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Use of a potential probiotic *Lactococcus lactis* AR21 strain for the enhancement of growth in the rotifer *Brachionus plicatilis* (Müller)

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Abstract

The effect of a potential probiotic on the growth performance of a rotifer and its inhibition against *Vibrio anguillarum* was studied. Probiotic strain AR21 had no significant observable effect on the growth rate of rotifers under optimal culture conditions in three consecutive experiments. In the first and second experiments, the AR21 strain exhibited an inhibitory effect against the *V. anguillarum* strain when rotifer cultures were maintained at a suboptimal feeding regime. The growth rate of the rotifers in suboptimal feeding conditions was significantly higher in the treatment receiving AR21 and *V. anguillarum* than in the treatment where only *V. anguillarum* was added.

Introduction

Various hygienic precautions are recommended to ensure optimal rotifer cultures; for example, water filtration, disinfection with sodium hypochlorite, ultraviolet radiation, ozonation, frequent water exchange and the use of antibiotics. However, the occurrence of opportunistic pathogenic microorganisms can not be excluded with these treatments, and consequently, low rotifer quality and larval mortality is often recorded (Nicolas, Robin & Ansquer 1989; Blanch, Simon, Jofre & Minkoff 1991; Sorgeloos 1994). In a bacteriological analysis carried out in two different marine fish hatcheries, Verdonck, Grisez, Sweetman, Minkoff, Sorgeloos, Ollevier & Swings (1997) reported that *Vibrio anguillarum* and *V. alginolyticus* were the dominant *Vibrio* species in rotifer samples taken after enrichment and rinsing.

Recently, the application of probiotics in aquaculture for primary disease control has become a promising new and important development. The term probiotics has been used in several ways, but in the present paper, the authors follow Fuller's (1989) definition: 'A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.' The use of beneficial bacteria in human, pig, cattle and poultry nutrition is well documented (Gilliland 1979; Conway 1989; Jong 1993). *Lactobacillus* bacteria are commonly used to control and prevent infection by *Escherichia coli* and other pathogenic microorganisms in the intestinal tract of many terrestrial animals (Tortuero 1973; Sissons 1989).

Douillet & Langdon (1994) reported a bacterial strain that enhanced growth in larval Pacific oyster, *Crassostrea gigas* (Thunberg), whereas Gatesoupe (1991b) described the use of *Bacillus* sp. spores as a tool to reduce bacterial infections and to obtain significant increases in weight in turbot, *Scophthalmus maximus* (Mitchill). Attempts have also been made to modify microbial flora in rotifer cultures by inoculation with beneficial bacterial strains. Gatesoupe (1991a, 1993) reported that commercial preparations of live *Lactobacillus plantarum* and *Bacillus* spores decreased the amount of Vibrionaceae in rotifer cultures. Gatesoupe (1991a, 1993) found that the addition of probiotic bacterial strain resulted in a more stable performance

of rotifers. In a recent study, Dutka-Gianelli, Kennedy, Fernandez, Gensler, Tucker (1997) demonstrated that *Bacillus* strain HBOI no. 48, isolated from healthy cultures of common snook, *Centropomus undecimalis* (Bloch), larvae modified the microflora and the production rate of rotifers.

The aim of the present study was to investigate the effect of *Lactococcus lactis* AR21 on the growth performance of the rotifer *Brachionus plicatilis* in two feeding regimes (optimal and suboptimal feeding) and also to demonstrate possible inhibitory effects of that strain against a potential pathogenic *V. anguillarum* strain.

Material and methods

Experimental procedure

The rotifers, *Brachionus plicatilis,* used in the present study were obtained from a stock culture maintained on an algal diet (*Chlorella* sp.) at 25 °C, 25 g L^{-1} salinity and a light intensity of 3000 lux. The experiments were performed in batch cultures in glass cones of 1000 mL filled with 600 mL of culture water. The culture water consisted of diluted sea water (25 g L^{-1} salinity) prefiltered over two membrane filters (1.00 and 0.22 µm, Sartorius, Göttingen, Germany). The culture water had previously been disinfected with sodium hypochlorite for 24 h. After disinfection, the excess of sodium hypochlorite was neutralized with sodium thiosulphate just before start of each experiment. All experiments were performed in a temperature-controlled water bath at 25 °C and at an initial rotifer density of 150–250 individuals mL^{-1}. Soft aeration was provided at the bottom of the cones to ensure good oxygenation and a uniform distribution of the diet. The air was filtered through a 0.2-µm in-line filter which was located between the main air supply and the individual aeration lines. Experiments were performed in darkness. Each experiment consisted of three consecutive reproduction cycles. One reproduction cycle consisted of a 3-day culture period, after which the water was renewed before starting the next reproduction cycle. During the first and the second cycle of the experiments, the rotifers were adapted to the optimal and suboptimal feeding regimes on Culture Selco (CS®, INVE Aquaculture, Dendermonde, Belgium). In the optimal feeding regime, the rotifers were fed on CS according to Lavens, Dhert, Merchie, Stael, & Sorgeloos (1994) (feeding regime as a function of rotifer density), while in the suboptimal feeding regime, the amount of food was reduced by 55% (Shiri Harzevili, Van Duffel, Defoort, Dhert, Sorgeloos & Swings 1997). The CS was suspended in distilled water and mixed in a kitchen blender for 2–3 min.

