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Analysis of the evolution of microbial communities associated with different cultures of rotifer strains belonging to different cryptic species of the *Brachionus plicatilis* species complex

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ABSTRACT

The evolution of the composition of microbial communities associated with cultures of 3 different strains belonging to different cryptic species of the rotifer *Brachionus plicatilis* was monitored during four subsequent cycles of batch cultivation using denaturing gradient gel electrophoresis, cluster analysis, multidimensional scaling and principal component analysis. The data suggest that the evolving microbial communities are different with different *B. plicatilis* strain cultures. Moreover, large changes in rotifer growth rate were found to be associated with large changes in the microbial community composition, suggesting that there might be a causal link. Finally, Lorenz curves and Gini-coefficient analysis revealed that good performing *B. plicatilis* cultures showed a more even microbial community structure.

1. Introduction

Rotifers (*Brachionus* spp.) have been used as a live food for feeding larval marine fishes for over 30 years (Yúfera, 2001). Today, more than 60 marine fish species and 18 crustacean species require adequate and reliable production of high-quality, nutritious rotifers. The success of rotifer mass cultures is determined not only by reproduction rate and density, but also by their nutritional composition and their associated microbiota (Dhert, 1996; Dhert et al., 2001).

Batch cultivation, due to its simplicity is probably the most common type of rotifer production in marine fish hatcheries (Lubzens et al., 2001). Initially, rotifers are introduced at low density into tanks or ponds. The culture strategy consists of either the maintenance of a constant culture volume with an increasing rotifer density or the maintenance of a constant rotifer density by increasing the culture volume. Rotifers are fed with microalgae, bakers' yeast or an artificial diet. A total harvest of the rotifers is applied with part of the rotifers used as food for fish larvae and part used as inoculum for the next culture (Lubzens et al., 1987). Using an artificial diet (e.g. Culture Selco®), the density at harvest time is about 600 rotifers ml^{-1} after four days of culture starting from 200 to 250 rotifers ml^{-1} (Suantika et al., 2000).

Although they are frequently used, batch culture systems generate highly variable conditions that can have an influence on growth performance that also affects the composition of associated microbial communities (Rombaut et al., 2001). Bacteria are always associated with mass production of rotifers and may cause unexpected mortality or suppressed growth of rotifers. Moreover, since they are used as the first food of larvae, rotifers are often suspected as vectors of potential harmful bacteria to the cultured animals (Dhert et al., 2001). Using conventional culture-based methods, the dominant bacterial groups in rotifer cultures were classified as *Pseudomonas*, *Vibrio* and *Aeromonas* spp. (Nicolas et al., 1989; Skjermo and Vadstein, 1993; Verdonck et al., 1997). Large variations in the number of rotifer-associated ($1.8–7.6 \times 10^3$ CFU $rotifer^{-1}$) and free-living bacteria ($0.6–25 \times 10^7$ CFU ml^{-1}) have been observed (Skjermo and Vadstein, 1993). Rombaut et al. (2001) described the evolving microbial community present in rotifer batch and recirculation systems by means of the molecular biological technique DGGE. The authors found that in a recirculation system, subsequent to a high-variable period, a climax community was established, which remained more or less stable and was characterized by the dominance of one bacterial genus, i.e. *Marinomonas*. In contrast to the recirculation system, the profiles of the microbial community present in the batch system were more susceptible to variation.

Especially in relation to microbial communities associated with live food, it is important to study microbial communities associated with closely related hosts. A recent study found that different cryptic *B. plicatilis* strains exist within aquaculture hatcheries (Papakostas

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et al., 2006). It is likely that many previous studies were done with unknown and different strains of the *B. plicatilis* complex or even mixtures of species. Therefore, the characterization of microbial communities of rotifer cultures needs to be done with clear knowledge of the rotifer species.

To address the questions whether cultures of different cryptic *B. plicatilis* strains have different associated microbial communities, and how the microbial communities are evolving in consecutive batch cultures, DGGE fingerprints of PCR-amplified 16S rRNA gene fragments were made and analyzed with a series of ecological tools.

2. Materials and methods

2.1. Rotifer strains

Experiments were performed with 3 different cryptic *B. plicatilis* strains, namely *B. plicatilis sensu strictu*, *B. plicatilis* Cayman and *B. plicatilis* Nevada. Before the start of the experiment, rotifer strains were kept in non-sterile cultures at the Laboratory of Aquaculture and Artemia Reference Center (Gent, Belgium). The rotifer stocks were regular examined by microscopy for the presence of ciliates and other protists and maintained at controlled culture conditions: 28 °C, light intensity 2000 lx, salinity 25 g l^{-1}, and fed with yeast-based Culture Selco® following the culture procedure described by Dhert (1996).

