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Selection of lactic acid bacteria as candidate probiotics and in vivo test on *Artemia* nauplii

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Abstract:

Lactic acid bacteria (LAB) were isolated from rotifer cultures in a marine hatchery to search for potential probiotics for marine animals. Fifteen strains were first selected among a total of 55, according to antibacterial activity against *Vibrio* sp. Among eight strains identified as *Lactobacillus casei*, four were highly adhesive, suggesting some ability for surface colonization. The other strains were identified as *Lactobacillus plantarum*, *Lactobacillus dextrinicus*, and *Leuconostoc* sp. To validate probiotic potential, *Artemia* were challenged against pathogenic *Vibrio alginolyticus*, with or without one of six selected LAB strains. The six strains protected *Artemia* against the pathogen, to some extent on condition that nutrient enrichment was provided. *La. casei* BR51 and X2 were preferred, as they were efficient even in the absence of nutrient supply. *La. casei* X2 was finally selected as candidate probiotic, due to the best growth performances of *Artemia*, with or without the pathogen.

Keywords: 16S Rdna ; Antagonism ; Artemia ; Lactic acid bacteria ; Vibrio

Abbreviations: ARDRA: amplified ribosomal DNA restriction analysis;
Art: *Artemia*;
Art axe: axenic *Artemia*;
bp: base pair;
La.: Lactobacillus;
Le.: *Leuconostoc*;
FASW: filtered and autoclaved sea water;
LAB: lactic acid bacteria;
MRS: De Man, Rogosa, Sharpe;
OD570: optical density at 570 nm;
TSA: tryptic soy agar;
TSB: tryptic soy broth;
V.: *Vibrio*;
VA: *Vibrio alginolyticus*;
WHO: World Health Organization.

1. Introduction

Due to the risks of high mortality and infection spread, it is essential to develop disinfection and antimicrobial strategies that could prevent and control bacterial diseases in aquaculture. The massive use of antibiotics may lead to the emergence of resistant bacteria, which can spread in the environment and jeopardize human health (Nomoto, 2005; WHO, 2006). Probiotics are among the most promising alternatives to antibiotics, and the application to aquaculture is now widely accepted (Gomez-Gil et al., 2000; Wang, 2007). Probiotics may enhance health performances, for example by improving the intestinal microbial balance, colonizing the gut, depriving pathogens for adhesion sites, or competing for nutrients (Gatesoupe, 1999). Merrifield et al. (2010) stressed that probiotics must not be pathogenic and should exhibit antagonistic properties towards one or more key pathogens. Most lactic acid bacteria (LAB) are generally recognized as safe, and some species have been extensively studied (Holzapfel et al., 1995). The importance of LAB in fish culture was emphasized by Ringø and Gatesoupe (1998). A strain presumptively characterized as *Lactobacillus plantarum* increased the resistance of turbot larvae against pathogenic *Vibrio* sp. (Gatesoupe, 1994). The interest for LAB as probiotics in aquaculture hatcheries is growing with the recurring evidences of their contribution to maintain a healthy microbial environment in the larval rearing tanks. The choice of an appropriate LAB strain demands critical attention to strain identification and characterization. The application of probiotics must be based on understanding the mechanisms involved and the putative consequences. In this regard, effective probiotics should be aimed against pathogens encountered in hatchery conditions. Marques et al. (2004) reported that *Artemia* – the most common live food organism – is also particularly suitable as test organism to study the host–microbe interactions. Under gnotobiotic conditions, it is possible to manage microbiota associated to *Artemia* nauplii, and to evaluate the impact for improving growth and survival (Gordon and Pesti, 1971; Marques et al., 2006).

The aim of this work was to select LAB isolated in our fish hatchery as candidate probiotics for fish larvae. To this end, a first screening of the isolates was based on the protocol proposed by Vine et al. (2006) for selecting intestinal probiotics in marine larviculture. The antimicrobial activity was evaluated in vitro against pathogenic *Vibrio*. The selected strains were characterized taxonomically, checked for the absence of haemolysin as virulence factor, and tested for adhesiveness to abiotic surfaces. Fjellheim et al. (2010) stressed the importance to combine such methods of screening in vitro with testing in vivo, and the potential of the strains as probiotics was further investigated with a bioassay on *Artemia*, which was already applied to *Bacillus* sp. by Mahdhi et al. (2012).

2. Materials and methods

2.1. Antimicrobial activity of the LAB isolates

LAB were isolated from cultures of *Brachionus plicatilis* in the larval rearing facilities of Ifremer, Centre de Brest. 55 isolates selected after their distinct phenotypic characteristics were tested with an agar well diffusion method, as described by Reinheimer et al. (1990). The bacteria chosen as pathogenic indicators were *Vibrio alginolyticus* (ATCC 17749; ATCC 33787), and *Vibrio parahaemolyticus* (ATCC 17802). Six other putative pathogenic isolates were also tested, after characterization as *V. alginolyticus* (32J; 30J; 55J) and *V. parahaemolyticus* (53V; 12pv; 30v) with API 20E strips (Bio-Mérieux). The indicator strains were re-activated overnight in tryptic soy broth (TSB) at 30°C, and then grown for 24 hours on agar (TSA) at 30°C. The colonies from pure culture were suspended in 10 mL of physiological medium and well mixed for 5 min. The suspensions were spread in new TSA plates (1 mL per plate), which were let dry at 27°C for 15 min. A sterile 7-mm-diameter cork borer was used to cut uniform wells in the agar. The LAB isolates were grown in MRS broth for 24h at 37°C, and after centrifugation and filter sterilization (0.2 µm), 70 µL of the spent culture media were introduced into the wells of the test plates. All the assays were carried out in triplicates. The plates were held at 4°C for 2 h, and then incubated at 30°C. The diameters of the clear zones around the wells were measured after 24 h of culture.

2.2. Biochemical characterization of the selected LAB

The isolates that presented antagonistic activity were characterized by Gram stain, motility, indole production, spore forming, catalase and oxidase activity. Salinity tolerance was evaluated on MRS broths containing 2%, 3% and 6% NaCl. Exoenzymes production was tested on MRS agar, to which one of the following substrates was added: 0.2% (wt vol^{-1}) starch for amylase, 1% (wt vol^{-1}) skim milk for caseinase, 1% (wt vol^{-1}) Tween 80 for lipase, 5% (vol vol^{-1}) egg yolk for phospholipase (lecithinase) and 5% (vol vol^{-1}) fish blood for haemolysin (Ben Kahla-Nakbi et al., 2007). The enzymatic activities were further characterized with the API Zym System (Bio-Mérieux) as described by Papamaloni et al. (2002).

2.3. Adherence assay

LAB strains that were cultured in MRS broth were characterized for their ability to form biofilm with a semi-quantitative adherence assay in 96 polystyrene microplates according to Chaieb et al. (2007), with some modifications. An overnight culture in MRS broth at 37°C was diluted hundredfold with 2% glucose in MRS solution (wt vol^{-1}). Aliquots of 200 µl of the cell suspensions were transferred to U-bottomed 96 wells Microtitre plates (Nunc, Roskilde, Denmark). Each strain was tested in triplicate. Wells with sterile MRS alone were used as controls. The plates were incubated aerobically at 37°C for 24 h. The supernatant was discarded and the wells were washed twice to remove the nonadherent cells with PBS (7 mM Na2HPO4, 3 mM NaH2PO4, 130 mM NaCl; pH 7.4). The plates were dried in inverted position. Adherent bacteria were fixed with 95% ethanol, and stained for 5 min

with 100 µl of crystal violet solution (1%, Merck). The wells were washed and poured off three times with 300 µl of sterile distilled water, and then the plates were air-dried again. The optical density of bacteria at 570 nm (OD570) was measured with an automated Multiskan reader (Gio de Vita E C., Italy). Adhesion ability was interpreted as highly positive (OD570 ≥ 1), moderately to weakly positive (1 > OD570 > 0.1), or negative (OD570 ≤ 0.1).

