Probiotic and formaldehyde treatments of Artemia nauplii as food for larval pollack, Pollachius pollachius

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Abstract:

Formaldehyde was used to disinfect *Artemia* cysts and nauplii, while introducing two probiotics in the enrichment process: Bactocell (*Pediococcus acidilactici*) and Levucell (*Saccharomyces cerevisiae*). The disinfectant was selected due to its potential compatibility with probiotics, since it was more effective against Gram-negative bacteria than against lactic acid bacteria and yeast. However, the presence of formaldehyde reduced the intake of *P. acidilactici* in *Artemia*. Consequently, the disinfection was stopped before Bactocell supplementation to the nauplii fed to pollack (*Pollachius pollachius*) larvae. The mean weight of pollack was higher with this probiotic treatment. Growth was even better with the combination of Levucell and Bactocell, but the yeast should be introduced circumspectly. A high bacterial load was found in the nauplii enriched with Levucell, but not treated with formaldehyde. In the absence of Bactocell, the discontinuation of disinfecting *Artemia* after Levucell enrichment caused poor growth of pollack.

Resistant strains had emerged after 3 months of daily cyst incubation with 50 mg l^{-1} formaldehyde, rising from 1.4 CFU per newly hatched nauplius before the experiments, up to 3.4×10^4 CFU nauplius^{-1} by the end of 3 months. The phenotypes and genotypes of these opportunistic resistant strains were quite different from the initial resistant strains. Particular attention was paid to *Vibrio alginolyticus*-like isolates that were not detected before the experiments. These isolates were compared by amplified ribosomal DNA-restriction analysis (ARDRA) and RAPD to two reference strains previously isolated in turbot larvae, indicating some genotypic distance between the new isolates and the reference strains. Thus, the formaldehyde treatment cannot be recommended due to the risk of resistance spread.

It was concluded that *P. acidilactici* is a promising probiotic for fish larvae. Its combination with *S. cerevisiae* may be valuable, but on the condition that a concentrated form that would not jeopardize the bacterial balance in the absence of disinfectant should be prepared.

1. Introduction

A preliminary study has shown an interest of pollack as candidate species for aquaculture in the temperate Atlantic coast of Europe (Suquet, 2001). The first attempts of larval rearing were promising (Suquet et al., 1996), and it could serve as a model to improve the rearing technique for cod and other Gadidae considered to be difficult to rear like saithe (*Pollachius virens*; van der Meeren and Lønøy, 1998).

Schrøder et al. (1980) isolated a lactic acid bacterium from the gut of saithe, which inhibited *in vitro* the growth of *Vibrio* sp. This pioneering study pointed out the ecological importance of such bacteria in gut microbiota of Gadidae. Gildberg et al. (1997), then Gildberg and Mikkelsen (1998) evidenced the probiotic effect of lactic acid bacteria in cod fry.

In aquaculture, autochthonous probiotics have been generally preferred to commercial preparations designed for man and terrestrial livestock (Gatesoupe, 1999). However, there is a renewal of interest for such products already documented as safe foodstuff, and immediately available for every fish farms and hatcheries. For instance, Nikoskelainen et al. (2001a) investigated the potential probiotic properties of seven lactic acid bacteria intended for human or land animal use, by studying their capacities of adhesion and penetration into fish mucus, their inhibitory effect against fish pathogens, and their resistance to fish bile. The same authors showed the protective effect of one of these strains, *Lactobacillus rhamnosus*, against furunculosis in rainbow trout (Nikoskelainen et al., 2001b).

The present study aimed to study the effects of two commercial probiotic strains that were introduced into pollack larval microbiota via *Artemia* nauplii. These products designed for land animals contained either lactic acid bacteria, *Pediococcus acidilactici*, or yeast, *Saccharomyces cerevisiae*. Both species have been also described as being harbored in fish microbiota, where they may present some probiotic features, like the bacteriocin production

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of *P. acidilactici* (Halami et al., 1999), and the colonization potential of *S. cerevisiae* (Andlid et al., 1999). Both can survive in seawater, and non-autochthonous strains of *S. cerevisiae* adhered to the gut of European sea bass larvae (Tovar et al., 2000). Moreover, the combination of probiotics with different mode of action is theoretically interesting (Filho-Lima et al., 2000). To my knowledge, the synergistic effect of lactic acid bacteria and yeast has not been demonstrated, though commercial mixtures are available. Such a preparation with *Lactobacillus coagulans* and *S. cerevisiae* was efficient to increase the growth rate of tropical freshwater fish larvae and fry (Mohanty et al., 1996; Swain et al., 1996).

