Veterinary Microbiology 185 (2016) 41–48

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Vibrio lentus protects gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae against challenge with *Vibrio harveyi*

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ARTICLE INFO

Article history:
Received 5 November 2015
Received in revised form 11 January 2016
Accepted 30 January 2016

Keywords:
Probiotics
Aquaculture
European sea bass
Vibrio
Gnotobiotic
Challenge

ABSTRACT

Due to the mounting awareness of the risks associated with the use of antibiotics in aquaculture, treatment with probiotics has recently emerged as the preferred environmental-friendly prophylactic approach in marine larviculture. However, the presence of unknown and variable microbiota in fish larvae makes it impossible to disentangle the efficacy of treatment with probiotics. In this respect, the recent development of a germ-free culture model for European sea bass (*Dicentrarchus labrax* L.) larvae opened the door for more controlled studies on the use of probiotics.

In the present study, 206 bacterial isolates, retrieved from sea bass larvae and adults, were screened *in vitro* for haemolytic activity, bile tolerance and antagonistic activity against six sea bass pathogens. Subsequently, the harmlessness and the protective effect of the putative probiotic candidates against the sea bass pathogen *Vibrio harveyi* were evaluated *in vivo* adopting the previously developed germ-free sea bass larval model.

An equivalence trial clearly showed that no harmful effect on larval survival was elicited by all three selected probiotic candidates: *Bacillus* sp. LT3, *Vibrio lentus* and *Vibrio proteolyticus*. Survival of *Vibrio harveyi* challenged larvae treated with *V. lentus* was superior in comparison with the untreated challenged group, whereas this was not the case for the larvae supplemented with *Bacillus* sp. LT3 and *V. proteolyticus*. In this respect, our results unmistakably revealed the protective effect of *V. lentus* against vibriosis caused by *V. harveyi* in gnotobiotic sea bass larvae, rendering this study the first in its kind.

1. Introduction

With most capture fisheries worldwide considered fully exploited or overexploited, aquaculture will become central to meet the future fish demand, which will continue to increase with population growth, rising incomes and incrementing urbanization (Msangi and Batka, 2015). However, unpredictable larval and juvenile fish mortality impede the sustainable development of global marine aquaculture. Different factors have been proposed to cause this growth constraint, including inadequate nutrition and detrimental fish–microbe interactions (Vadstein et al., 2013). While juvenile and adult fish may be vaccinated, as they have a mature immune system, the vulnerable larval stages are most prone to bacterial infections (Vázquez and Muñoz-Cueto, 2014). Due to the mounting awareness of the risk associated with the use of antimicrobial agents, treatment with beneficial or probiotic bacteria has recently emerged as the preferred environmental-friendly prophylactic approach in larval disease management and production enhancement (Gatesoupe, 1999).

Although a substantial amount of studies has confirmed the potential of probiotics in improving growth performance and/or avoiding pathogen-induced disease, to date there are only few experiments evaluating potential probiotics for European sea bass larvae (*Dicentrarchus labrax*, Linnaeus 1758), which is the most important commercial fish species cultured in the Mediterranean (FAO, 2015). Carnevali et al. (2006) observed positive effects on larval welfare, higher growth performance and decreased cortisol levels in larval sea bass when treated with *Lactobacillus delbrueckii*

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http://dx.doi.org/10.1016/j.vetmic.2016.01.024
0378-1135/

subsp. *delbrueckii*. Picchietti et al. (2009) provided evidence that early feeding with a probiotic-supplemented diet stimulated the larval gut immune system, through an increase in intestinal T-cells and granulocytes and a lowered transcription of key pro-inflammatory genes. Several studies have shown that diets containing live yeast (*Debaryomyces hansenii*) are beneficial for gut maturation, enhancement of antioxidant response, increased survival and growth and skeletal development at the first stages of sea bass development (Tovar-Ramírez et al., 2004, 2010).

However, to date the probiotics concept remains highly controversial in aquaculture because a judicious and scientifically supported commercial application of a probiotic treatment warrants a thorough testing of its efficacy and safety, using standardized *in vivo* experiments (Ninawe and Selvin, 2009). This is challenging in an aquaculture environment, since the naturally occurring bacterial microbiota in larval fish are highly dynamic, making it impossible to define the exact role of the probiotics (Vadstein et al., 2013). However, with the recent development of a germ-free model system for European sea bass larvae, the door was opened for more controlled studies on host-microbe interactions and the effects of pre- and probiotics (Dierckens et al., 2009; Schaeck et al., 2016).

In view of this, the objective of the present study was to retrieve probiotic candidates from the intestinal microbiota in sea bass, and to evaluate their probiotic potential *in vitro* and subsequently *in vivo* by means of a gnotobiotic sea bass larval challenge model with *V. harveyi*. To our knowledge, this is the first study testing probiotic effectiveness *in vivo* while adopting a gnotobiotic aquatic model.