2.4. Phylogenetic characterization

The LAB were cultivated in MRS agar and incubated at 37°C for 24 h. The colonies were collected, and DNA was extracted as described by Lambert et al. (1998), with some modifications (Supplementary Material, Fig. S3). To characterize the amplicons by amplified ribosomal DNA restriction analysis (ARDRA), aliquots of each PCR product were treated with two restriction enzymes, *Hae* III or *Cfo* I (Gatesoupe, 2002). One isolate per each dominant cluster was selected, and purified with PCR Clean-Up System kit (Amersham Biosciences, NJ, USA) before partial sequencing the 16S rRNA gene (partial sequences of 896–1037 bp from primer SA-dir) by MilleGen Biotechnologies (Labège, France). The nucleotide sequences obtained were checked with a sequence alignment editor (BioEdit), and homologies were searched with BLAST (NCBI). The sequences were deposited in the EMBL nucleotide sequence database.

2.5. Challenge test of candidate probiotics with *Artemia*

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture NV, Belgium). *Artemia* cysts were decapsulated as described by Sorgeloos et al. (1977). Decapsulated cysts were washed nine times with filtered and autoclaved sea water (FASW) over a 50-µm sterile filter net. Washed decapsulated cysts were transferred to a sterile falcon containing 30 mL of FASW. The Falcon were placed on incubator shaker (28°C, 120 rpm) and exposed to constant incandescent light. After 18–20 h, ten axenic nauplii were picked up and transferred to sterile falcons containing 30 mL of FASW. All manipulations were carried out under sterile conditions according to Marques et al. (2004).

The bacterial suspension was added at a density of 10^6 and 10^7 colony forming units mL^{-1} for the candidate probiotic and the pathogen, *Vibrio alginolyticus* E3 (ATCC 17749), respectively. Each treatment consisted of three sterile 60-mL falcons. To evaluate the effect of the LAB strains on *Artemia*, six series of six tests were applied. Each series corresponded to one strain selected for the challenge: 611, BR743, BR51, X2, BXI, and B31. The series included six conditions of culture: (1) axenic *Artemia* (Art axe); (2) gnotobiotic culture with the test LAB; (3) culture with the test LAB and a sterilized commercial enrichment product (Red Pepper, Bern Aqua); (4) simple challenge with *V. alginolyticus* (Art+VA); (5) challenge with VA in the presence of the test LAB*;* (6) challenge with VA in the presence of the test LAB and the enrichment product. The tests lasted 6 days. The commercial enrichment (27.6 µg mL^{-1}) and the candidate probiotic were added daily from day 1 to day 3. The pathogenic *Vibrio* strain was inoculated once at day 3.

During the challenge tests, the percentage of survival was calculated by counting the number of swimming larvae. At the end of each experiment, *Artemia* were fixed with Lugol's solution to measure their individual length under binocular magnifying glass fitted with a micrometer (Nikon Eclipse 50i, Japan). The absence of bacteria in the axenic group was monitored at the beginning and at the end of each run of the procedure.

2.6. Statistical analysis

Normal distribution and homoscedasticity were obtained in *Artemia* survival (%) and length with arcsine and square-root transformation, respectively. The differences between means were compared by analysis of variances and Fisher's test of protected least significant difference by using Statview software package.

3. Results

3.1. Bacterial characterization and antimicrobial activity

Among the 55 strains of LAB examined for antibacterial activity, 15 were selected for their strong antagonism to *Vibrio* sp. (Table 1). The diameters of the inhibition zones corresponding to the 15 strains were variable, depending on the indicator strain, but the average was around 13-14 mm for every LAB, except for strain TGO, which appeared poorly antagonistic to some biovars, with the higest cell-free supernatant pH (4.5). With the other strains however, there was not obvious relationship between antagonistic activity and pH range (3.9-4.3; Table 1).

A second step of selection was done with the plate test for adhesion. Four strains were highly adhesive to polystyrene, with scores between 1.04 and 1.12 (X6, X4, X2, and O32, Table 1), while four other strains were moderately adherent, and the remaining seven strains were weakly or not adherent.

The 15 LAB were also checked for general phenotypic characterization. All the strains were Gram positive, indole negative, catalase negative, non-motile and non-spore-forming. They grew on MRS broths supplemented with 2, 3 and 6% NaCl, except strain TGO with 6% NaCl. They produced caseinase, but they did not produce amylase, lipase, lecithinase, and haemolysin. The enzymatic activity profiles were discriminated with the API Zym System (Supplementary Material, Table S1). The similarity of the enzymatic profiles was estimated after Bray-Curtis index. The highly or moderately adherent strains gathered in the same cluster, while the weakly or not adherent strains were retrieved in a second cluster, except TGO that presented the most dissimilar profile (Supplementary Material, Fig. S2).

3.2. Bacterial identification

The genotypes of the 15 LAB were characterized by the restriction profiles that were obtained with *HaeIII* and *Cfo I* (Supplementary Material, Fig. S3). Based on this screening, seven strains were selected for sequencing, in comparison with *Lactobacillus plantarum* B3G, which was previously tested on turbot larvae (Gatesoupe, 1994; Table 2). The restriction fragments delineated a main cluster including strains B3P and X2 (Supplementary Material, Fig. S4), while strain BR743 was less similar. The main cluster corresponded to *Lactobacillus casei*, and strain BR743 corresponded to *Lactobacillus dextrinicus* (Haakensen et al., 2009). A second cluster included strain 611, identified as *La. Plantarum*, and less similar strains: *Leuconostoc mesenteroides* B31, *Leuconstoc* sp. TGO, and *La. casei* BR51, the ARDRA profile of which was apparently dissimilar from those observed with the other representatives of the species (Supplementary Material, Fig. S3), unlike its enzymatic profile that matched within the main cluster of *La. casei* (Supplementary Material, Fig. S2).

3.3. *Artemia* survival and growth

A mortality peak was observed in axenic *Artemia* at day 5, without any survivor at day 6 (treatment Art axe; Figure 1). When LAB were not introduced in the culture medium during the first 3 days, the inoculation of *V. alginolyticus* at day 3 advanced the mortality peak at day 4 (treatment Art + VA). In the absence of nutrient enrichment, mean survival rates of 30 and 20 % were obtained at day 6 in the groups treated for the first 3 days with *La. casei* BR51 and X2, respectively. An average of 10 % survival was also observed at day 6 with *Le. mesenteroides* B31, but without significant difference with the other cultures that resulted in total mortality in the absence of nutrient supply. The addition of nutrient enrichment increased dramatically the survival of *Artemia*, which was maintained between 100 and 90% at day 6 with five of the six LAB strains tested without the pathogen. The survival at day 6 was only 50% in the group treated with *La. Plantarum* 611 and the enrichment mix. In the trials with one LAB and *V. alginolyticus*, but without enrichment, the mortality peak was delayed in comparison with the group Art + VA. The most significant differences were observed at day 5, as survival was still 60% with *La. casei* BR51, and 30% with *La. casei* X2 or with *La. dextrinicus* BR743 (Supplementary Material, Table S5). However, every challenge test without enrichment resulted in total mortality at day 6. By contrast, the enrichment supply maintained the survival at day 6 between 15 and 50% in the challenges including one of the LAB – 611, B31, BXI, BR51, BR743, and X2, by growing order of survival.

