres better to intestinal mucus (30%) \((P < 0.05)\) than unspecific substrates—BSA-coated (8.75%) or untreated polystyrene (9.65%), suggesting that microbial adhesion process can be due to specific structures, such as external appendages covered by lectins\(^9\). Isolate also shows ability to exclude *V. anguillarum* by competing for binding sites on the intestinal mucus of sea bass, showing a rate of exclusion of 66.2% against *V. anguillarum* 975–1.

On the other hand, *E. gallinarum* L1 was established in the gut mucosa for several weeks, and was also able to survive easily the coating on the feed, maintaining its original concentration, at least for 20 days while food was stored at 4°C.

*Enterococcus gallinarum* L1 does not produce biofilm, which has been described for some *Enterococcus* species as a factor contributing with their virulence\(^8\). Although some species of the Genus *Enterococcus* have been reported to cause damage to host\(^9\), but *E. gallinarum* L1 was harmless to sea bass and mice in inoculation test, and no mortality and specific damage were observed in the internal organs. Indeed, *E. gallinarum* has been isolated in our laboratory as normal flora in the intestine of healthy fish.

In the fish protection assay, *V. anguillarum* 975–1 was recovered in pure culture from the internal organs of the dead or moribund fish that showed typical signs of vibriosis. Fish protection assay showed a mortality rate of 24% in the probiotic group previously fed with the isolate, compared to 30.66% of mortality in the positive control group and 0% in the negative control group.

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**Fig. 1.** Production of antagonistic effect (a) and antibacterial substances (b) against *V. anguillarum*.

Isolate L1 produce a mix of lactic acid (24.12%), acetic acid (21.7%) and ethanol (7.6%). Similar result against different species of the Genus *Vibrio* was found in a previous study\(^7\), by using lactic acid bacteria.

This isolate L1 was also subjected to different tests, as bile and pH resistance, to simulate the passage of bacterial strains across the digestive tract. The isolate showed a good level of survival in the range of pH between 7 and 5 (higher than 50%), but a difference of survival rate was obtained at pH 4 (40%). The susceptibility of the isolate in acid pH does not mean that *E. gallinarum* L1 is unable to survive and colonize the intestine because this does not occur *in vivo* where bacteria will mix with food and the action of the acid pH will not be direct\(^6\). The isolate showed a satisfactory survival rate in the fish bile assay (75.7%).

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**Fig. 2.** Cumulative mortality of sea bass previously fed with *E. gallinarum* L1 and challenged with *V. anguillarum*. 

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(Fig. 2), and these were statistically different between the group ($P < 0.05$), resulting in a 21.73% of relative percent survival (RPS). This data is similar to those reported previously with the probiotic Roseobacter 27–44, resulting in a RPS of 22%–25% against *V. anguillarum* infection. Other species in the Genus *Enterococcus* such as *E. Faecium*, have been successfully used as probiotics for fish, can improve growth rate, tolerance to stress and survival rate of cultured fish21–23.

This is the first study that reports the probiotics effect of *E. gallinarum* against *V. anguillarum* infection, an important disease among marine aquaculture fish. Further experiments will be needed to evaluate potential of the isolate *E. gallinarum* L1 as probiotic including safety for fish and humans, as well as to identify the antimicrobial substances produced by the isolate.

References

ウイルス性神経壞死症の防除を目的とした電解海水によるクエおよびマハタ受精卵の消毒条件の検討
渡邉研一・井手健太郎・岩崎隆志
佐藤・森・広一郎・米加田徹

クエおよびマハタにおけるウイルス性神経壞死症（VNN）原因ウイルス（ベータノウイルス）の垂直感染を防除することを目的として、電気分解した海水（電解海水）による受精卵の適切な消毒条件を検討した。0.1～0.5 mg/Lの電解塩素を含む電解海水に受精卵を浸漬したところ、両魚種ともに0.3～0.5 mg/Lでは5分、1.0 mg/Lでは3分。1.5 mg/Lでは1分浸漬しても、電解海水による影響はなかった。ベータノウイルスを遊離塩素濃度0.3～1.0 mg/Lで3分または1.5 mg/Lで1分処理すると、99.4%以上のウイルスが不活化された。