The bacterial load is quite high in newly hatched *Artemia* nauplii (Lavens and Sorgeloos, 2000). Verschuere et al. (1999) proposed to control the microbial environment of *Artemia* by cyst disinfection, then by preemptive inoculation of selected bacterial strains into the rearing water. Sahul Hameed and Balasubramanian (2000) recommended to disinfect the nauplii with formaldehyde before introduction into the rearing systems. In case of probiotic treatment, a particular interest of this disinfectant might be its efficiency stronger against gram-negative bacteria than against gram-positive bacteria and fungi (Spicher and Peters, 1976). Most bacteria are gram-negative in marine fish and shellfish microbiota, and formaldehyde could favor the artificial colonization by probiotic yeast or lactic acid bacteria. We have thus tried to use formaldehyde in combination with the introduction of probiotics. However, some gram-negative bacterial strains are known to resist to formaldehyde disinfection because of a plasmidic resistance gene (Kümmerle et al., 1996). It was therefore important to check whether the formaldehyde treatment could cause resistance spread.

The experimental design was thus split into three steps: (1) the bacterial characterization of *Artemia* nauplii treated with formaldehyde and with the probiotics, (2) the application of the treatments to pollack larvae, and (3) a bacteriological survey of formaldehyde resistant strains before and after the experiments.

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2. Materials and methods

2.1. Microbial study of Artemia cysts and nauplii

Artemia cysts (INVE, AF lot 01.27C) were incubated in strongly aerated seawater at 28°C, while disinfecting with 50 mg l^{-1} formaldehyde, unless other specified. After 20 h incubation, the nauplii were harvested by sieving between two plankton nets (100 μm and 212 μm mesh). The initial effect of formaldehyde disinfection on bacterial load was analyzed by counting bacteria associated with nauplii, after a cyst incubation test with 0, 50 and 100 mg l^{-1} formaldehyde, with four replicates per treatment. After three months of daily cyst disinfection, the bacteria were counted in a similar incubation test, with an additional treatment at 75 mg l^{-1} formaldehyde. To this end, the cysts were dispatched into 500 ml flasks with aeration, at the rate of 1 g l^{-1}. The bacteria were counted in newly hatched nauplii with the method used previously for rotifers (Gatesoupe, 1990). In addition to Petrifilm counts (Aerobic Count Plates, 3M Microbiology Products), the population of *Vibrio* was approximated by counting thiosulfate-citrate-bile salt agar plates(TCBS, AES Laboratoire, dissolved in half-strength seawater equivalent to 18 practical salinity units).

The initial and final resistant strains were sampled by isolating colonies on Petrifilm. The isolates were cultivated on plate count agar (PCA, AES Laboratoire, enriched with 18 g l^{-1} NaCl, with the pH adjusted to 7.8), and then they were stored under liquid nitrogen. The phenotypes were compared with API 20 E strips according to the manufacturer's instructions (bioMérieux). The API 20 E profiles were computed with Mix and Drawtree of Phylip (Felsenstein, 1996).

The genotypes were compared by amplified ribosomal DNA-restriction analysis (ARDRA, Dees and Ghiorse, 2001). Briefly, DNA was prepared for PCR, then the 16S rDNA gene was amplified with the universal primers SA-dir (5'-AGAGTTTGATCATGGCTCAG-3') and S17-rev (5'-GTTACCTTGTTACGACTT-3'), as described by Lambert et al. (1998). Two aliquots of the PCR product were incubated for 2 h at 37°C with (1) *Hae* III and (2) *Cfo* I, plus the respective buffers provided by the manufacturer (Sigma). The *Vibrio* isolates were compared to 2 reference strains that were closely related: VM2, formerly "vibrio E" (Gatesoupe, 1997), and V16 which was isolated once from healthy turbot larvae at 8 day post-hatching (dph) during an experiment of rotifer treatment with VM2 (unpublished data). These *Vibrio* strains were compared by RAPD (Gatesoupe et al., 1999), and they were cultured on PCA with 0, 50 and 100 mg l^{-1} formaldehyde.

The two probiotic preparations tested were Levucell SB 20 (*Saccharomyces cerevisiae* I-1079, CNCM, Institut Pasteur, Paris), and Bactocell (*Pediococcus acidilactici* CNCM MA 18/5M). Newly hatched nauplii were introduced into 500 ml bottles containing 400 ml seawater (100 nauplii ml^{-1}). The nauplii were starved for 6 h, then they were enriched for 14 h with the self-emulsifying concentrate of essential fatty acids DHA Selco (INVE, 150 mg l^{-1}), with or without 50 mg l^{-1} formaldehyde, and with or without 50 mg l^{-1} of one of the probiotic preparations, corresponding to ca. 10^9 CFU l^{-1} of *S. cerevisiae*, or ca. 10^{10} CFU l^{-1} of *P. acidilactici*. After enrichment, the bacteria associated with *Artemia* were counted on Petrifilm and TCBS plates. Two other specific media were used to count the probiotics: Sabouraud agar (AES Laboratoire) supplemented with antibiotics (chloramphenicol, 1 mg l^{-1}; Polymixin B sulfate, 0.8 mg l^{-1}; Amoxicillin, 1.25 mg l^{-1}) to count the yeast after 48 h of incubation at 30°C, and MRS agar (AES Laboratoire) to count *P. acidilactici* after 48 h of incubation at 45°C.