Under axenic conditions, the mean length of dead *Artemia* at day 6 was 0.41 ± 0.02 mm (SD). Growth was stimulated by the introduction of bacteria, even *V. alginolyticus*, which induced a slight growth gain compared to the axenic control (0.52 ± 0.03 mm). The growth was further increased with LAB (between 0.67 ± 0.01 mm and 0.85 ± 0.01 mm with BR51, X2, B31, BXI, BR743, and 611, by growing order). The mean weights were significantly different between treatments for each LAB, with few exceptions (Supplementary Material, Table S6). The addition of nutrient enrichment to LAB raised the growth of *Artemia* between 0.93 ± 0.02 mm and 1.11 ± 0.01 mm with BXI, BR743, B31, BR51, 611, and X2, by growing order. In the presence of *V. alginolyticus*, the six LAB strains increased growth between 0.65 ± 0.08 mm and 0.85 ± 0.01 mm with BXI, 611, B31, BR51, BR743, and X2

by growing order. The addition of nutrient enrichment to the challenges with one LAB and the pathogen increased further growth between 0.78 ± 0.01 mm and 0.93 ± 0.02 mm with BR51, B31, BR743, BXI, 611, and X2 by growing order.

4. Discussion and conclusion

The selection of candidate probiotics among LAB isolated in the hatchery appeared feasible with the simplified screening process that was derived from the scheme proposed by Vine et al. (2006). The production of antimicrobial compounds in LAB cell-free supernatants was tested by diffusion on agar plate cultures of pathogenic *Vibrio* sp. A variety of strains was thus selected, most of them belonging to the genus *Lactobacillus*, with representatives of three species, *La. casei*, *La*. *dextrinicus*, and *La. plantarum.* Two selected isolates belonged to the genus *Leuconostoc*, but strain TGO was less antagonistic to some test pathogens. The nature of the antimicrobial compounds was not characterized in this study. Most antimicrobial activities of LAB are due to organic acids (Reinheimer et al., 1990) and consequent lowering pH (Tejero-Sariñena et al., 2012), but hydrogen peroxide and bacteriocins are also produced (Dimitonova et al., 2007).

The second step of the selection process was the adhesion test to abiotic surface, which may indicate the potential of LAB to colonize the gut, and to further antagonize pathogens (Servin and Coconnier, 2003). The strains identified as *La. casei* were adherent except BX1, while the others were weakly or not adherent, except *La. plantarum* sp. O32 that appeared strongly adherent.

The survival of LAB in seawater is an important factor for candidate probiotics in marine larviculture (Vazquez et al., 2003). All the selected strains grew at 2-6% NaCl, except *Leuconostoc* sp. TGO that could not grow at 6 % NaCl. Abriouel et al. (2012) showed that most *Lactobacillus* and *Leuconostoc* strains can grow at 6.5% NaCl.

The enzymatic profiles matched generally with the characteristics of the species *La. plantarum* (Samolada et al., 1998) and *La. casei* (Charteris et al., 2001), while confirming the atypical phenotypes of strains BXI and O32, whose distinctive adhesiveness was already noted. The genotypic characterization by ARDRA indicated that these two strains belonged to *La. casei*, and *La. plantarum*, respectively. Surprisingly, the ARDRA profile of strain BR51 appeared close to those of *La. plantarum*, though the partial nucleotidic sequence of its 16S rRNA gene was identical to that of *La. casei* B3P, likely due to artifact. The genera *Lactobacillus* and *leuconostoc* are recognized as safe, and the attention to virulence factors was limited to check the absence of haemolysin.

LAB are known to produce extracellular products that can inhibit the growth of *V. alginolyticus* in *Artemia* cultures, likely in synergy with organic acids (Villamil et al., 2003). To further validate the probiotic potential, LAB strains were tested on *Artemia*. Six strains were selected as representatives of the species and biovars, and they protected *Artemia* against *V. alginolyticus*, to some extent on condition that nutrient enrichment was provided. The beneficial effect of the enrichment was likely independent from that of the probiotic, as the fatty acids and vitamins brought by the feed are required by *Artemia*, but

not by LAB. *La. plantarum* 611 was the least efficient strains, and *Artemia* survived less well with this strain, compared to the others. *La. plantarum* was ruled out, even though strain B3G improved survival in turbot larvae that were challenged against pathogenic *Vibrio* sp. in a previous experiment (Gatesoupe, 1994). In the present screening bioassay, the other LAB were equivalent in terms of survival of enriched *Artemia*. *La. casei* BR51 and X2 were preferred, as they were efficient even in the absence of nutrient supply. *La. casei* X2 was finally selected due to the best growth performances of *Artemia*, with or without the pathogen.

These results confirmed the usefulness of screening strategies for rational selection of candidate probiotics (Vine et al., 2006; Fjellheim et al., 2010; Mahdhi et al., 2012). The present selection process was followed by an application to sea bass larvae (Lamari et al., 2013). *La. casei* X2 appeared efficient to promote growth in fish larvae, but the incidence on conformation was detrimental, compared to the results obtained with commercial probiotics. Consequently, the present way of selecting candidate probiotics may be valid, but the final evaluation in hatchery conditions remains critical.

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References

Abriouel H, Benomar N, Cobo A, Caballero N, Fernández Fuentes MA, Pérez-Pulido R, Gálvez A (2012) Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiol 32:308-316.

Ben Kahla-Nakbi A, Besbes A, Bakhrouf A, Alcaide E (2007) Characterisation and virulence properties of *Vibrio* isolates from diseased gilthead sea bream (*Sparus aurata*) cultured in Tunisia. Bull Eur Ass Fish Pathol 27:90–99.

Chaieb K, Chehab O, Zmantar T, Rouabhia M, Mahdouani K, Bakhrouf A (2007) In vitro effect of pH and ethanol on biofilm formation by clinical *ica*-positive *Staphylococcus epidermidis* strains. Ann Microbiol 57:431-437.

Charteris WP, Kelly PM, Morelli L, Collins JK (2001) Quality control *Lactobacillus* strains for use with the API 50CH and API ZYM systems at 37 °C. J Basic Microbiol 41:241-251.

Dimitonova SP, Svetla TD, Julia PS, Boris VB (2007) Antimicrobial activity and protective properties of vaginal lactobacilli from healthy Bulgarian women. Anaerobe 13:178-184.

Fjellheim AJ, Klinkenberg G, Skjermo J, Aasen IM, Vadstein O (2010) Selection of candidate probionts by two different screening strategies from Atlantic cod (*Gadus morhua* L.) larvae. Vet Microbiol 144:153-159.

Gatesoupe FJ (1994) Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic vibrio. Aquat Living Resour 7:277-282.

Gatesoupe FJ (1999) The use of probiotics in aquaculture. Aquaculture 180:147–165.

Gatesoupe FJ (2002) Probiotic and formaldehyde treatments of *Artemia nauplii* as food for larval pollack *Pollachius pollachius*. Aquaculture 212:347-360.

Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259–270.

Gordon H, Pesti L (1971) The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev 35:390–429.

Haakensen M, Dobson CM, Hill JE, Ziola B (2009) Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) Back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*. Int J Syst Evol Microbiol 59:615-621.

Holzapfel WH, Geisen R, Schillinger U (1995) Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int J Food Microbiol 24:343–362.

Lambert C, Nicolas JL, Cilia V, Corre S (1998) *Vibrio pectenicida* sp. nov., a pathogen of scallop (*Pecten maximus*) larvae. Int J SystBacteriol 48:481-487.

Lamari F, Castex M, Larcher T, Ledevin M, Mazurais D, Bakhrouf A, Gatesoupe FJ (2013) Comparison of the effects of the dietary addition of two lactic acid bacteria on the development and conformation of sea bass larvae, *Dicentrarchus labrax*, and the influence on associated microbiota. Aquaculture 376–379:137–145.

Mahdhi A, Esteban MA, Hmila Z, Bekir K, Kamoun F, Bakhrouf A, Krifi B (2012) Survival and retention of the probiotic properties of *Bacillus* sp. strains under marine stress starvation conditions and their potential use as a probiotic in *Artemia* culture. Res Vet Sci 93:1151-1159.

Marques A, Dhont J, Sorgeloos P, Bossier P (2004) Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically-grown brine shrimp *Artemia franciscana*. J Exp Mar Biol Ecol 312:115–136.

Marques A, Thanh TH, Verstraete W, Dhont J, Sorgeloos P, Bossier P (2006) Use of selected bacteria and yeast to protect gnotobiotic *Artemia* against different pathogens. J Exp Mar Biol Ecolo 334:20–30.

Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RR, Bogwald J, Castex M, Ringø E (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302:1-18.

Nomoto K (2005) Prevention of infections by probiotics. J Biosci Bioeng100:583–592.

Papamaloni E, Kotzekidou P, Tzanetakis N, Litopoulou-Tzanetaki E (2002) Characterization of *Micrоccocaceae* isolated from dry fermented sausage. Food Microbiol 19:441–449.

Reinheimer JA, Demkow MR, Candioti MC (1990) Inhibition of coliform bacteria by lactic acid cultures. Aust J Dairy Tech 45:5-9.

Ringø, E, Gatesoupe FJ (1998) Lactic acid bacteria in fish: a review. Aquaculture 160:177-203.

Samolada M, Litopoulou-Tzanetaki E, Xanthopoulos V, Tzanetakis N (1998) Changes in microbial flora during manufacture of a traditional fermented milk from ewe's milk. Food Microbiol 15:43-50.

Servin AL, Coconnier MH (2003) Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract Res Clin Gastroenterol 17:741-754.

Sorgeloos P, Bossuyt E, Laviña E, Baeza-Mesa M,Persoone G (1977) Decapsulation of *Artemia* cysts: A simple technique for the improvement of the use of brine shrimp in aquaculture. Aquaculture 12: 311-315.

Tejero-Sariñena S, Barlow J, Costabile A, Gibson GR, Rowland I (2012) *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. Anaerobe 18: 530-538.

Vazquez JA, Cabo ML, Gonzalez MP, Murado MA (2003) Survival of lactic acid bacteria in seawater: A factorial study. Curr Microbiol 47:508-513.

Villamil L, Figueras A, Planas M, Novoa B (2003) Control of *Vibrio alginolyticus* in *Artemia* culture by treatment with bacterial probiotics. Aquaculture 219:43-56.

Vine NG, Leukes WD, Kaiser H (2006) Probiotics in marine larviculture. FEMS Microbiol Rev 30:404-427.

Wang YB (2007) Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259–264.

WHO (World Health Organization) (2006) Report of a joint FAO/OIE/WHO expert consultation on antimicrobial use in aquaculture and antimicrobial resistance: Seoul, Republic of Korea, 13-16 June 2006, WHO Press, Geneva.

Tables

Table 1. Antagonistic activity and adhesion score of the LAB selected as candidate probiotics. The strains were ranked by descending order of adhesion score, which was estimated by the Microtitre-plate test (optical density at 570 nm). The antagonistic activity was expressed as the diameter (mm ± s.d.) of the mean inhibition zone in the agar plates inoculated with *Vibrio* sp. indicator strains.

LAB	Cell-free	Antagonistic activity versus *Vibrio* sp. indicator strains									Adhesion
	supernatant	*Vibrio alginolyticus*					*Vibrio parahaemolyticus*				Average	score
	pH	E1[§]	E3*	55j	32j	30j	E2[$]	30v	53v	12pv	activity	
X6	4.00	14.6 ± 0.5	14.0 ± 0.0	14.3 ± 0.5	12.3 ± 0.5	13.0 ± 0.5	12.6 ± 0.5	14.6 ± 0.5	15.0 ± 1.0	12.6 ± 0.5	13.7 ± 1.0	1.12 ± 0.11
X4	3.96	14.0 ± 0.0	13.6 ± 0.5	15.0 ± 0.0	14.3 ± 0.5	14.6 ± 0.5	13.3 ± 0.5	13.0 ± 0.0	17.0 ±1.0	13.6 ± 0.5	14.0 ± 0.6	1.07 ± 0.13
X2	3.93	14.0 ± 0.0	14.6 ± 0.5	14.3 ± 0.5	13.3 ± 0.5	14.6 ± 0.5	13.6 ± 0.5	13.3 ± 0.5	15.0 ± 0.0	13.6 ± 0.5	14.3 ± 1.2	1.08 ± 0.10
O32	3.94	14.3 ± 0.5	14.0 ± 0.0	13.0 ± 0.0	13.3 ± 0.5	13.0 ± 0.5	13.0 ± 1.0	13.6 ± 0.5	14.6 ± 0.5	13.3 ± 0.5	13.6 ± 0.6	1.04 ± 0.05
BR51	3.97	15.0 ± 1.0	14.0 ± 0.0	13.0 ± 1.0	13.3 ± 0.5	12.6 ± 0.5	13.0 ± 1.0	12.6 ± 0.5	14.6 ± 0.5	12.0 ± 1.0	13.3 ± 1.0	0.93 ± 0.02
X5	4.07	14.6 ± 0.5	13.6 ± 0.5	14.6 ± 0.5	13.3 ± 0.5	13.3 ± 0.5	12.6 ± 0.5	14.6 ± 0.5	15.6 ± 1.1	12.6 ± 0.5	13.9 ± 1.0	0.84 ± 0.09
F2P	3.97	15.0 ± 0.0	13.6 ± 0.5	14.0 ± 1.0	12.0 ± 1.0	12.6 ± 1.0	13.6 ± 0.5	13.3 ± 0.5	15.6 ± 1.1	12.6 ± 0.5	13.6 ± 1.2	0.76 ± 0.02
B3P	4.27	15.3 ± 0.5	13.3 ± 0.5	15.0 ± 0.0	14.0 ± 1.0	13.6 ± 1.0	13.3 ± 0.5	14.0 ± 1.0	15.0 ± 1.0	12.3 ± 0.5	14.0 ± 1.0	0.74 ± 0.07
B31	4.13	15.6 ± 0.5	13.3 ± 0.5	14.3 ± 0.5	13.3 ± 0.5	13.0 ± 0.5	13.3 ± 0.5	13.3 ± 0.5	14.3 ± 0.5	12.0 ± 1.0	13.6 ± 1.0	0.22 ± 0.01
BR743	3.91	14.0 ± 0.0	13.6 ± 0.5	14.3 ± 0.5	14.3 ± 0.5	14.6 ± 0.5	13.3 ± 0.5	14.0 ± 1.0	15.3 ± 0.5	13.3 ± 0.5	14.1 ± 0.6	0.11 ± 0.02
BR611	4.02	10.3 ± 0.5	10.6 ± 0.5	15.3 ± 0.5	12.6 ± 0.5	13.0 ± 0.5	9.3 ± 0.5	14.6 ± 0.5	17.0 ± 1.0	13.0 ± 1.0	12.9 ± 2.5	0.09 ± 0.02
611	3.88	14.0 ± 0.0	14.0 ± 0.0	15.0 ± 1.0	14.6 ± 0.5	14.6 ± 0.5	13.3 ± 0.5	15.0 ± 1.0	15.6 ± 1.1	13.6 ± 0.5	14.4 ± 0.7	0.08 ± 0.02
XIG	3.90	14.6 ± 0.5	13.6 ± 0.5	14.0 ± 1.0	12.3 ± 0.5	12.3 ± 0.5	13.0 ± 0.0	14.3 ± 0.5	16.0 ± 1.0	13.0 ±0.0	13.7 ± 1.2	0.08 ± 0.03
BXI	4.03	15.6 ± 0.5	13.6 ± 0.5	15.0 ± 0.0	14.0 ± 1.0	13.3 ± 1.0	13.0 ± 0.0	14.3 ± 1.1	15.0 ± 1.0	12.0 ± 1.0	14.0 ± 1.1	0.06 ± 0.01
TGO	4.48	15.3 ± 0.5	13.3 ± 0.5	10.6 ± 0.5	8.3 ± 0.5	8.3 ± 0.5	13.0 ± 0.0	8.6 ± 0.5	8.3 ± 0.5	8.3 ± 0.5	10.4 ± 2.7	0.04 ± 0.01

