Probiotic Use of *Lactobacillus* spp. for Black Tiger Shrimp, *Penaeus monodon*

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A mixture of *Lactobacillus* species and strains (designated L-P) was isolated from chicken gastrointestinal tracts and mixed with a formulated shrimp diet. Growth and survival of *Penaeus monodon* juveniles fed the L-P diet for 100 days was significantly greater ($p<0.05$) than those shrimp fed a control diet without L-P added. A 10-day immersion challenge test with *Vibrio harveyi* D331 resulted in 74% mortality in the control group, while none of the treatment group shrimp died.

Key words: Probiotics, shrimp health, *Lactobacillus* spp., *Penaeus monodon*, *Vibrio harveyi* D331.

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การใช้ *Lactobacillus* spp. เป็นโพรไบโอติกในการเลี้ยงกุ้งกุลาดำ *Penaeus monodon*

วรรนิภา เพี้ยนภักตร์, ศิริรัตน์ เร่งพิพัฒน์, สมเกียรติ ปิยะธีรธิติวรกุล
และ เปี่ยมศักดิ์ เมนะเศวต (2542)

วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **24 (1)**

Lactobacillus spp. หลายสายพันธุ์ (L-P) แยกได้จากทางเดินอาหารไก่ นำมาผสมกับอาหารกุ้ง ในการเลี้ยงกุ้งกุลาดำ *Penaeus monodon* ด้วยอาหารปกติ (กลุ่มควบคุม) และอาหารผสม L-P พบว่า การเจริญเติบโต และการรอดชีวิตของกุ้งกุลาดำที่เลี้ยงด้วยอาหารผสม L-P เป็นเวลา 100 วัน มีค่ามากกว่า ในกลุ่มกุ้งกุลาดำที่เลี้ยงด้วยอาหารปกติอย่างมีนัยสำคัญ ($p<0.05$) เมื่อนำกุ้งทั้งสองกลุ่มมาเหนี่ยวนำให้เกิดโรคด้วย *Vibrio harveyi* D331 โดยวิธีการแช่เป็นเวลา 10 วัน กุ้งในกลุ่มควบคุมมีการตาย 74 % ในขณะที่กลุ่มได้รับอาหารผสม L-P รอดชีวิต 100 %

คำสำคัญ โพรไบโอติก, สุขภาพกุ้ง, *Lactobacillus* spp., *Penaeus monodon*, *Vibrio harveyi* D311

INTRODUCTION

Metchnikoff first used the term "probiotics" in 1907, where it was defined as a microorganism feed supplement which is non-pathogenic but which propagates in the host animals, gastrointestinal (GI) tract thus serving as a growth promotor and/or providing resistance to infectious disease.[1] Probiotic bacteria and other microbes include *Lactobacillus* spp., *Bifidobacterium* sp., *Clostridium* sp., *Enterococcus* sp., *Escherichia coli*, *Bacillus* sp., *Streptococcus* sp., yeast, and mixed cultures. Probiotics are widely used commercially as feed supplements for terrestial animals such as pigs[2-5] and chickens.[6-7] Probiotic growth promotion, health enhancement, and infectious disease inhibition greatly reduce investment risks and costs. At the same time, probiotics have not yet been used widely in aquaculture, although a substantial market currently exists in the agro-industry for these products.[8] However, some successful probiotics trials have been reported with *Vibrio alginolyticus* in salmon.[9] Recently, Sugita *et al*. (1996)[10] isolated bacterial flora from the GI tract of marine crabs and fish which have potential uses as probiotics. Futhermore, Rengpipat et al. (1998)[11] clearly demonstrated that *Bacillus* S11 could be used as a probiotic for *Penaeus monodon*.