2.2. Larval rearing experiments

Pollack (*Pollachius pollachius*) larvae were reared in 150 l tanks with conical bottom. The newly hatched larvae were introduced into 18 tanks at the concentration of 28 larvae l^{-1}. The initial temperature was 11.8° C, and it was progressively increased up to 15.1 ± 1° C at 9 day post hatching (dph) onwards. The water supply was introduced at the bottom of the tanks at the rate of 1 l mn^{-1} from 1 to 11 dph, according to the recommendation of Gaignon et al. (1998). A tangential water supply was then showered at the surface at the rate of 500 ml mn^{-1} from 8 to 11 dph, then 1.5 l mn^{-1} from 12 dph onwards. The water surface was cleaned with the device of Chatain and Ounais-Guschemann (1990).

The food sequence was adapted from Suquet et al. (1996). The larvae were fed with rotifers *Brachionus rotundiformis* from 3 to 11 dph at the rates of 20-200 rotifers per 1 dph larva. The rotifers were cultured with live bakers yeast (60 mg l^{-1} day^{-1}, on a dry matter basis), DHA Selco (18 mg l^{-1} day^{-1}), and T-*Isochrysis affinis galbana* (ca. 2×10^5 cells mL^{-1} day^{-1}). Then, they were transferred into clean seawater (200 rotifers ml^{-1}), enriched with DHA Selco (18 mg l^{-1}), and distributed continuously into the larval rearing tanks with a peristaltic pump (Gatesoupe, 1990).

Newly hatched *Artemia* nauplii were introduced into the larval rearing tanks from 8 to 12 dph (1-30 nauplii per 1 dph larva).

From 13 dph onwards, the nauplii were further treated for one day with six experimental enrichments. To this end, the nauplii were introduced into enrichment tanks at the rate of 20 nauplii ml^{-1}, and treated with formaldehyde (50 mg l^{-1}). After 6 h of starvation, the nauplii were enriched for 14 h with DHA Selco (150 mg l^{-1}), and either brewers dried yeast (inactive food ingredient, 50 mg l^{-1}, treatments BC, BL and BP) or the probiotic preparation Levucell

(50 mg l^{-1}, treatments LC, LL and LP). This 14 h phase was designated as the first step of enrichment. The 1 dph *Artemia* were then distributed continuously with a peristaltic pump, and further enriched with DHA Selco (50 mg l^{-1}). Depending on the experimental treatment, this second step of the enrichment process included no probiotic (treatments BC and LC), or either Levucell (50 mg l^{-1}, treatments BL and LL) or Bactocell (50 mg l^{-1}, treatments BP and LP). When Levucell was used during the second step of enrichment, the distribution medium was again treated with formaldehyde (50 mg l^{-1}, treatments BL and LL).

The amounts of 1dph *Artemia* were adjusted daily for each treatment, according to the amounts remaining in the larval rearing tanks (from 5 to 120 nauplii per 1 dph larva). The food amounts could not be adjusted independently for each replicate, because the larval tanks were fed from one enrichment tank per treatment. For each treatment, the total amount of 1 dph *Artemia* distributed to the larvae from 13 dph onwards was added up, and then divided by the number of larvae surviving at 29 dph.

The six enrichment treatments were dispatched at random in triplicates among the 18 larval rearing tanks. Thirty larvae were sampled per tank at 13 and 29 dph, to estimate the mean weights before and by the end of the experimental feeding period. At 28 dph, five larvae were sampled per tank. They were transferred to a solution of 0.1% benzalkonium chloride for surface disinfection, then rinsed with seawater under sterile conditions (Skjermo and Vadstein, 1993). Then associated microbes were counted as described for *Artemia*.

The data were compared by analysis of variance (ANOVA), after checking normality and variance homogeneity. When both tests were passed, the effects of the individual treatments were assessed by an a priori test (Sokal and Rohlf, 1969), in case of significant difference. When one or both tests failed, ANOVA on ranks (Kruskall-Wallis) was applied, followed by the Student-Newman-Keuls test for significant differences.

3. Results

3.1. Microbial study of Artemia cysts

There was a strong initial effect of formaldehyde treatment of *Artemia* cysts on bacterial counts, but not significant effect on hatching rate (Table 1). Only 0.7 CFU nauplius^{-1} was recovered on Petrifilm after cyst incubation with 100 mg l^{-1} formaldehyde. Thirteen colonies were isolated for further characterization. The bacterial concentration was double with 50 mg l^{-1} formaldehyde, but this was still negligible in comparison with the 2 x 10^4 CFU nauplius^{-1} retrieved with the control incubation without formaldehyde. Colonies growing on TCBS agar were retrieved only in the control group.