Reference strains: [§]ATCC 33787; *ATCC 17749; [$]ATCC 17802

Table 2. Bacterial isolates selected as representatives of the different clusters obtained after restriction analysis of the 16S rRNA gene*, and subsequent partial sequencing

Lactobacilli isolates	Identification	Accession number	% identity
BR51[§]	*Lactobacillus casei* BR51	HF562841	
X2	*Lactobacillus casei* X2	HF562840	
			99.8
BR743	*Lactobacillus dextrinicus* BR743	FM877685	
611	*Lactobacillus plantarum* 611	HF562838	
B3G*	*Lactobacillus plantarum* B3G	HF562839	
			99.9
B31	*Leuconostocmesenteroides* B31	HF562842	
TGO	*Leuconostoc* sp. TGO	HF562843	
			99.1

* *Lactobacillus plantarum* B3G was added as a reference strain, which was previously tested (Gatesoupe, 1994)

[§]Sequence 100% identical with that of strain B3P on a read length of 913 bp

Figures

Figure 1 : Survival of *Artemia* under axenic (Art axe) and gnotobiotic conditions (%± SD). Six LAB strains were tested: 611, BR743, BR51, X2, BX1, and B31 (figures 1a, 1a, 1c, 1d, 1e and 1f, respectively), with or without addition of enrichment feed (A). The nauplii were challenged with *V. alginolyticus* (VA), in the presence or absence of the LAB, with or without the enrichment feed.

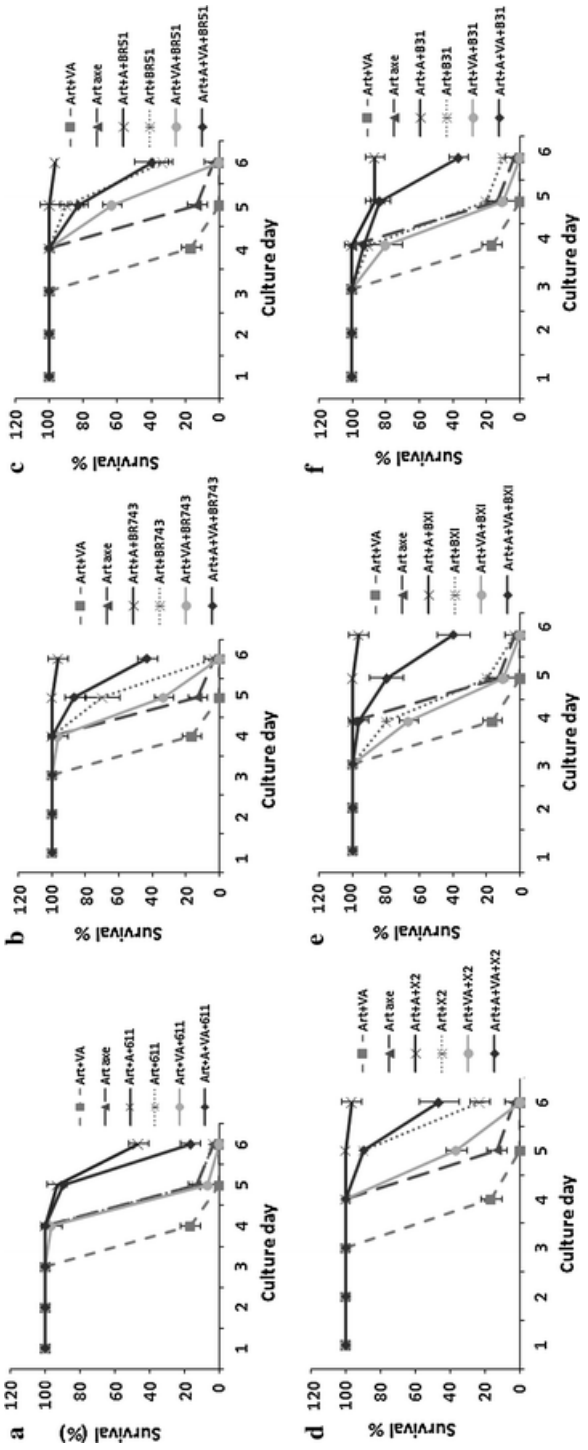
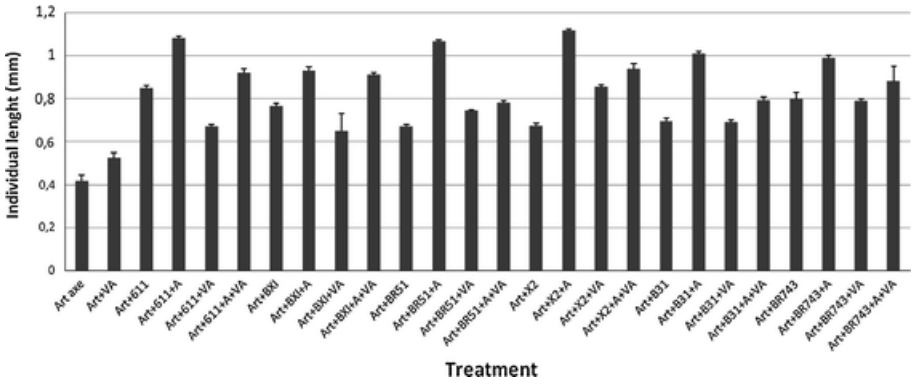


Figure 2 : Mean length (mm ± SD) of 6 days-old Artemia in axenic (Art axe), monoxenic cultures (LAB strain 611, BR743, BR51, X2, BX1, or B31, or *V. alginolyticus* VA) , and dixenic cultures (LAB strain + VA), with or without nutrient enrichment (A).