2. Methods and materials

2.1. Bacterial isolates included in the study

2.1.1. Probiotic candidates

Ten clinically healthy cultured European sea bass larvae from both 10 days post hatching (dph) and 33 dph were retrieved from the Ecloserie Marine de Gravelines (Gravelines, France). All larvae were sacrificed with an overdose of MS222 (tricaine methanesulfonate, Sigma–Aldrich, Diegem, Belgium), surface disinfected for 30 s (0.1% benzalkonium chloride), rinsed with autoclaved artificial sea water (AASW) and homogenized in 1 ml AASW. In addition, three clinically healthy wild adult sea bass were obtained from the Belgian part of the North Sea, sacrificed with an overdose of MS222 and surface disinfected with 70% ethanol, whereafter the intestine was dissected under antiseptic conditions. Intestinal samples were homogenized in 1 ml AASW. Serial tenfold dilutions of the larval and adult sea bass intestinal content homogenates were plated on Marine Agar 2216 (MA, Scharlab S.L., Sentmenat, Spain), de Man, Rogosa and Sharpe agar (MRS; Oxoid Ltd., Hampshire, UK) and Thiosulfate Citrate Bile Sucrose Agar (TCBS; Sigma–Aldrich, Diegem, Belgium). The inoculated plates were incubated at 17 °C with 5% CO_2 for 72 h.

Bacillus sp. LT3, a putative probiotic strain (Niu et al., 2014), isolated from the intestinal tract of clinically healthy pacific white shrimp (*Penaeus vannamei*, Linneaus 1758), was provided by the Laboratory of Aquaculture & Artemia Reference Center (Defoirdt et al., 2011).

2.1.2. Bacterial pathogens

Vibrio ordalii (LMG 10951) was obtained from the BCCM/LMG Bacteria Collection. *Vibrio harveyi* HHS was isolated from the liver of a hammerhead shark (*Sphyrnidae*) exhibiting signs of septicemia. *Vibrio anguillarum* strains HI610, CNEVA NB 11008 and 43 were isolated from diseased cod at the Parisvatnet research facility and from a sea bass farm in France and the UK, respectively. These three strains were kindly provided by the Laboratory of Aquaculture & Artemia Reference Center (Ghent University). *Vibrio harveyi* SB was procured from a disease outbreak in a French sea bass farm.

2.1.3. Cultivation practices

All bacterial isolates were cultured on MA, incubated for 48 h at 17 °C in 5% CO_2 and subcultured in tryptic soy broth (TSB, Becton, Dickinson and Company, New Jersey, USA) supplemented with 2% NaCl and grown overnight at 17 °C in 5% CO_2. For the *in vivo* assays the cultivated broth was centrifuged (3000 rpm for 10 min) and the resulting pellet re-suspended in filtered artificial autoclaved sea water, adjusted to a salinity of 33 ppt and a temperature of 16 ± 1 °C (FAASW; Instant Ocean®; 0.2 μm filter, Sartopore Pt MidiCaps, Sartorius). The stock culture was adjusted to the desired bacterial concentration by using an ATB 1550 densitometer (bioMérieux, Marcy-l'Etoile, France). Bacterial titres were verified by making tenfold dilution series of the stock cultures on MA.

2.2. In vitro *selection of probiotic candidates*

2.2.1. Antagonistic activity

The Kirby–Bauer disk diffusion method, issued by the National Committee on Clinical Laboratory Standards (1997) for susceptibility testing, was adjusted for testing the antagonism of the retrieved probiotic candidates against six selected fish pathogens as listed above: *V. ordalii*, *V. harveyi* HHS, *V. harveyi* SB and three *V. anguillarum* strains (HHI610, CNEVA NB 11008, 43). Marine agar plates were streaked with cultivated broth of the pathogens, so that confluent bacterial growth would be encountered on the agar after 24 h incubation at 17 °C in 5% CO_2. Sterile paper discs (Whatman® Antibiotic Assay Discs diam 6 mm, Maidstone, UK) were immersed into the cultivated broth of the probiotic candidate isolate, and mounted onto the agar surface already seeded with pathogen, resulting in eight discs per plate. Blank discs immersed in TSB + 2% NaCl were used as a negative control to exclude any non-specific activity. Antagonism was detected by recording the presence or absence of a zone of inhibition surrounding the disc after 24 h incubation at 17 °C in 5% CO_2. Antagonistic activity was classified according to the number of pathogens that were inhibited, resulting in the following classes: weak (1–2), moderate (3–4) or strong (5–6) inhibitory.

2.2.2. Haemolytic activity

Haemolytic activity was evaluated on Marine agar plates, supplemented with 5% sheep blood (Oxoid Ltd., Hampshire, UK), which were streaked with the cultivated broth of the probiotic candidate isolates. Haemolytic activity was examined after 48 h incubation at 17 °C in 5% CO_2.

2.2.3. Bile tolerance

Bile tolerance was evaluated for all probiotic candidate isolates exhibiting strong or moderate antagonism and absence of haemolytic activity, according to Lin et al. (2007) with minor modifications. Twenty microliter of cultivated broth was transferred to 1 ml of TSB + 2% NaCl with 0%, 0.5%, 1% or 2.0% oxgall (Oxoid Ltd., Hampshire, UK) and incubated for 24 h at 17 °C in 5% CO_2. Bacterial growth was monitored by measuring absorbance with a Multiscan MCC 340 spectrophotometer (Labsystem) at 620 nm. The percentage of bile tolerance was calculated by comparing the OD values between cultivated broth with and without oxgall (Lin et al., 2007).

2.3. In vivo *screening of probiotic candidates*

All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience-Engineering, Ghent