After the cysts had been daily disinfected with 50 mg l^{-1} formaldehyde for three months, the bacterial concentration on Petrifilm was 3.4 x 10^4 CFU per nauplius hatched from cysts treated with 50 mg l^{-1} formaldehyde, and still 161 CFU nauplius^{-1} with 75 mg l^{-1} formaldehyde. No culturable bacterium was detected with 100 mg l^{-1} formaldehyde (detection threshold: 0.7 CFU nauplius^{-1}).

Among the colonies recovered after the treatment with 50 mg l^{-1} formaldehyde, 21 isolates were further characterized, and compared with the 13 initial isolates resistant to 100 mg l^{-1} formaldehyde.

The phenotypes of both series of isolates were quite different, as illustrated by the dendrogram obtained with their API 20 E profiles (Fig. 1). A particular attention was paid to the swarming strains VA75S3 and VAPFS4. Their profiles corresponded to *Vibrio alginolyticus*, and they were compared to the reference strain V16 that matched profile

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VAPFS4. The second reference strain VM2 had a distinct profile. The four strains were completely inhibited by 100 mg l^{-1} formaldehyde, but they were differently affected by half concentration. The swarming of VM2 was completely inhibited at 50 mg l^{-1} formaldehyde, V16 was still slightly swarming, whereas VA75S3 and VAPFS4 were not sensitive to this concentration (Fig. 2).

The genotypes were characterized by ARDRA (Fig. 3). Two groups of genotypes were observed among the initial isolates. A310, A315 and A317 had the same band pattern with the both restriction enzymes *Cfo* I and *Hae* III. The second group was also homogeneous, but A331 and A309 were distinct in one of the profiles obtained either with *Cfo* I or *Hae* III, respectively. Two types were also obtained with the final isolates, which were quite different from the initial ones. The first group was composed of PF1, PF3, 50F6 and PF18, with exactly the same pattern, plus 75F2 and PF23, which gave an additional restriction fragment of ca. 800 bp with *Hae* III. The second group corresponded to VAPFS3 and VAPFS4, which were similar to the *V. alginolyticus*-like reference strains. However, these two isolates had the same RAPD profiles but one obtained with Primer 5, whereas those of the reference strain V16 were quite different (Fig. 4). The profile of VM2 was only showed for Primer 2, but all the RAPD primers gave PCR products of VM2 that were much closer to those of V16 than those of the present isolates (data not shown, the RAPD characterization of VM2 will be published elsewhere).

3.2. Effect of treatments on Artemia *nauplii and pollack larvae*

Two hundred CFU were recovered on Petrifilm per *Artemia* nauplius fed without probiotic and not treated with formaldehyde (Table 2). This bacterial level was not significantly affected by feeding the nauplii with Bactocell, but it was tenfold increased with Levucell. The

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TCBS counts were not significantly affected by the probiotic treatments. The formaldehyde treatment inhibited completely the bacteria growing on TCBS, whereas the CFU on Petrifilm were decreased at least tenfold. *P. acidilacti* did not grow on Petrifilm, but its colonies were selectively retrieved on MRS agar incubated at 45° C, with an average of 69 CFU per nauplius fed Bactocell. An additional treatment with formaldehyde decreased fivefold this amount. The recovery of *S. cerevisiae* from Levucell on the selective agar was not affected by the formaldehyde treatment, and that corresponded to ca. 2×10^2 CFU per nauplius fed with Levucell.

The survival of pollack larvae was not significantly affected by the treatments (Table 3). The best growth rates were obtained when *Artemia* had been fed with Bactocell during the second step of enrichment. The combination of this enrichment with previous feeding with Levucell increased further the growth rate in comparison with the diet composed of inactive brewers yeast. However, the worse growth rate was observed when the nauplii had been fed with Levucell only during the first enrichment step. The growth rate obtained by feeding *Artemia* without probiotic was not significantly different from those obtained when Levucell was used at least during the second step of enrichment. The intake of *Artemia* enriched with Bactocell was more than 4000 nauplii per 29-dph larva, whereas the intake of the other groups was between 2000 and 3000 nauplii larva^{-1}.

There was no significant difference among the bacterial counts that averaged out to 10^5 CFU larva^{-1} and 5×10^4 CFU larva^{-1} on Petrifilm and TCBS agar, respectively. *P. acidilactici* was retrieved at a level of 10^5 CFU per larva fed with nauplii enriched with Bactocell, but few tens of CFU larva^{-1} were also recovered on MRS agar after enrichment with Levucell. Though the means were not significantly different, between 2×10^3 CFU larva^{-1} and 1.4×10^4 CFU larva^{-1} were retrieved on the selective yeast agar, when Levucell had been brought either only during the first enrichment step, or also during the second enrichment step, respectively.

4. Discussion

In previous experiments, the treatment of *Artemia* nauplii with lactic acid bacteria and *Bacillus* sp. spores did not improve the growth rate of turbot larvae (Gatesoupe, 1991, 1993). The recovery of the lactic acid bacteria was not studied (Gatesoupe, 1991), but *Bacillus* sp. was retrieved in turbot larvae at a low level (Gatesoupe, 1993). In the present experiment, the amount of *P. acidilactici* was even higher than the Petrifilm counts in the larvae fed with the enriched nauplii. This artificial dominance was likely decisive for the effect of the probiotic on larval growth.