Supplementary material

Table S1. Enzymatic activities corresponding to the LAB strains tested on API ZYM strips; the cluster analysis based on the Bray-Curtis similarity of these profiles was illustrated in Figure S2.

Enzymes / **Strains**	Phosphatase alcaline	Estérase (C4)	Estérase lipase (C8)	Lipase (C14)	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsine	α-Chymotrypsine	Phosphatase acide	Naphtol phosphohydrolase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	N-acétyl-béta-glucosaminidase	α-mannosidase	α-fucosidase
B31	2	1	1	0	5	5	1	0	0	4	2	2	5	0	5	5	5	0	0
611	2	1	0	0	5	5	3	0	0	5	3	0	5	0	5	4	5	0	0
X2	1	2	1	0	5	5	4	0	4	5	4	0	5	0	5	5	1	0	1
F2p	2	2	2	0	5	5	4	0	3	5	5	2	5	3	5	4	0	0	1
BR611	2	1	1	0	5	5	4	0	0	5	4	0	5	0	5	5	5	0	0
X6	1	1	1	0	5	5	3	0	3	4	4	0	5	0	5	4	3	0	2
O32	3	3	3	0	5	5	4	0	3	5	4	0	5	0	5	4	1	0	2
BXI	3	0	0	0	5	4	0	0	0	5	3	4	5	0	5	4	5	0	0
X4	3	3	2	0	5	5	3	0	3	5	4	0	4	0	5	4	0	0	2
XIG	3	1	1	0	5	5	4	0	0	5	4	0	5	0	5	5	5	0	0
BR743	2	0	0	0	5	5	3	0	0	4	3	2	5	0	4	4	5	0	0
B3p	1	1	1	0	5	5	2	0	3	5	4	0	5	0	5	4	1	0	1
X5	2	2	2	0	5	5	5	0	4	5	4	3	5	3	5	4	1	0	1
BR51	2	2	2	0	5	5	4	0	3	4	4	0	5	0	5	2	0	0	2
TGO	2	2	0	0	2	2	0	0	0	5	3	0	5	0	5	2	0	0	2

0: no activity; 1: liberation of 5 nmol; 2: 10 nmol; 3: 20 nmol; 4: 30 nmol and 5: 40 nmol.

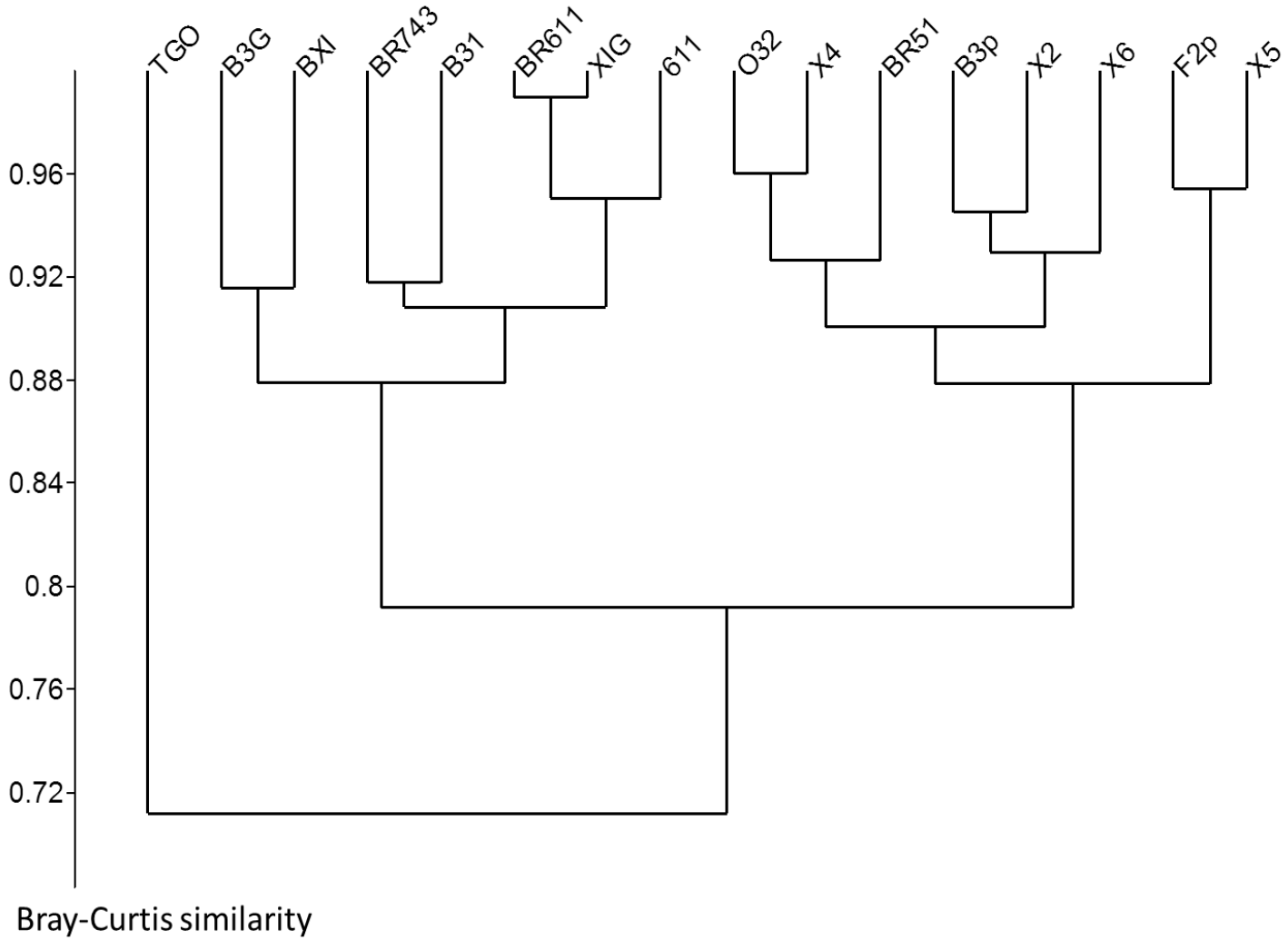
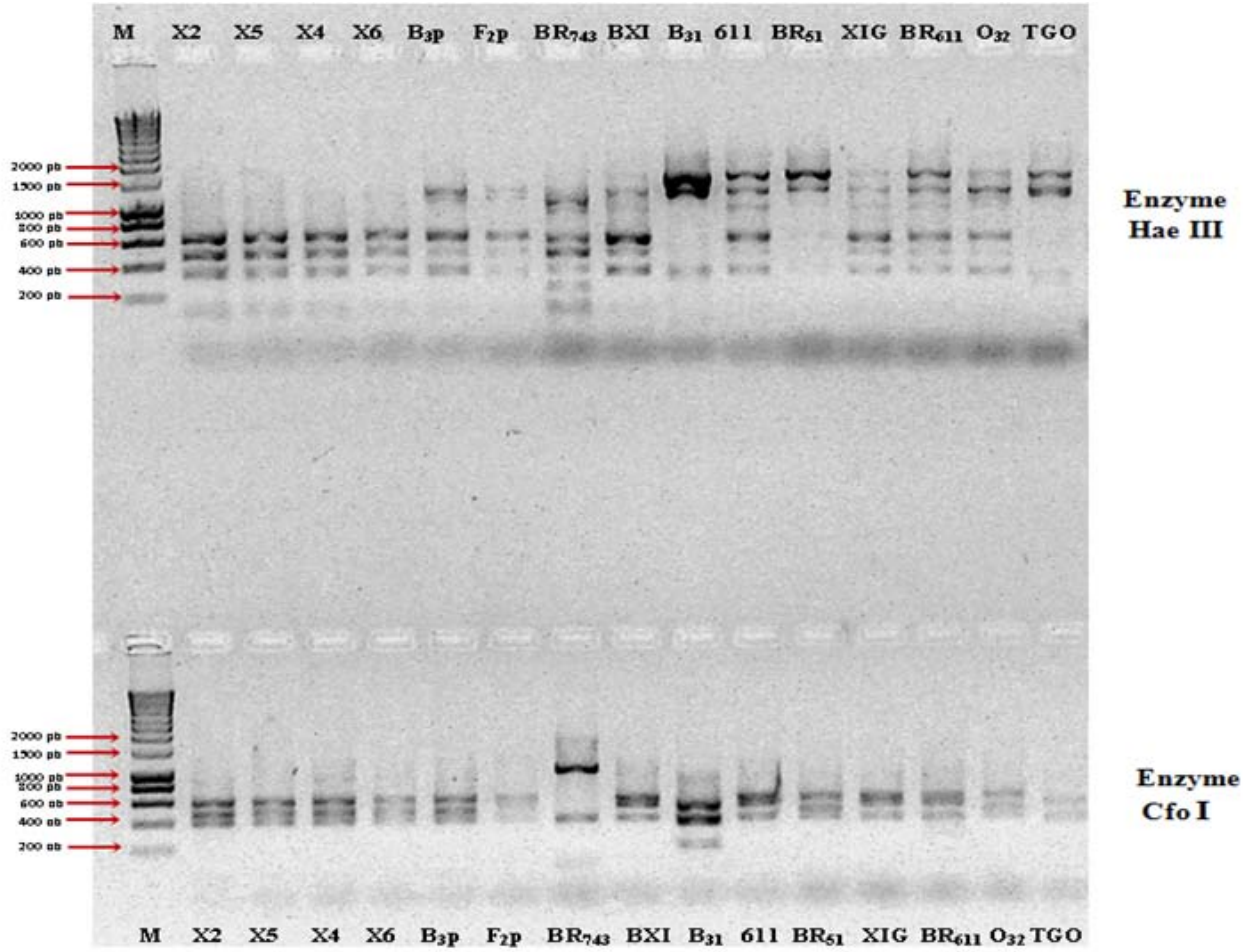