Experimental design

Three experiments were conducted to study the effect of a potential probiotic AR21 strain in rotifer cultures. In each experiment, eight treatments were applied: (1) control 100% CS, the optimal feeding regime; (2) control 45% CS, the suboptimal feeding regime; (3) AR21-100% CS, the optimal feeding regime + AR21; (4) AR21-45% CS, the suboptimal feeding regime + AR21; (5) VA-100%CS, the optimal feeding regime + *V. anguillarum*; (6) VA-45% CS, the suboptimal feeding regime + *V. anguillarum*; (7) AR21 + VA-100% CS, the optimal feeding regime + AR21 on day 0 and *V. anguillarum* 6 h later; and (8) AR21 + VA-45% CS, the suboptimal feeding regime + AR21 on day 0 and *V. anguillarum* 6 h later. All experiments were performed in triplicate.

Bacterial strains

The *L. lactis* AR21 strain was provided by the Laboratory of Microbiology, University of Gent, Gent, Belgium. The strain was isolated from a rotifer mass culture in the Laboratory of Aquaculture and Artemia Reference Centre in 1994 which showed an *in vitro* inhibitory effect against *V. anguillarum* Q19. This strain, showing non-sporing rods, oxidase and katalase negative, could not be identified by API nor by BIOLOG using the commercial Microlog database for identification. Based on these initial characteristics, the strains was further characterized by using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) on whole-cell protein and by comparison with a large laboratory database of lactic acid bacteria. Following numerical analysis, strain AR21 clustered together with reference strains of *L. lactis* ssp. *lactis*. The AR21 strain produces diplococcin, which shows slight inhibition against *Lactobacillus acidophilus*. Diplococcin was also tested against *V. anguillarum* Q19 and other *Vibrio* sp., and exhibited no inhibition. The AR21 strain has an acidifying effect on brain heart infusion (BHI) and reduces pH in the liquid medium (L. Grisez, personal communication).

The *V. anguillarum* strain used for the infection test was isolated from a rotifer culture in a commercial hatchery and was obtained from the Laboratory of Microbiology.

Preparation of the bacterial suspension

The *L. lactis* AR21 and *V. anguillarum* strains were preserved in liquid nitrogen and thawed before use. Three drops were plated in duplicate on Marine Agar (MA; Difco, Detroit, MI, USA). Plates were incubated at 25 °C for 24 h. Colonies were removed and a number of plates were inoculated with sterile cotton-bud applicators, depending on the required concentration of bacteria in the suspension. Freshly grown bacteria were taken from the plates and suspended in sterile saline solution (1.5% NaCl). To assess bacterial numbers, the density of the suspension was measured by means of a photospectrometer (550 nm) and a dilution of the suspension was plated on MA.

Bacterial inoculation

At day 0 of the third reproduction cycle of the rotifers, 1 mL of a suspension of the *L. lactis* AR21 strain (10^7–10^8 CFUs mL^{-1}) was added to the culture water in order to achieve a final concentration of 10^6–10^7 CFUs mL^{-1}. In each experiment, a suspension of the *V. anguillarum* strain (10^6–10^7 CFUs mL^{-1}) was added to the culture water 6 h after the addition of AR21 in order to demonstrate a possible inhibitory effect of *L. lactis* AR21 against *V. anguillarum*.