The amount of *Artemia* distributed to each experimental group may bring some information about feed efficiency, though there was no statistics. The superior growth rate observed with Bactocell seemed due a higher food consumption rather than due to a better feed efficiency. However, feed efficiency may account for the extra weight gain obtained with the combination Levucell-Bactocell that did not increase further food intake in comparison with Bactocell only. Live yeast are known to stimulate gut maturation in larval sea bass (Tovar et al., 2002), and the strain of *S. cerevisiae* may have improved the digestion efficiency of nauplii by larval pollack. Dietary live *S. cerevisiae* improved also feed efficiency in Israeli carp, but to a lesser extent than *Streptococcus faecium* (Noh et al., 1994). The feed conversion ratio of mrigal fry was also increased with a dietary combination of live *S. cerevisiae* and *Lactobacillus coagulans* (Swain et al., 1996).

In the absence of *P. acidilactici*, the yeast did not increase larval growth. Its concentration was inferior to Petrifilm and TCBS counts in the larvae. When the yeast treatment was discontinued during the second enrichment step, the growth rate was even lower than in the control group. This may be due to the concomitant discontinuation of formaldehyde

disinfection, allowing bacteria to proliferate. Though the means were not significantly different, the highest Petrifilm counts were observed in the group fed with Levucell only during the first enrichment step.

The formaldehyde treatment was required when the nauplii were enriched with Levucell. Indeed this feed additive increased dramatically the bacterial load in *Artemia* without disinfectant. Yeast and *Artemia* survived well in 50-100 mg l^{-1} formaldehyde, by contrast with *P. acidilactici*. However, Bactocell did not increase bacterial load in comparison with the control treatment without probiotic. That may be due to the concentrated form of the bacterial additive, whereas Levucell contains excipient. *Artemia* nauplii digest poorly baker's yeast (Coutteau et al., 1991). It was thus possible to introduce the probiotic yeast early during the first enrichment step, resulting in a significant final yeast intake by the larvae, even without Levucell during the second enrichment step. Bactocell was reserved for the second step without disinfection, enabling thus a combination treatment in spite of the formaldehyde sensitivity of *P. acidilactici*.

However, the treatment used to disinfect *Artemia* in the present experiment appeared inadvisable, because of the emergence of bacterial strains that could proliferate during cyst incubation in 50 mg formaldehyde l^{-1} disinfection. These strains were quite different from those initially resistant to 100 mg formaldehyde l^{-1}, and they were still sensitive to this twofold concentration. It could not be recommended to double the concentration of formaldehyde, since some strains were still resistant, and a higher concentration would be detrimental to *Artemia* survival (Sahul Hameed and Balasubramanian, 2000). There is a risk of transmission of resistance gene to opportunistic strains. Plasmids have been isolated in antibiotic-resistant strains of *V. alginolyticus* (Li et al., 1999; Zanetti et al., 2001). The *V. alginolyticus*-like isolates of the present experiment were genetically distinct from those previously isolated in the same facilities and used as reference. Such bacteria were not

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detected in the initial samples of formaldehyde-treated newly hatched nauplii, since there was no colony growing on TCBS. The selection pressure exerted by the formaldehyde treatment caused likely the emergence of strains more resistant than those previously dominant. Sodium hypochlorite remains the best disinfectant for *Artemia* cysts (Agh et al., 2001), but it cannot be used during the enrichment process.

Ghioni et al. (1998) tested the effect of formaldehyde on rainbow trout skin cells *in vitro*. A dose of 20 mg l^{-1} was sufficient to reduce the lipid content of the cells, with other detrimental effects on their lipid and fatty acid composition. A direct effect of formaldehyde on the larvae was unlikely in the present experiment, due to high dilution, but a detrimental effect on the dietary value of *Artemia* cannot be excluded.

Though the present disinfection treatment was not advisable, *P. acidilactici* is a promising probiotic for fish larvae in view of its effect as growth promoter. Its combination with *S. cerevisiae* may be valuable, but on condition to prepare a concentrated form that would not jeopardize the bacterial balance in the absence of disinfectant.

Acknowledgements

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Table 1

Initial effect of formaldehyde treatment of *Artemia* cysts on hatching rate (in nauplii per mg of cysts) and bacterial counts after hatching (CFU nauplius^{-1}); $\pm$ standard error; n.d. not detected; n.s. not significant; parametric ANOVA, unless KW specified for Kruskall-Wallis procedure.