Figure S2 - Cluster analysis obtained after Bray-Curtis similarity of the API ZYM profiles of the LAB strains (see Table S1)

Figure S3. Restriction patterns obtained after digestion with *Hae* III and *Cfo* I of PCR products from the 16S RNA gene of the LAB strains (M: SmartLadder, Eurogenetec).

Method:
The LAB were cultivated in MRS agar and incubated at 37°C for 24 h. The colonies were collected, and suspended in 200 µL lysis solution (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100), heated for 5 min at 100°C, and immediately cooled on ice. After adding 200 µL glass beads (Sigma G4649), the tubes were beaten for 10 min. DNA was then purified by chloroform extraction and ethanol precipitation. The air-dried DNA pellet was resuspended in DNAse-free MiliQ water.
The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with the universal primers SA-dir (5'-AGAGTTTGATCATGGCTCAG-3') and S17-rev (5'-GTTACCTTGTTACGACTT-3') as described by Lambert et al. (1998), using a thermocycler Techne TC-512. The PCR conditions were as follows: initial denaturation at 95oC for 3 min, followed by 25 cycles of denaturation at 94oC for 30 s; annealing at 52oC for 1 min; extension at 72oC for 1.5 min, and a final extension at 72oC for 5 min. The PCR products were deposited into wells of agarose gel (1.5%, wt vol-1) with ethidium bromide for staining, plus one well with DNA base-pair (bp) ladder, and the electrophoresis was run at 100 V. The resulting migration was visualized under UV light.
To characterize the amplicons by amplified ribosomal DNA restriction analysis (ARDRA), 4-µl aliquots of each PCR product were added to 5.5 µl of buffered premix with one of the two restriction enzymes, Hae III or Cfo I (Gatesoupe, 2002). The incubation was carried out for 4 h at 37° C. The restriction products were separated by electrophoresis on agarose gel, in conditions similar to those for controlling PCR products. The selective migration was visualized under ChemiDoc XRS System (BioRad.) The sizes of DNA fragments were estimated using a 100-bp DNA ladder (SmartLadder, Eurogenetec).