魚病研究，48 (1)，5～8 (2013)

Vibrio anguillarum 感染に対する Enterococcus gallinarum のブロバオイティクス効果
L. Sorroza・F. Real・A. Acosta・S. Deniz
L. Román・F. E. Aamri・D. Padilla

海水魚養殖用で用いるブロバオイティクス細菌の探索を目的に研究を行った。海水魚4種の消化管から分離した195株のV. anguillarumに対して強い抗生物質作用を示す細菌が1株見つかった。本分離株はE. gallinarumと同定され、in vitroでV. anguillarumに対して優れた病気予防作用を示した。本分離株を機に20日間海水に投与してからV. anguillarumで感染を試験したところ、弱いかかった感染症を示した。本分離株は長期間保存に耐え、本分離株を腸内接種されたシーパスに異常が認められなかった。以上のことから、本分離株E. gallinarum L1はブロバオイティクスとして使用できる可能性がある。

魚病研究，48 (1)，9～12 (2013)

シロサケから分離された冷水病原体の病原性の差異
三坂源行・細山 誠・小出久男・鈴木邦夫

北海道内の無症状のシロサケから分離された冷水病原体10株および標準株について、シロサケ稚魚（体長1.01～1.36 g）に対して腸内注射による感染試験を行い、毒力を比較した。さらに過去の報告から毒力との関連があるとされるエラスチン分解活性を各株について調べ、毒力との関連を検討した。シロサケから分離された10株のLD₅₀は体重1 gあたり2.64×10⁵から4.74×10⁴ CFUと大きな差異を示した。株のエラスチン分解活性と毒力の間に明確な関係は認められなかった。

魚病研究，48 (1)，17～20 (2013)

アサリに寄生する Perkinsus 属原虫2種の栄養体増殖と遊走子発達に及ぼす温度と塩分の影響
浦田明佑・下川 潤・永良知義

Perkinsus olseni とP. hoshunensisの栄養体の増殖と遊走子発達の指標としての遊走子放出率に対する温度と塩分の影響をin vitroで調べた。これらの環境要因の影響は、2種間で大きな差はなかった。栄養体は両種とも28℃でよく増殖し、P. hoshunensisは塩分21～33%で最もよく増殖した。前遊走子からの遊走子の放出は、P. olseni, P. hoshunensisともに25～30℃、25～35℃で最も高かった。日本各地のアサリにおける Perkinsus 属原虫の寄生に関しては、2種が識別される以前の研究では河口域（低塩度）で感染が低いことが示され、また、最近、2種間ではP. olseniが優占種であることが報告されている。しかし、本研究では、これらの現象を説明できるような Perkinsus 属原虫2種の増殖・発達特性を見出すことはできなかった。

魚病研究，48 (1)，13～16 (2013)

イシダイ脾腫細胞からのqPCRによるRSIVゲノムの定量的検出法の最適化
 prey 昭映・西澤豊彦

イシダイの脾腫細胞からのPst I fragment遺伝子を標的としたqPCRによるRSIVゲノムの定量的検出法の最適化を検討した。RSIVゲノムの定量的検出用基準直線は、y = -0.266x + 10.95（y: RSIV遺伝子数, x: Ct値）であり、56.6%の増幅効率（84.5%）となった。本基準直線では、Ct値8～33においてRSIVゲノムの定量的検出が可能であり、定量的検出限界は約100 RSIV genomes/reaction（10⁻⁵ genomes/mg）と推定された。本基準直線に基づき、RSIVで人為感染させたイシダイの脾腫細胞からRSIVゲノムの定量的検出を試みたところ、RSIV接種後7日目までRSIVゲノムは検出限界以下であったが、接種後10日目以降では≥10²±1.77 genomes/mg（mean ± SD）のRSIVゲノムが検出された。

魚病研究，48 (1)，21～24 (2013)