Formaldehyde dose	0 mg l^{-1}	50 mg l^{-1}	100 mg l^{-1}	Significance
Hatching rate	152 ± 34	199 ± 15	181 ± 10	n.s.
Petrifilm counts	22710 ± 4103^a	1.4 ± 0.3^b	0.7 ± 0.1^c	***P<0.001 (KW)
TCBS counts	7547 ± 2290	n.d.	n.d.	**P<0.05 (KW)

Table 2

Effect of probiotic and formaldehyde treatments of *Artemia* nauplii on microbial counts (CFU nauplius^{-1} ± standard error; n.a. not applicable; n.d. not detected; n.s. not significant; KW specified for Kruskall-Wallis procedure; § parametric ANOVA after exclusion of nil groups).

Agar	Petrifilm	TCBS	MRS	Sabouraud + antibiotics
Control	202 ± 99^b	30 ± 11^a	n.d.	n.d.
Formaldehyde	17 ± 17^c	n.d.	n.d.	n.d.
Bactocell	109 ± 38^b	36 ± 9^a	69 ± 31^a	n.d.
Bactocell + formaldehyde	0.05 ± 0.02^c	n.d.	14 ± 5^b	n.d.
Levucell	1877 ± 959^a	72 ± 46^a	n.d.	235 ± 82^a
Levucell + formaldehyde	0.3 ± 0.3^c	n.d.	n.d.	255 ± 95^a
Significance (KW)	*P<0.05	*P<0.05	**P<0.01	**P<0.01
ANOVA§	n.a.	n.s.	*P<0.05	n.s.

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Table 3

Effect of the probiotic treatment of *Artemia* nauplii on the final survival rate, mean weight, prey intake, and microbial counts of pollack larvae (± standard error; n.a. not applicable; n.d. not detected; n.s. not significant; parametric ANOVA, , unless KW specified for Kruskall-Wallis procedure).

Nauplii enrichment	BC	LC	BL	LL	BP	LP	Significance
1st step of enrichment	brewers yeast	Levucell	brewers yeast	Levucell	brewers yeast	Levucell	
2nd step of enrichment	no probiotic	no probiotic	Levucell	Levucell	Bactocell	Bactocell	
Survival (%)	12 ± 4	12 ± 3	33 ± 7	25 ± 5	15 ± 5	24 ± 5	n.s.
Weight (mg)	4.7 ± 0.2^c	3.3 ± 0.2^d	5.1 ± 0.2^c	5.3 ± 0.3^c	6.1 ± 0.3^b	8.1 ± 0.3^a	***$P<0.001$
Artemia per 29-dph larva§	3.0×10^3	3.1×10^3	2.2×10^3	2.8×10^3	4.7×10^3	4.4×10^3	n.a.
Petrifilm counts ($\times 10^4$ CFU larva^{-1})	11 ± 0.6	18 ± 9	8 ± 5	12 ± 11	2 ± 0.8	8 ± 4	n.s.
TCBS counts ($\times 10^4$ CFU larva^{-1})	2 ± 2	10 ± 5	3 ± 2	11 ± 11	3 ± 2	2 ± 2	n.s.
MRS counts (CFU larva^{-1})	n.d.	n.d.	18 ± 10^b	27 ± 23^b	$(1.3 \pm 1^a) \times 10^5$	$(2.1 \pm 0.7^a) \times 10^5$	*$P<0.05$ (KW)
Sabouraud + antibiotics ($\times 10^3$ CFU larva^{-1})	n.d.	2 ± 1^a	14 ± 6^a	14 ± 5^a	n.d.	2 ± 0.4^a	**$P<0.01$ (KW)

§ Computed as the total amount 1 dph *Artemia* distributed to the larvae from 13 dph onwards, and divided by the number of larvae that survived at 29 dph.
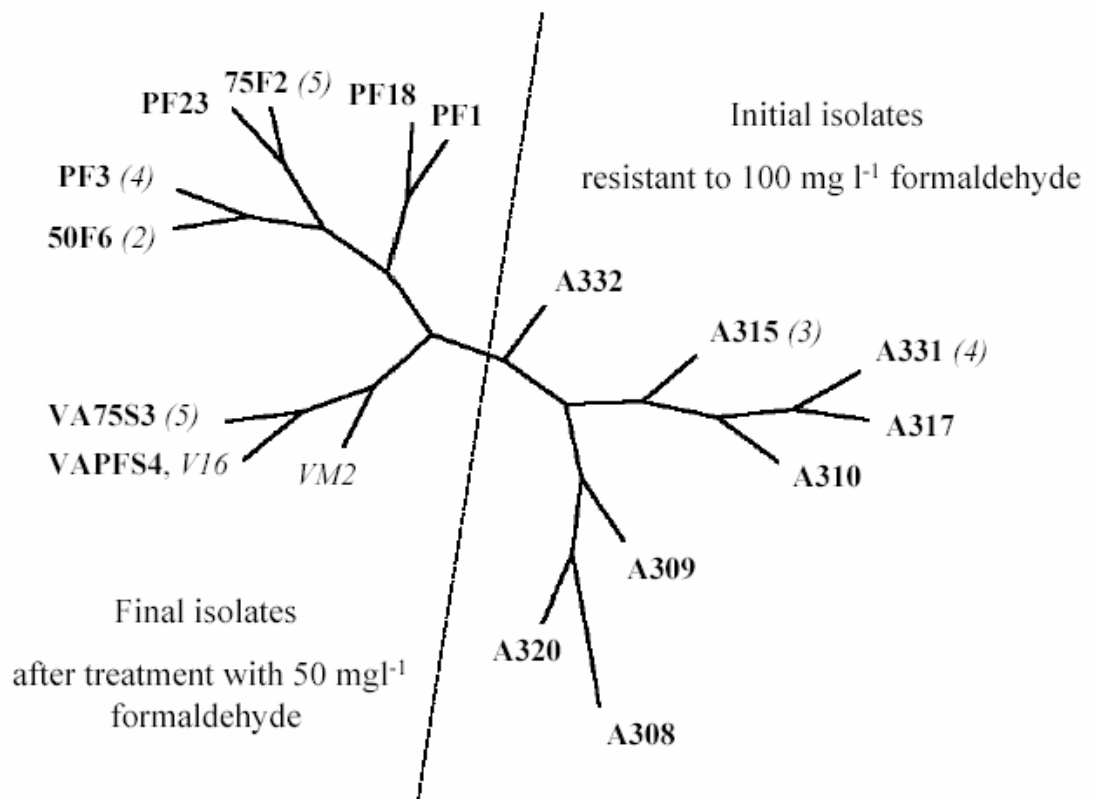
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Figures