Microbiological sampling and analysis

Rotifer samples were taken for microbiological analysis 24 h after inoculation to trace back the initial bacteria added. Five millilitres of rotifer suspension was taken from each replicate with a sterile pipette and pooled (15 mL) in a sterile vial. Ten millilitres from each pooled sample was filtered on a sterile nylon mesh (50 μm) mounted in a plastic filter holder (Nalgene®). The rotifers remained on the mesh and were rinsed with sterile saline solution (NaCl, 1.5%). The nylon mesh containing the rotifers was aseptically transferred into a sterile plastic bag (Seward Medical Stomacher®, London, UK) containing 10 mL of sterile saline solution (1.5%) in which the rotifers were homogenized by means of a laboratory stomacher blender (Seward Medical Stomacher®). For plating, ten-fold dilutions were prepared in sterile saline solution (1.5%) from the homogenized suspension in the stomacher bags. Out of four different dilutions, 100 μL was plated in duplicate on MA (Difco), thiosulphate citrate bile sulphate agar (TCBS; Difco) and *V. anguillarum* medium (VAM), according to Alsina, Matrez-Picado, Joffre & Blanch (1994). The plates were incubated at 25 °C for 24–48 h. For identification of the AR21 and the *V. anguillarum* strain, fatty acid methyl ester (FAME) fingerprinting was applied. Qualitative analysis of the cellular fatty acid composition was performed using gas–liquid chromatography, as described by De Boer & Sasser (1986). The bacterial cultures were grown for 24 h at 28 °C on trypticase soy agar (TSA, Difco) supplemented with 1.5% (w/v) sodium chloride. Approximately 70 mg of cells was added to 1 mL of 3.75-M NaOH in 50% (v/v) aqueous methanol and heated for 10 min at 80 °C. After cooling to room temperature, fatty acid methyl esters were extracted with a 1:1 mixture of hexane and methyl-iso butylether. The methyl esters were analysed with a Hewlett Packard model 5898 A gas chromatograph and identified using the Microbial Identification System (MIS) software package (version no. 3.9, MIDI Inc., Newark, DE, USA). The strains were compared to the laboratory database of reference *Vibrio* strains (Laboratory for Microbiology) for identification. Clustering of the *Vibrio* strains was performed by numerical analysis using the Euclidean distance coefficient and the unweighted pair-group method of averages (UPGMA) (Sneath & Sokal 1973).

In vitro antagonism test

The inhibition of growth of a *V. anguillarum* Q19 strain by AR21 was investigated *in vitro*. Both the pathogenic strain and AR21 were grown on brain–heart infusion agar medium (BHIA) containing 1.5% NaCl for 24 h. A few colonies of AR21 were suspended in 5 mL of saline solution (1.5%), and 10 μL of the suspension was again plated out on BHIA and incubated for 24 h. The colonies of AR21 on the BHIA medium were killed by exposure to chloroform vapour for 45 min. A suspension of *V. anguillarum* Q19 was prepared in 2–3 mL of trypticase soy broth (TSB) by adding a few colonies and 5 μL of this was again suspended in 7–8 mL of

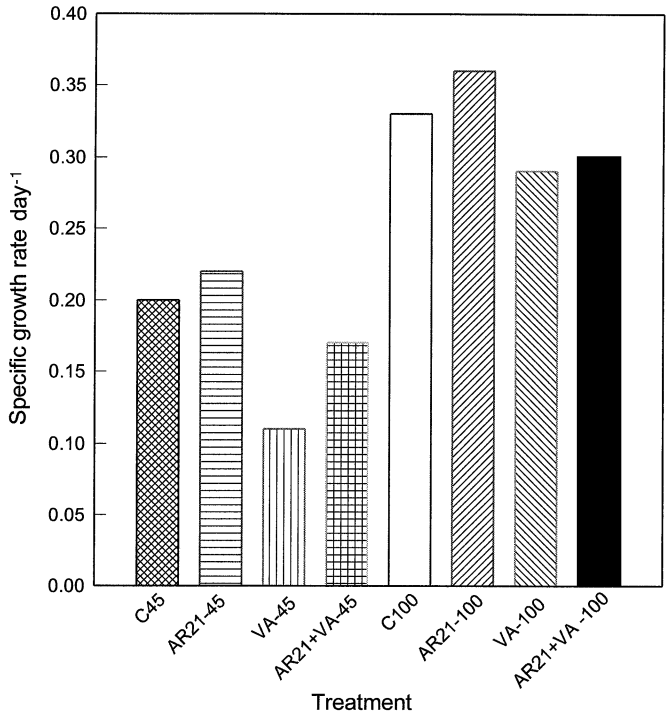

Figure 1 Mean values and standard deviations of the specific growth rates of rotifers after the addition of *Lactococcus lactis* AR21 in three repeated experiments in time: (C100) controls fed 100% Culture Selco (CS); (C45) controls fed 45% CS; (AR21–100) AR21 receiving 100% CS; (AR21–45) AR21 receiving 45% CS; (VA-100) *V. anguillarum* receiving 100% CS; (VA-45) *V. anguillarum* receiving 45% CS; (VA + AR21-100) AR21 and *V. anguillarum* receiving 100% CS; and (AR21 + VA-45) AR21 and *V. anguillarum* receiving 45% CS.

soft TSA containing 0.5% agar. The whole content of the soft TSA was poured over the BHIA plates containing dead colonies of AR21. The double-layer dishes, prepared in triplicate, were incubated for 48 h at 25 °C, and observed for growth and zones of growth inhibition (L. Grisez, personal communication).