2.2. Preparation of microbial communities (MCs)

MCs were prepared following the methods described by Tinh et al. (2006). Briefly, MCs were isolated from normal performing rotifer *B. plicatilis sensu strictu* cultures. For isolation of MCs, the culture water collected from rotifer culture was filtered through 250 μm and 60 μm meshes to remove big food particles and all rotifers, respectively, and was subsequently centrifuged at 1600 g for 5 min to remove the algal cells, thus only retaining the MCs in the supernatant. These MCs were preserved for further experiments in 1 ml eppendorfs containing 20% glycerol and 80% bacterial suspension and kept at −80 °C. Before starting each experiment, the Eppendorf tubes were thawed. Subsequently, 50 μl of the MC suspension was spread plated on Marine Agar (MA). After 24 h of incubation at 28 °C, the bacteria were harvested by swabbing the MA plate and suspending in autoclaved Nine Salt Solution (NSS). Cell density of the suspensions was calculated according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), based on optical density measurements (OD_{550} = 1.000 corresponds to 1.2×10^9 cells ml^{-1}). Subsequently, the appropriate volume to be added to each treatment was calculated in order to have a density of 10^6 cells ml^{-1} in the rotifer culture water at the start of each experiment independent of the *Brachionus* strains.

2.3. Experimental set-up of rotifer batch cultures

The separate runs of experiments of each rotifer strain were performed in 50 ml sterile falcon tubes (TRP®, γ-irradiated) with four replicates for rotifer *B. plicatilis* Nevada and three replicates for *B. plicatilis sensu strictu* and *B. plicatilis* Cayman. Rotifers were harvested by filtration from stock cultures, rinsed three times with 0.22 μm-filtered and autoclaved natural seawater (FASW) to remove most of the bacterial load, and distributed into falcon tubes containing 32.5 ml of 25 g l^{-1} FASW, to have a density of 30 rotifers ml^{-1} at the start of each experiment. The MCs were added once to each tube to have a density of 10^6 cells ml^{-1} at the start of experiment. The falcon tubes were put on a rotor (4 rpm) which was placed inside a temperature-controlled room (28 °C, light intensity 2000 lx). Each experiment was run for 4 batch cycles and each cycle consisted of a 3-day culture period. At the end of each batch, rotifers were harvested, rinsed and re-distributed at a density of around 30 rotifers ml^{-1} in fresh FASW before starting the next batch culture.

2.4. Rotifer diet

The rotifer diet consisted of a commercial diet Culture Selco 3000®, CS 3000 (INVE), Belgium. The rotifers were fed daily following a standard feeding regime for Culture Selco 3000® according to manufacturer's instructions below:

$$\text{CS 3000} = 0.0168D^{0.415}V$$

where: CS 3000 = the weight of experimental diet (g); D = rotifer density (individuals ml^{-1}); V = culture water volume (L).

2.5. Sampling, counting and growth data analysis

Three samples of 0.5 ml were taken from the rotifer cultures. The rotifers in each sample were killed by adding three drops of lugol, and were counted. Empty and transparent lorica belonging to dead rotifers were not taken into consideration.

The specific growth rate was calculated using the following equation described by Rombaut et al. (2001): $\mu = (\ln N_t - \ln N_0)/t$, where: μ = specific growth rate; N_t = rotifer density after culture period t (individuals ml^{-1}); N_0 = initial rotifer density (individuals ml^{-1}); t = culture period (day).

Data of the growth rates on day 3 were evaluated using Levene's test for homogeneity of variances and Shapiro–Wilk's test for normality. As data were normal-distributed and homoscedastic, the growth rates on day 3 for batch 1 and batch 4 were compared between experiments using one-way ANOVA, followed by Tukey test. All the tests were performed using the computer program SPSS release 12.0 (SPSS, USA).

2.6. Microbial analyses

2.6.1. Sampling procedure

One-milliliter culture water with rotifers was collected from the rotifer culture after inoculation at the start of experiment. Samples were centrifuged at 5000 g for 5 min and stored at −20 °C.

2.6.2. DNA extraction

Total DNA from the samples was obtained by a modified DNA extraction method as described previously by Rombaut et al. (2001). To obtain bacterial DNA, the samples were centrifuged for 30 min at 5000 g. The pellet was dissolved in 0.2 ml Milli-Q water, transferred to 0.4 ml of 10 mM Tris–HCl (pH 9) and 0.3 g of glass beads (0.10–0.11 mm diameter) was added. This mixture was homogenised three times for 30 s using a bead beater at 2000 rpm (B. Braun Biotech International, Melsungen, Germany). After this, 16 μl of 50 mg ml^{-1} lysozyme was added, and then the suspension was incubated at 37 °C for 15 min on a shaker (200 rpm). Chemical lysis of the bacterial cells was achieved by adding 30 μl of 20% SDS after which the suspension was slowly mixed for 5–10 min. Subsequently, 0.1 ml of 8 M ammonium acetate was added. DNA was obtained from the lysates using standard phenol–chloroform extraction and isopropanol precipitation procedures (Boon et al., 2000). The total DNA extracted was quantified by a spectrophotometer ND-1000 at 280 nm (NanoDrop Technologies, Wilmington, USA).

2.6.3. Amplification of 16S rRNA genes

DNA extracted from samples was amplified with primers gc338f and 518r spanning the V3 region of the 16S rRNA gene (Muyzer et al., 1993) using a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). PCR amplification was carried out in 24 μl reaction volumes to which 1 μl of DNA extract was added.

The PCR master mix contained 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 μl of thermophilic DNA polymerase 10× reaction buffer ($MgCl_2$-free), 2.5 U of Taq