Fig. 1. Phenotypic dendrogram of bacterial isolates obtained from *Artemia* nauplii after cyst disinfection with formaldehyde. The dotted line separated the initial isolates (right) from the final ones (left). When several isolates corresponded to the same profile, their number was indicated in brackets. VM2 and V16 were added to the dendrogram, as reference strains.
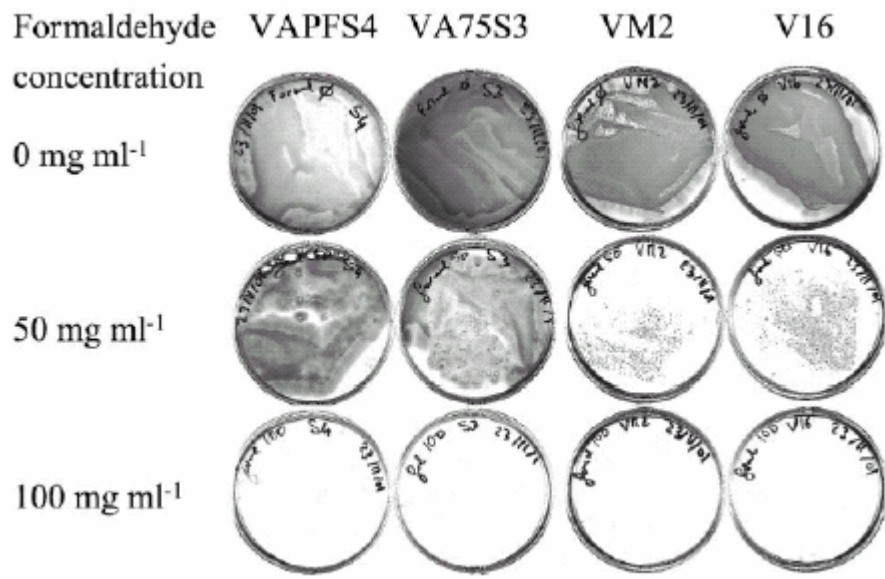