Lactobacillus, a group of lactic acid bacteria, has long been used as starter culture for several milk products,[12,13] and other fermented foods.[14] The end product of this fermentation is lactic acid which is a safe food preservative and flavor enhancer.[15] Several *Lactobacillus* spp. strains are used as probiotics for terrestial animals, including, *Lactobacillus lactis*, *L. bulgaricus*, *L. fermentum, L. acidophilus* and *L. reuteri.*[2,4,5,16] These *Lactobacillus* spp. reduced *Escherichia coli* in feces,[4] guts[5] and stomachs[2] of small pigs, and they suppressed and neutralized *E. coli* toxin in calves.[16] *Lactobacillus* spp. feed additives also benefited host metabolism, including reduced serum cholesterol[17] and amine[3] in pigs, and they produced hydrolytic enzymes which improved digestion in rats,[18] chicks[6,7] and pigs.[19] The main purpose of our study was to evaluate potential benefits of *Lactobacillus* spp. mixed cultures with black tiger shrimp, *Penaeus monodon.*

MATERIALS AND METHODS

Five strains of *Lactobacillus* spp., including *Lactobacillus acidophilus* TISTR 1338, *L. bulgaricus* TISTR 1339, *L. casei* TISTR 1340, *L. casei* subsp. *tolerans* TISTR 1341, and *L. jensenii* TISTR 1342 were used in this study. They were provided by Bangkok MIRCEN, Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. They were originally isolated from intestines of healthy chickens obtained from local markets in Bangkok. Prior studies by Thanaruttikannont (1996)[20] confirmed that they possessed good probiotic properties for rearing chickens. Each *Lactobacillus* spp. was grown in MRS broth (DIFCO) at 37□C for 24 h and then harvested by centrifuging at 5,000 rpm for 30 min. Cells were immediately washed twice with sterile normal saline solution. Cells of each strain were mixed in equal amounts to achieve final concentration of ~10^{10} cells g^{-1} wet weight. This *Lactobacillus* spp. mixture was designated L-P. *Lactobacillus* spp. confirmations were performed on MRS agar by picking isolated colonies for examination, using Gram staining, a catalase test, and a biochemical test. Confirmation was by standard techniques.[21]

The shrimp diet was prepared consisting of 32% fish meal, 25% soybean, 10% shrimp head meal, 1% lecithin, 20% wheat flour, 5% wheat gluten, 1% vitamin complex, 1% mineral complex, and 5% fish oil, all by weight. These ingredients were mixed, extruded, heated at 100□C for 10 min, and then dried at 80□C overnight. After cooling to room temperature, feed was kept at -20□C until used. Total bacterial count revealed mostly *Bacillus* spp. (10^2 CFU g^{-1}). Prior to use, L-P was added to feed in a 1:3 ratio, thoroughly mixed, and kept at -20□C until use. L-P in this aliquot was at approximately 10^{10} CFU g^{-1}. Feed without any addition (non-treated feed) was used as a control diet.

Healthy black tiger shrimp aged PL-15 were acclimatized in six concrete test tanks (each measuring 80x74x87 cm) until PL-30 (0.7-0.8 g), and were fed non-treated feed three

times daily. The shrimp culture procedure was as described by Menasveta *et al.* (1991),[22] including a closed, recirculating water system. The experiment was conducted in a completely randomized design with two treatments: a treatment group (shrimp fed L-P diet); and a control group (shrimp fed non-treated diet). Three replications with 40 shrimps per replicate were used in each treatment. Feeding was conducted three times daily at 0800, 1200 and 1600 h. Total daily feed was ~10% of total body weight. Total shrimp weights and the number of shrimp in each tank were measured every three weeks.

Water samples (120 ml) were collected weekly from the center of each tank, along with shrimp feces (~200 mg), and one live shrimp once every three weeks for bacterial determination starting from the first day of the feeding trials. Water quality was monitored weekly, including pH, dissolved oxygen, temperature, salinity, ammonium, nitrate, nitrite and phosphate as described by Strickland and Parsons (1972).[23] Shrimp were dissected using sterile surgical scissors to remove the hepatopancreas and intestines for microbial enumeration and identification. All samples for bacterial determination were serially diluted in NSS and plated on nutrient agar, tryptic soy agar, MRS agar, and thiosulfate citrate bile salt agar (TCBS). All agar media included NaCl at 1% w/v. Reagents were from DIFCO Laboratories, USA. Colonies formed on plates after 24-48 h of incubation at 37□C were counted and recorded. Confirmation of each strain was microbiologically re-examined by Gram staining, spore staining, and selected biochemical tests using standard techniques.[21]