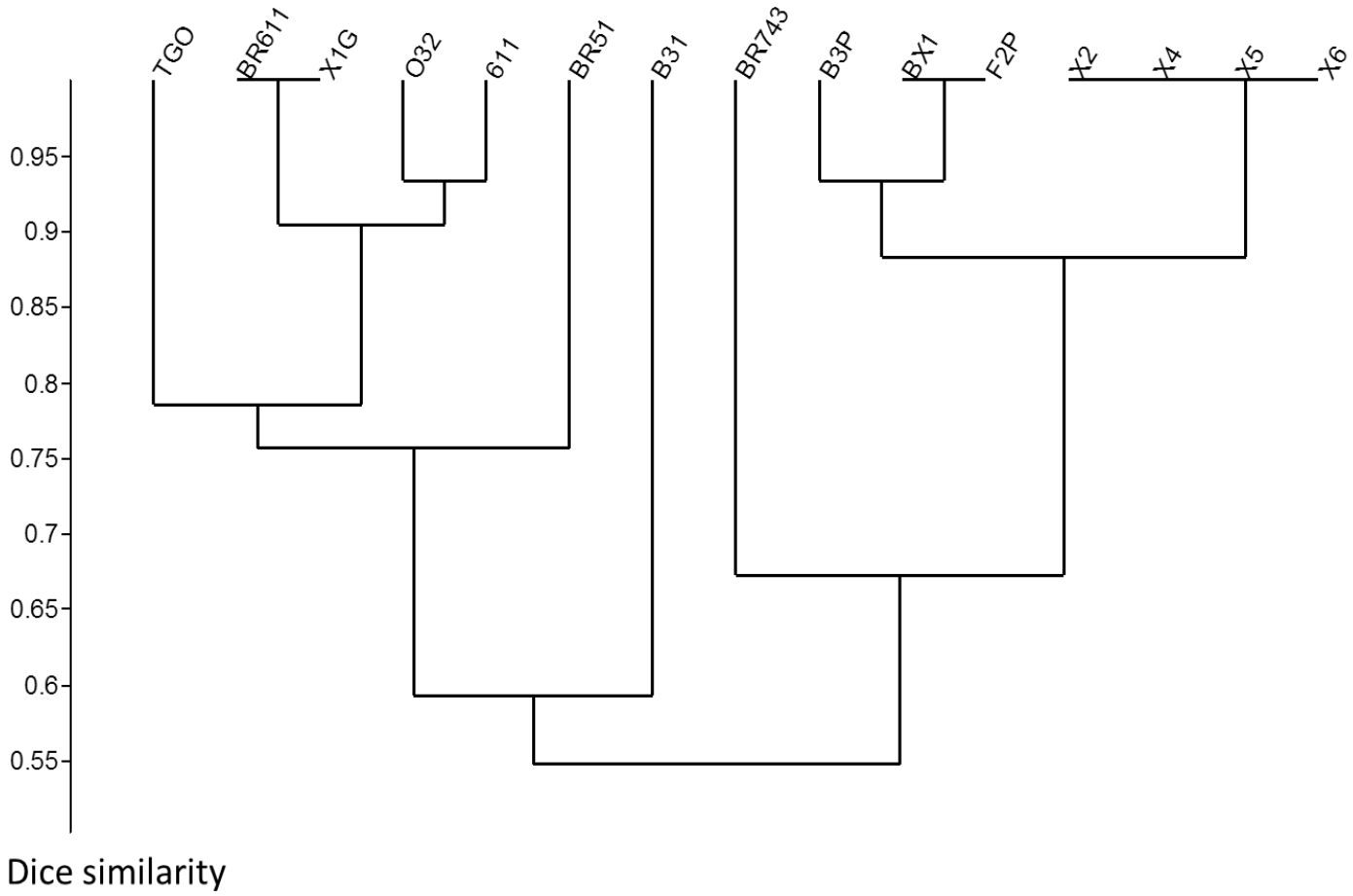


Figure S4 - Cluster analysis obtained after Dice similarity of the ARDRA profiles of the LAB strains (see Figure S3)

Table S5. Pairwise comparisons among the survival rates of *Artemia* measured at day 5 with the different treatments, with or without each strain lactic acid bacteria (611; X2; BR743: BXI; BR51 and B31; Fig. 1).

		Art+VA	Art axe	Art+A+611	Art+611	Art+VA+611	Art+A+VA+611
Strain 611	**Art+VA**	-	0.2012	<0.0001*	0.2012	0.5118	<0.0001*
	Art axe		-	<0.0001*	0.000	0.5118	<0.0001*
	Art+A+611			-	<0.0001*	<0.0001*	0.1541
	Art+611				-	0.5118	<0.0001*
	Art+VA+611					-	<0.0001*
	Art+A+VA+611						-
		Art+VA	**Art axe**	**Art+A+X2**	**Art+X2**	**Art+VA+X2**	**Art+A+VA+X2**
Strain X2	**Art+VA**	-	0.0006*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+X2			-	<0.0001*	<0.0001*	<0.0001*
	Art+X2				-	<0.0001*	0.000
	Art+VA+X2					-	<0.0001*
	Art+A+VA+X2						-
		Art+VA	**Art axe**	**Art+A+BR743**	**Art+BR743**	**Art+VA+BR743**	**Art+A+VA+BR743**
Strain BR743	**Art+VA**	-	0.700	<0.0001*	<0.0001*	0.0003*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	0.104*	<0.0001*
	Art+A+BR743			-	<0.0001*	<0.0001*	<0.0001*
	Art+BR743				-	<0.0001*	0.0015*
	Art+VA+BR743					-	<0.0001*
	Art+A+VA+BR743						-
		Art+VA	**Art axe**	**Art+A+BXI**	**Art+BXI**	**Art+VA+BXI**	**Art+A+VA+BXI**
Strain BXI	**Art+VA**	-	0.0758	<0.0001*	0.0130*	0.1706	<0.0001*
	Art axe		-	<0.0001*	0.3504	0.6358	<0.0001*
	Art+A+BXI			-	<0.0001*	<0.0001*	<0.0001*
	Art+BXI				-	0.1706	<0.0001*
	Art+VA+BXI					-	<0.0001*
	Art+A+VA+BXI						-
		Art+VA	**Art axe**	**Art+A+BR51**	**Art+BR51**	**Art+VA+BR51**	**Art+A+VA+BR51**
Strain BR51	**Art+VA**	-	0.2889	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	0.0006*	<0.0001*
	Art+A+BR51			-	0.0106*	<0.0001*	0.0004*
	Art+BR51				-	0.0010*	0.1012
	Art+VA+BR51					-	0.0254*
	Art+A+VA+BR51						-
		Art+VA	**Art axe**	**Art+A+B31**	**Art+B31**	**Art+VA+B31**	**Art+A+VA+B31**
Strain B31	**Art+VA**	-	0.0910	<0.0001*	0.0174*	0.1934	<0.0001*
	Art axe		-	<0.0001*	0.3764	0.6542	<0.0001*
	Art+A+B31			-	<0.0001*	<0.0001*	0.4000
	Art+B31				-	0.1934	<0.0001*
	Art+VA+B31					-	<0.0001*
	Art+A+VA+B31						-

*Significant difference, after *p* computed from arcsine transformed data, with post-hoc Fisher's test

Table S6. Pairwise comparisons among the mean weights of *Artemia* measured at day 6 with the different treatments, with or without each strain lactic acid bacteria (611; X2; BR743: BXI; BR51 and B31; Fig. 2).

		Art+VA	Art axe	Art+A+611	Art+611	Art+VA+611	Art+A+VA+611
Strain 611	**Art+VA**	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+611			-	<0.0001*	<0.0001*	<0.0001*
	Art+611				-	<0.0001*	<0.0001*
	Art+VA+611					-	<0.0001*
	Art+A+VA+611						-
		Art+VA	**Art axe**	**Art+A+X2**	**Art+X2**	**Art+VA+X2**	**Art+A+VA+X2**
Strain X2	**Art+VA**	-	0.001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+X2			-	<0.0001*	<0.0001*	<0.0001*
	Art+X2				-	<0.0001*	<0.0001*
	Art+VA+X2					-	0.3624
	Art+A+VA+X2						-
		Art+VA	**Art axe**	**Art+A+BR743**	**Art+BR743**	**Art+VA+BR743**	**Art+A+VA+BR743**
Strain BR743	**Art+VA**	-	0.0031*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+BR743			-	<0.0001*	<0.0001*	0.0025*
	Art+BR743				-	0.8213	0.0137*
	Art+VA+BR743					-	0.0089*
	Art+A+VA+BR743						-
		Art+VA	**Art axe**	**Art+A+BXI**	**Art+BXI**	**Art+VA+BXI**	**Art+A+VA+BXI**
Strain BXI	**Art+VA**	-	0.0043*	<0.0001*	<0.0001*	0.0013*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+BXI			-	0.0002*	<0.0001*	0.5226
	Art+BXI				-	0.0023*	0.0005*
	Art+VA+BXI					-	<0.0001*
	Art+A+VA+BXI						-
		Art+VA	**Art axe**	**Art+A+BR51**	**Art+BR51**	**Art+VA+BR51**	**Art+A+VA+BR51**
Strain BR51	**Art+VA**	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+BR51			-	<0.0001*	<0.0001*	<0.0001*
	Art+BR51				-	0.0002*	<0.0001*
	Art+VA+BR51					-	0.0215*
	Art+A+VA+BR51						-
		Art+VA	**Art axe**	**Art+A+B31**	**Art+B31**	**Art+VA+B31**	**Art+A+VA+B31**
Strain B31	**Art+VA**	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art axe		-	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	Art+A+B31			-	<0.0001*	<0.0001*	<0.0001*
	Art+B31				-	0.8322	<0.0001*
	Art+VA+B31					-	<0.0001*
	Art+A+VA+B31						-

*Significant difference, after *p* computed from square-root transformed data, with post-hoc Fisher's test