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Isolation of putative probionts from cod rearing environment

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Abstract

Survival problems are encountered at early stages of intensive fish rearing and antibiotics are widely used to remedy the situation. Probiotics may provide a potential alternative method to protect larvae from opportunistic and pathogenic bacteria and promote a balanced environment. This study was designed to search for new probiotics to target this critical period in cod rearing. Potential probionts were selected from the natural microbiota of cod aquacultural environment. The selection was based on several criteria: pathogen inhibition potential, growth characteristics, strain identification, metabolite production and adhesion to fish cell lines. Our study demonstrated that 14% of screened bacteria ($n = 188$) had antagonistic properties towards fish pathogens. The majority of these isolates were Gram-positive (81%), belonging to Firmicutes (69.2%) and Actinobacteria (11.5%) phyla based on 16S rRNA gene sequencing. Only 6 (3.2%) of 188 isolates could inhibit all three pathogens tested: *Vibrio anguillarum*, *Aeromonas salmonicida* subsp. *achromogenes* and *Vibrio salmonicida*. Differences observed in activity intensity and spectrum among inhibitory isolates emphasise the need to develop probiotic mixtures for efficient prophylactic methods. Comparison of growth behaviour of inhibitory isolates and pathogens at cod rearing temperatures, metabolite production and adhesion capacity were considered for final probiont selection. Four promising isolates that could be used as a mixed supplement to rearing water were identified as putative probiotic bacteria. This study emphasises the importance and potential of lactic acid bacteria in aquaculture.

Keywords: Cod aquaculture; Probiotic; Fish pathogens; Antagonism; Growth characteristics; Adhesion

1. Introduction

Proliferation of opportunistic and pathogenic microorganisms in intensive rearing systems is known to cause poor larval growth and high mortality rates (Munro et al., 1995). Antibiotics have been used to prevent and control bacterial

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diseases in aquaculture, but they can lead to the emergence of resistant bacteria and environmental problems (Serrano, 2005). Effective commercial vaccines against some fish pathogens are available, but not applicable to larvae due to their small size and immature immune system. There is an urgent need to control the microbiota in hatching facilities by using alternative approaches, like probiotics. ''Probiotics'' traditionally refers to a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). Such oral treatments may provide the most effects but, in aquaculture, surface treatments through rearing water may also be of importance (Gatesoupe, 1999). Also, a probiotic should have a proven safety and efficacy in the host (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf).

Available commercial probiotic products for aquaculture use are dedicated to warmwater species while none has been developed for coldwater species. Atlantic cod (*Gadus morhua* L.) is gaining importance as a farmed fish. It is important to ensure the sustainability of cod aquaculture by supporting its safe and effective development. In the search for probiotic candidates, the following criteria should be considered (Gatesoupe, 1999; Verschuere et al., 2000): the microorganisms should (1) be non-pathogenic to the host, (2) compete with or hinder the growth of undesirable microbes, (3) adhere to, develop within the host and (4) be indigenous to the environment to which it will be used. Many studies have demonstrated that the indigenous microbiota of fish or rearing environment can inhibit pathogen growth (Fjellheim et al., 2007; Joborn et al., 1997; Robertson et al., 2000; Spanggaard et al., 2001; Vijayan et al., 2006; Vine et al., 2004).

The purpose of this study was to isolate and characterize bacteria from healthy cod aquacultural environment for possible use as probiotics at early stages of cod rearing. A multi-level screening approach was selected to evaluate the inhibitory potential towards three fish pathogens, growth behaviour and metabolite production of inhibitory isolates as well as adhesion capacity to fish cell lines. To our knowledge, this is the first study reporting on a cod rearing microbiota screening for probiotics.

2. Materials and methods

2.1. Sampling of cod rearing systems

Sampling of cod rearing systems was performed during seven visits to the Aquaculture Centre of the Marine Research Institute (Grindavik, Iceland) during the pre- and post-hatching period of 2004. The pre- and post-hatching periods consisted of 12 days at 8 °C in 25 l incubators and up to 56 additional days at 8–14 °C in hatchery silos/tanks (150 and 3400 l), respectively. Algal supplement (*Nannochloropsis oculata*, Instant Algae® Nannochloropsis, Reed Mariculture, USA) was used to shade the rearing water in the hatchery silos/tanks, while clay (Glit, Iceland) was diluted in seawater, disinfected with Virkon (Antec, UK) and used in tanks from day 19. Live feed was given between days 3 and 26 (rotifers, *Brachionus plicatilis*) and days 22–54 (Artemia, *Artemia salina*), but dry feed (Gemma 0.5, Skretting, Norway) introduced from day 22. Samples analysed included rearing water, ova, larvae (mainly gastrointestinal (GI) tract), Artemia, rotifers, algal supplement, dry feed and juvenile gills. Juveniles (5–6 months) were sampled from three tanks during a visit in October 2004. All samples were aseptically prepared and diluted in cooled 50% artificial seawater (ASW, 20 g/l Sigma sea salts S9883, USA) supplemented with 0.1% peptone (Difco, BD, USA). Pools of fertilized ova (5–7 g) were transferred to the laboratory, trapped in a tea strainer and stored in rearing water from each respective incubator under chilled conditions. Surface rinsing (60 s) of ova was performed using 200 ml of chilled 50% ASW to remove non-adherent microorganisms. Larvae were similarly trapped and transferred to the laboratory, followed by anaesthetizing (0.17% hypnodyl for 30 s), surface disinfection (60 s in 200 ml chilled 0.1% benzalkonium chloride solution; Rodalon® 10%, Brenntag Disinfection, DK) and rinsing (3 × 200 ml chilled 1% NaCl solution for 30 s). This allowed for the isolation of the larval GI microflora. A pool of whole larvae (40–80 individuals) was mashed, diluted and mixed in a Petri dish. Artemia and rotifer samples were pipetted (10 ml) from their respective silo/tank and stored chilled until analysed. They were then vortexed for 30 s and diluted as required. Juvenile gills were aseptically cut, pooled from three individuals,