After feeding for 100 days, shrimp in each treatment were challenged with *Vibrio harveyi* D331, kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill Co. Ltd., Thailand. *Vibrio harveyi* D331 is a pathogenic bacterium causing luminescent disease in *P. monodon*. Both TCBS broth and agar were used as culturing and maintaining media for *V. harveyi* D331. Immersion techniques[9] (Austin *et al*., 1995) were used with *V. harveyi* D331 suspension and final concentrations of ~10^5 CFU ml^{-1} in tank waters at Day 0, with a second booster of 10^7 CFU ml^{-1} on Day 7. Water and shrimp samples were taken every two days from each tank for bacterial determination as described above, along with measurements of shrimp survival. After 10 days of the challenge test, shrimp in each treatment group were dissected and their internal organs were observed microscopically. Final survival was measured.

V. harveyi isolated from shrimp guts was purified and identified by examining their microbiological and biochemical characteristics, including. Gram staining, on oxidase test, and a motility test. These results were compared with the results from tests with *V. harveyi* D331. The identity of *V. harveyi* and *Vibrio* spp. were reconfirmed by following procedures described by Holt et al. (1986).[24]

The effects of L-P on shrimp growth and survival and *V. harveyi* D331 resistance, were analysed using analysis of variance and Duncan's multiple range tests.[25]

RESULTS AND DISCUSSION

All water quality values were within acceptable and safe ranges for shrimp culture (Table 1).[22] After 100 days, shrimp survival was quite low, averaging 34% in the treatment group and 16% in the control group (Fig. 1). Percent of shrimp survival calculated from the average of survival at each time point without subtacting the number of shrimp removed for microbiological study, in terms of percent. Cannibalism in clear water may have caused low survival. Furthermore, from our observations survival of shrimp in cement tank is generally quite low. No infection was found and shrimp were very healthy. Average shrimp weights of the the treatment group were significantly greater than those of the controls after 100 days (Fig. 2, $P<0.05$). It appears that L-P contributed to both increased yields and growth. This finding supports our earlier finding with other probiotics for the same purposes.[11,27]

The most useful probiotic micro-organisms for shrimp are those that grow and propagate well in the shrimp gastrointestinal tract, producing useful end products for their

host is benefit. Until now, *Lactobacillus* spp. was not known to inhabit shrimp GI tracts.[26,27] We have demonstrated, however, that *Lactobacillus* spp. (L-P) isolated from chicken intestines can colonize *P. monodon* GI tracts and function as probiotics to increase survival and growth of *P. monodon* (Fig. 1). Interestingly, only *L. casei* subsp. *tolerans* was the predominant strain in the GI tract, while other species disappeared (data not shown). *Lactobacillus* spp. were found in both tank water and shrimp guts and tank water in our control group (Figs. 3 and 4). This might be due to cross-contamination from using the same utensil for handling the diet on the first day. However, *Lactobacillus* spp. concentrations in the control group were insignificant compared with those of the treated groups. It is possible that *L. casei* subsp. *tolerans* can adapt and survive once introduced into a culture system.

