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Use of selected bacteria and yeast to protect gnotobiotic *Artemia* against different pathogens

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Abstract

To evaluate the potential probiotic effect of two bacterial strains towards *Artemia* cultured in different gnotobiotic conditions, challenge tests were performed with a virulent *Vibrio campbellii* or with an opportunistic *Vibrio proteolyticus* strain. For that purpose, three feed sources (different isogenic *Saccharomyces cerevisiae* mutant strains) were chosen, yielding distinct *Artemia* culture performances. Both bacterial strains, selected from previous well-performing *Artemia* cultures, were able to protect against the opportunistic *V. proteolyticus*, while, generally, these bacteria could not protect *Artemia* against *V. campbellii*. The quality of the feed provided (in the form of the isogenic mnn9 yeast mutant) to *Artemia* had a stronger influence on nauplii protection against the opportunistic and the virulent *Vibrio* than the addition of beneficial bacteria. This feed has a higher nutritional value for *Artemia*, but contains also more cell wall bound β-glucans and chitin. Data suggest that the change in the cell wall composition, rather than the overall better nutritional value, of the mnn9 strain is responsible for the protection against both *Vibrios*.
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Keywords: *Artemia*; Gnotobiotic culture; *Saccharomyces cerevisiae*; Probionts; Pathogens; *Vibrio campbellii*; *Vibrio proteolyticus*

1. Introduction

Live feeds, such as *Artemia*, play an important role in the dietary regime of fish and shellfish larvae produced in industrial hatcheries (Sorgeloos et al., 1986). However, these feeds can be a source of pathogenic bacteria in the hatchery environments (Vaseeharan and Ramasamy, 2003); hence, prevention of disease spreading through the feed animal is essential in aquaculture. Several environmental-friendly prophylactic and preventive methods can putatively be used to control pathogenic bacteria and to maintain a healthy microbial environment in aquaculture systems (e.g. probiotics, immunostimulants, antimicrobial peptides, and quorum sensing systems: Sakai, 1999; Verschuere et al., 2000; Bachère, 2003; Defoirdt et al., 2005). However, applications of these technologies must be based on thorough understanding of mechanisms involved and the putative consequences. An essential part of that understanding can be provided by looking in detail at host–microbial interactions.

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A key experimental strategy to study these interactions is to first define the functioning of the host in the absence of bacteria and then to evaluate the effects of adding a single or defined population of microbes, or certain compounds (i.e., under gnotobiotic conditions) (Gordon and Pesti, 1971). *Artemia* is particularly useful as a test organism to study the host–microbe interactions (e.g. to evaluate potential probiotic bacteria before testing in target-organisms), as it can easily be cultured in gnotobiotic conditions (Marques et al., 2004a,b). Furthermore, it is possible to bioencapsulate *Artemia* with probiotic bacteria in different gnotobiotic environments (e.g. Rico-Mora and Voltolina, 1995; Verschuere et al., 1999, 2000; Orozco-Medina et al., 2002). Nonetheless, most studies reported in literature have been performed with *Artemia* cultured in poor conditions, such as using only bacteria as feed (Rico Mora and Voltolina, 1995; Makridis et al., 2000), or using autoclaved (Douillet, 1987; Orozco-Medina et al., 2002) or irradiated inert feed (Verschuere et al., 1999, 2000). Such artificial environments in combination with poor feed negatively influence the overall condition of gnotobiotically grown *Artemia*. According to Marques et al. (2005), when medium/good-quality feeds are provided to *Artemia*, both direct (probiotic or pathogenic) and indirect (nutritional) effects of a bacterial strain are less visible than with poor-quality feeds.

The present study aims to evaluate the potential probiotic effect of two beneficial bacterial strains towards *Artemia* by application of a challenge test with a virulent and with an opportunistic pathogenic *Vibrio* strain. In addition, by setting different gnotobiotic conditions (through a combination of yeast isogenic mutants and bacteria as feed), it is possible to perform these challenges with *Artemia* in different nutritional status.

2. Material and methods

2.1. Axenic cultures of yeast

Two strains of baker's yeast (*Saccharomyces cerevisiae*) were used as feed for *Artemia*: the wild type strain (WT) (BY4741, genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and its mnn9 isogenic mutant (BY4741; genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YPL050c::kanMX4). The mnn9 yeast was previously shown to be a better quality feed to *Artemia* than the WT yeast (Marques et al., 2004b). This isogenic mutant presents a null mutation resulting in a lower concentration of mannose, linked to mannoproteins, and higher concentrations of chitin and glucans in the yeast cell wall (Magnelli et al., 2002; Marques et al., 2004b). Both strains were provided by the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF, University of Frankfurt, Germany). Both yeast strains (WT and mnn9 YEPD) were cultured in a complete Yeast Extract Peptone Dextrose medium (YEPD); in addition the mnn9 yeast was cultured in a minimal Yeast Nitrogen Based medium (YNB) (mnn9 YNB). The procedures used in the present study to culture both yeasts were identical to the methods described by Marques et al. (2004b). Both strains cultured in YEPD were harvested by centrifugation (±800×*g* for 10 min) in the stationary growth phase (using a spectrophotometer to measure the optical density–OD–of the yeast culture at a wavelength of 600 nm after 3 days of culture; OD_{600}: ±10.600 for WT and OD_{600}: ±7.300 for mnn9), while mnn9 cultured in YNB was harvested in the exponential growth phase (after 20 h of culture; OD_{600}: ±0.700). All handlings were performed in a laminar-flow hood to maintain sterility. These three feed sources were chosen according to their nutritional quality to *Artemia*: poor-quality feed (WT yeast—enabling low *Artemia* survival and low growth), medium-quality feed (mnn9 yeast cultured in YEPD—enabling intermediate values of *Artemia* survival and growth) and good-quality feed (mnn9 yeast cultured in YNB—enabling high *Artemia* survival and growth) (Marques et al., 2004a,b, 2005). Yeasts were resuspended in filtered and autoclaved seawater (FASW, 0.2 μm) and their densities were determined by measuring twice the cell concentration, using a Bürker haemocytometer. Suspensions were stored at 4 °C and used to feed *Artemia* until the end of each experiment.

2.2. Bacterial strains and growth conditions

Two bacterial strains (strain LVS 2—*Bacillus* spp.; and strain LVS 3—*Aeromonas hydrophila*) were selected for their positive effect towards *Artemia* (Verschuere et al., 1999, 2000; Marques et al., 2005) and examined for their ability to protect nauplii cultured in different gnotobiotic environments against two different pathogens. Furthermore, two bacteria pathogenic towards *Artemia*, namely *Vibrio proteolyticus* strain CW8T2 (Verschuere et al., 1999, 2000) and *Vibrio campbellii* strain LMG21363 (Soto-Rodriguez et al., 2003; Gomez-Gil et al., 2004) were also used in this study. Pure cultures of the 4 bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, and from the

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