Monitoring

Three samples of the rotifer culture were taken daily from each cone. After fixation with lugol solution, the animals were counted and the rotifer density (rotifer mL^{-1}) was determined. The specific growth rate (SGR) was estimated using the following equation:

$$SGR = (\ln N_t - \ln N_0)/t$$

where N_0 is the rotifer density at beginning of the experiment, N_t is the rotifer density at day t and t is the culture period in days.

Statistical analysis

The analysis of variance (ANOVA) was performed to determine any significant difference among the treatments. Significant differences between treatments were determined by Tukey's multiple range test ($P < 0.05$) (Zar 1996).

Results

The addition of *L. lactis* AR21 to rotifer cultures provided with an optimal feeding regime had no significant effect on the growth in three consecutive experiments (Fig. 1). The growth rates of rotifers fed on the suboptimal diet and receiving the probiotic strain were slightly higher, but no significant differences were observed in the control which did not receive the AR21 bacterial strain. In the first and the second experiments, the AR21 strain exhibited an inhibitory effect against the *V. anguillarum* strain in the suboptimal culture. The growth rates in the treatments where the *V. anguillarum* strain was added to the culture containing the AR21 were significantly ($P < 0.05$) higher than those in the treatments where only *V. anguillarum* was added. In the third experiment, the effect was not significant. The growth rates of rotifers fed on a suboptimal feeding regime and receiving *V. anguillarum* were significantly ($P < 0.05$) lower than the control suboptimal

cultures in the first and second experiments (Fig. 1). No significant differences in rotifer growth rates in the optimal feeding regime were found between the treatments receiving *V. anguillarum* and the treatment receiving a combination of AR21 and *V. anguillarum* strains in the three experiments. Neither AR21 nor *V. anguillarum* were recovered from the samples after 24 h.

Discussion

Gatesoupe (1989, 1991a,b, 1993) used the growth rate as a tool to evaluate the influence of the different probiotic bacteria in rotifer cultures. In his studies, the probiotics consisted of food additives containing live lactic bacteria. Some bacteria exhibited a positive effect on the performance of the rotifers and some other bacteria showed no effect on the rotifer performance. Douillet (1996) demonstrated that multiple probiotic applications in rotifer cultures can reduce the coefficient of variation in production compared to the control treatment.

There have been very few studies in aquaculture that focus on bacteria that prevent the growth of pathogenic organisms (Westerdahl, Olsson, Kjelleberg & Conway 1991; Nogami & Maeda 1992; Olsson Westerdahl, Conway & Kjelleberg 1992; Austin, Stuckey, Robertson, Effendi, & Griffith 1995; Bergh 1995; Riquelme, Hayashida, Araya, Uchida, Satomi & Ishida 1996). It is likely that the antagonistic relations of bacteria are as important a factor in the marine ecosystem as they are in soil, for example (Moriarty 1996). In the study by Lemos, Toranzo & Barja (1985), 38 out of 200 epiphytic isolates from intertidal seaweeds had the ability to inhibit growth of other bacteria. All these isolates belonged to the *Pseudomonas*/*Alteromonas* group, and the isolates showed growth inhibition against many fish pathogens, including *V. anguillarum* and *Aeromonas salmonicida* (Dopazo, Lemos, Lodeiros, Bolinches, Barja & Torango 1988). The application of microalgae to inhibit the growth of bacterial fish pathogens has also been suggested as a prophylactic strategy (Austin & Day 1990; Austin, Baudet & Stobie 1992).

In the present study, the *L. lactis* strain AR21 exhibited an inhibitory effect against the *V. anguillarum* strain in suboptimal cultures. A similar observation has been reported by Gatesoupe (1991a), who reported that the growth of *Aeromonas salmonicida* in a rotifer culture was inhibited by *L. plantarum*. Although the mechanisms of the bacterial interactions are poorly understood, the inhibitory effect of AR21 against *V. anguillarum* could be explained by the production of a vibriostatic agent or niche competition between the bacteria (Nogami & Maeda 1992). However, this inhibitory effect was only significant in two out of the three experiments performed. Although the rotifers were derived from the same stock cultures at the Laboratory of Aquaculture, rotifers did not perform in the same way. This was shown by the differences in growth rate between the different experiments for the control treatments (Fig. 1). Although the culture water and the set up had both been disinfected before use, the culture environment and the rotifers were not sterile. As a result, the microflora present differed from one experiment to the other. These factors, rotifer quality and present microflora, may be responsible for the variation in results between experiments.

A stricter method of standardization will be investigated in order to perform a more reliable screening of potential probiotics in the future. Further research is needed to establish the appropriate conditions for this research; for example, the genetic checking of rotifer stock, the correct preservation of rotifer stock material for challenge tests, bacterial incubation time, cell concentration and preservation of effective probiotics for practical use in aquaculture.

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