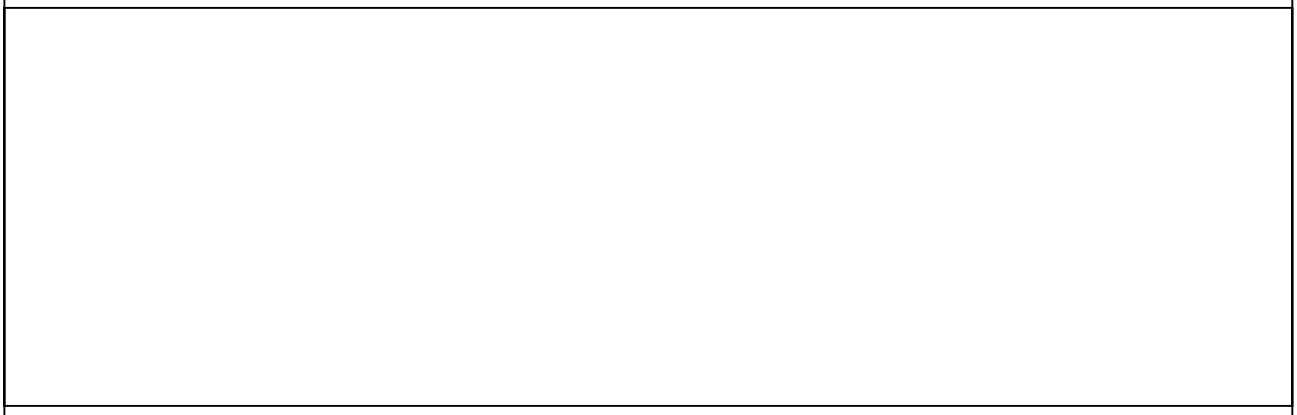

Figure 1. Average *Penaeus monodon* survival during 100 days of feeding trial. Shrimp fed non-treated diet (■) are compared with a treatment group fed a L-P diet (□). All values are means of three replicates per treatment.

Table 1. Range of water quality values in shrimp rearing tanks during 100 days of L-P diet applications.

pH	7.9-8.2
Dissolved oxygen	5-6 mg L^{-1}
Temperature	26-27 °C
Salinity	20 ppt
Ammonium	0-0.05 mg L^{-1}
Nitrite	0-2.5 mg L^{-1}
Nitrate	0-1.2 mg L^{-1}
Phosphate	2-3 mg L^{-1}

Figure 2. Average *Penaeus monodon* weights during 100 days of feeding trial. Shrimp fed non-treated diet (■) are compared with a treatment group fed a L-P diet (□). All values are means of three replicates per treatment.

It is possible that L-P could colonize and adhere to shrimp GI tract surface, leading to nearly compete exclusion of other microbes. Evidence for this conclusion includes the presence of *Vibrio harveyi* D331 (majority among *Vibrio* detection) in water and guts of the control group in much greater concentration than those of the treated group during challenge with *V. harveyi* D331 in the last 10 days (Figs. 3B and 4B). Futhermore, L-P was detected in both the guts (Fig. 3A) and feces (Fig. 6) of treatment group shrimp after feeding with the L-P diet. In addition, their end products of lactic acid (Gilliland, 1985), or some other anti-microbial substances[28-30] could tentatively interfere with or suppress the growth of *V. harveyi* D331 and lead to the indirect eradication of *V. harveyi* D331 infection. That could explain the lower numbers of *V. harveyi* D331 in guts and feces of treated shrimp compared with the controls. Obviously, *V. harveyi* D331 of 10^2 CFU g^{-1} in shrimp guts did not result in detectable disease symptoms.

During the 10 day challenge test with *V. harveyi* D331, no mortality occurred in the treatment group, but survival in the control group was only 26% (Fig. 5). Surviving shrimp in the control group looked unhealthy. These survival rates were significantly different ($p < 0.05$).

We still do not have a clear idea about exactly how probiotics protect shrimp and improve their survival and growth. We have demonstrated these benefits here and in our other findings, but we are uncertain whether the protective mechanism involves competitive exclusion of harmful microbes, or immune response by the shrimp.

We also need to evaluate *Lactobacillus* spp. applications during the entire culture cycle in ponds. Application times and dosages, shelf life of the probiotics, and other factors an in open cycle system need to be evaluated before large scale commercial applications are made. However, our work clearly demonstrates potential probiotic benefits of *Lactobacillus* spp. for control of certain serious diseases in *P. monodon*.