Fig. 2. The effect of formaldehyde concentration on growth and swarming of *V. alginolyticus*-like strains on PCA.
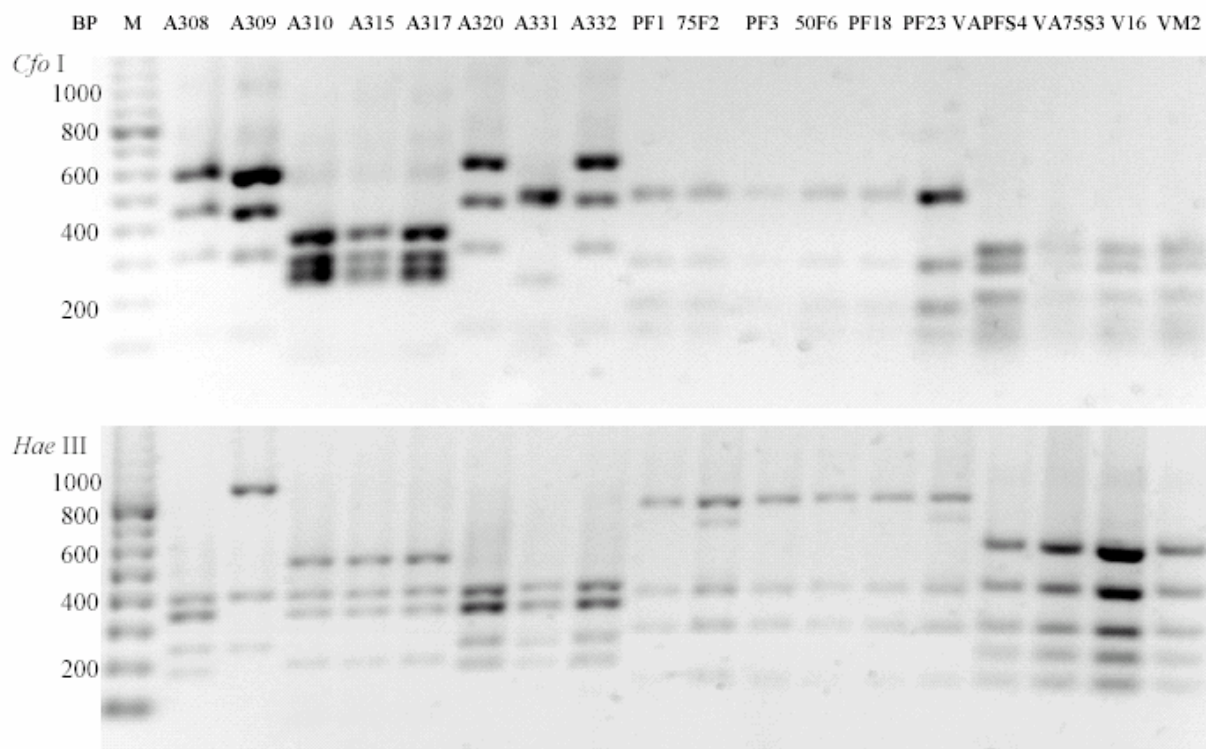
Fig. 3. ARDRA of the bacterial isolates obtained from *Artemia* nauplii after cyst disinfection with formaldehyde; M: Base-Pair ladder (BP).
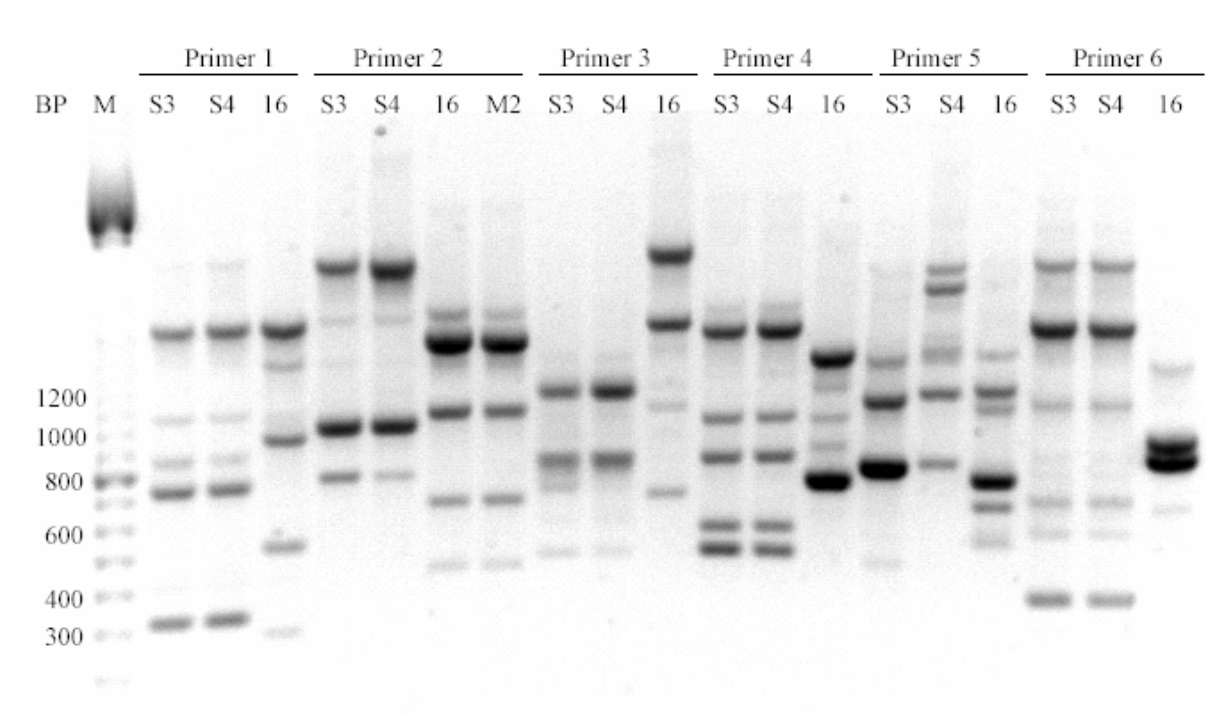
Fig. 4. RAPD analysis of *V. alginolyticus*-like strains; M: Base-Pair ladder (BP); S3: VA75S3; S4: VAPFS4; 16: V16; M2: VM2.