Figure 3. Average bacterial counts in shrimp guts (hepatopancreas and intestine) for: (A) shrimp cultured for the first 100 days on feeding trial and (B) shrimp cultured for the last 10 days during challenge with *V. harveyi* D331 (↕ 2nd booster with *V. harveyi* D331). Total bacteria (o), *Lactobacillus* spp. (□), *Vibrio* spp. (Δ), and *Vibrio harveyi* D331 (◊). Closed symbols represent mean values for controls whereas open symbols represent mean values for the treatment group. Each value is the mean of three replicates per treatment.

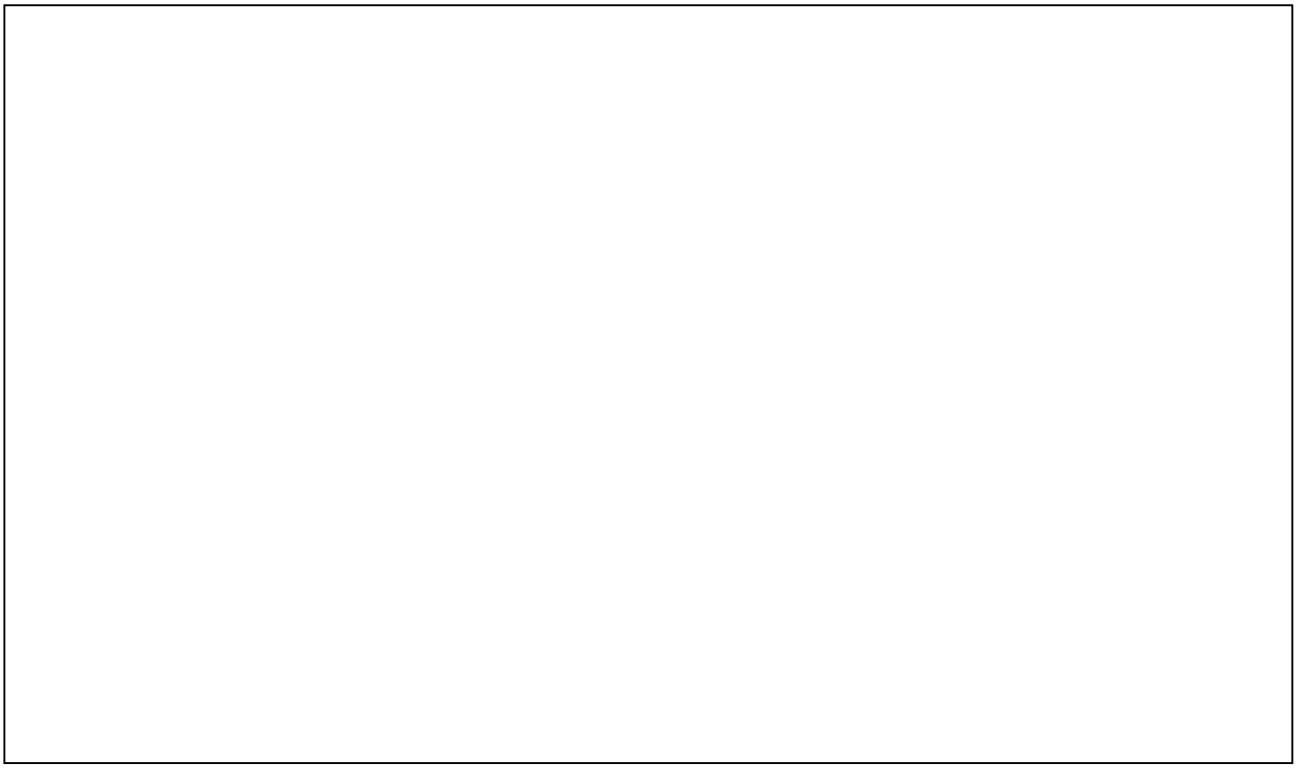

Figure 4. Bacterial counts in rearing tank water for: (A) shrimp cultured for the first 100 days on feeding trial; and (B) shrimp cultured for last 10 days during challenge with *V. harveyi* D331 (↑ 2nd booster with *V. harveyi* D331). Total bacteria (o) , *Lactobacillus* spp. (□) , *Vibrio* spp. (Δ) and *Vibrio harveyi* D331 (◊). Closed symbols represent mean values for controls whereas open symbols represent mean values for the treatment group. Each value is the mean of three replicates per treatment.

Figure 5. Average *Penaeus monodon* survival during 10-day challenge with *Vibrio harveyi* D331. Shrimp fed non-treated diet (■) are compared with a treatment group fed a L-P diet (□). All values are means of three replicates per treatment.

CONCLUSIONS

Lactobacillus spp. and especially *L. casei* ubsp. *torelans* can be used as probiotics for feeding black tiger shrimp, *Penaeus monodon*, leading to higher growth and survival. *Lactobacillus* spp. demonstrates potential of control *Vibrio harveyi* in the shrimp GI tract and provides highly healthy shrimp protected against such diseases.

Figure 6. Bacterial counts in shrimp feces during 100 days of feeding trial. Total bacteria (o), *Lactobacillus* spp. (□), and *Vibrio* spp. (Δ). Closed symbols represent mean values for controls whereas open symbols represent mean values for the treatment group. Each value is the mean of three replicates per treatment.

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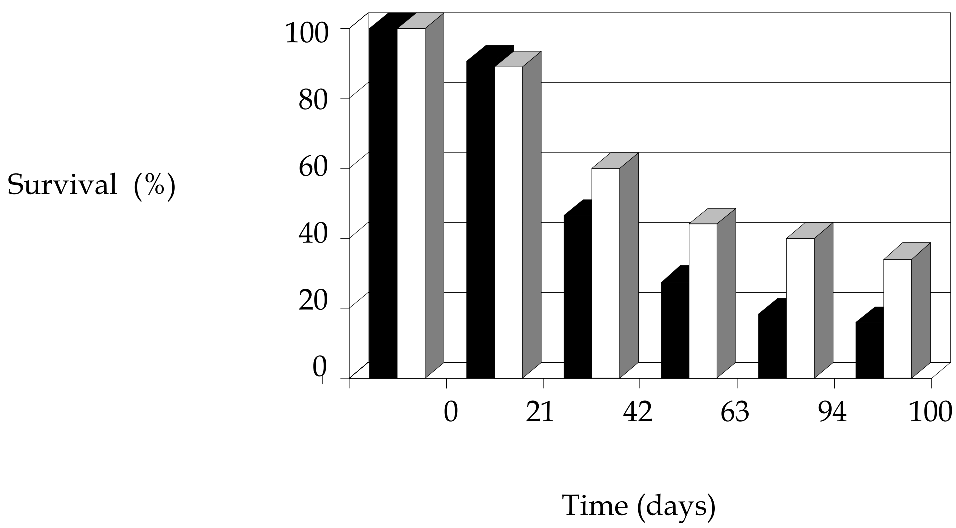
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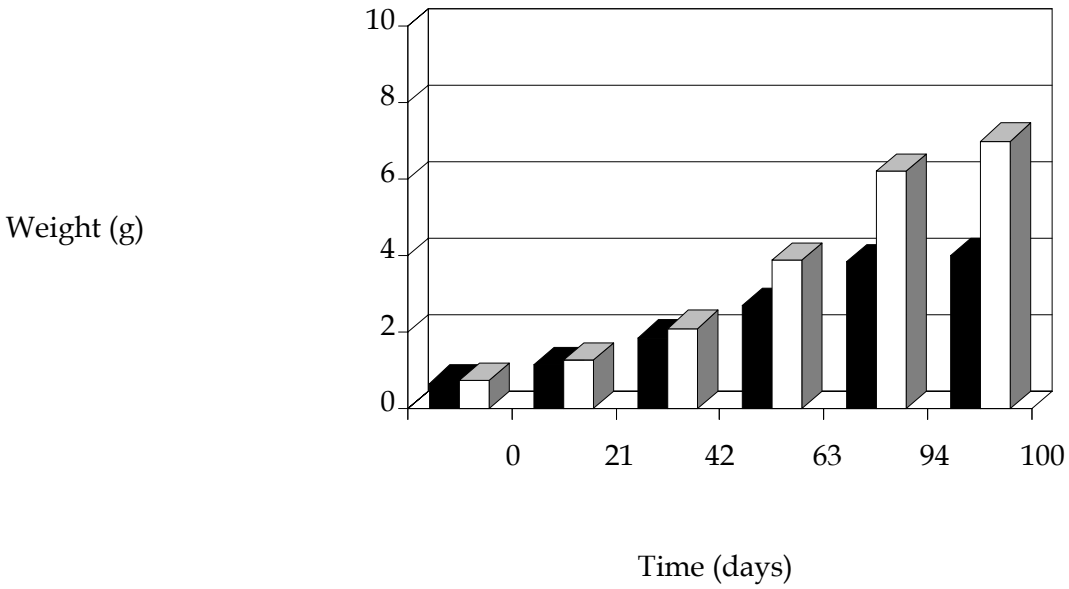
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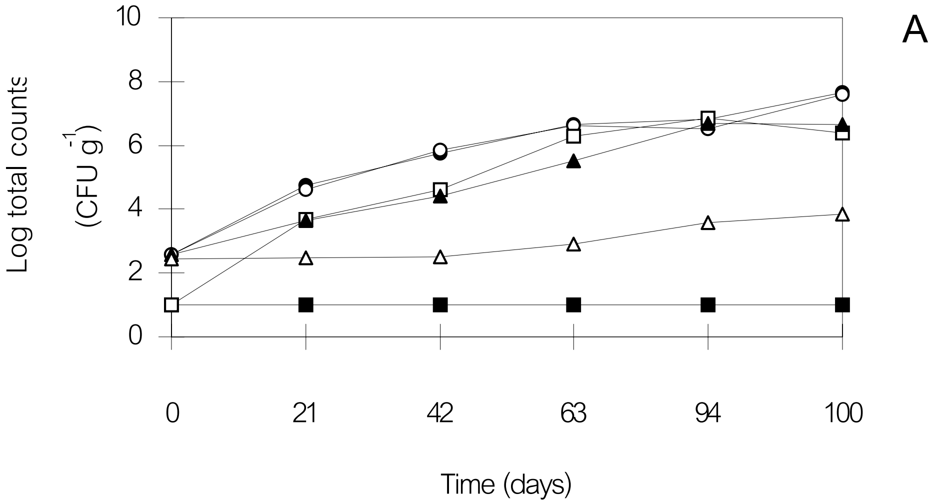
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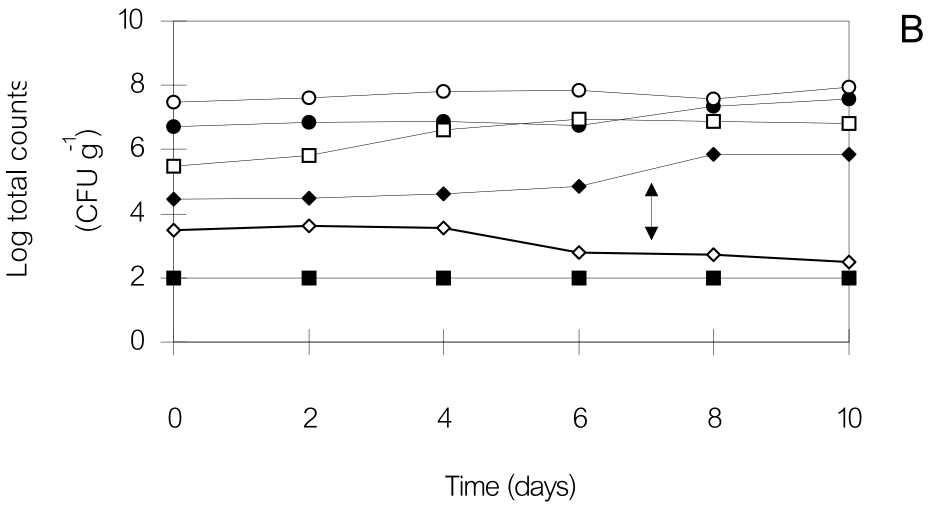
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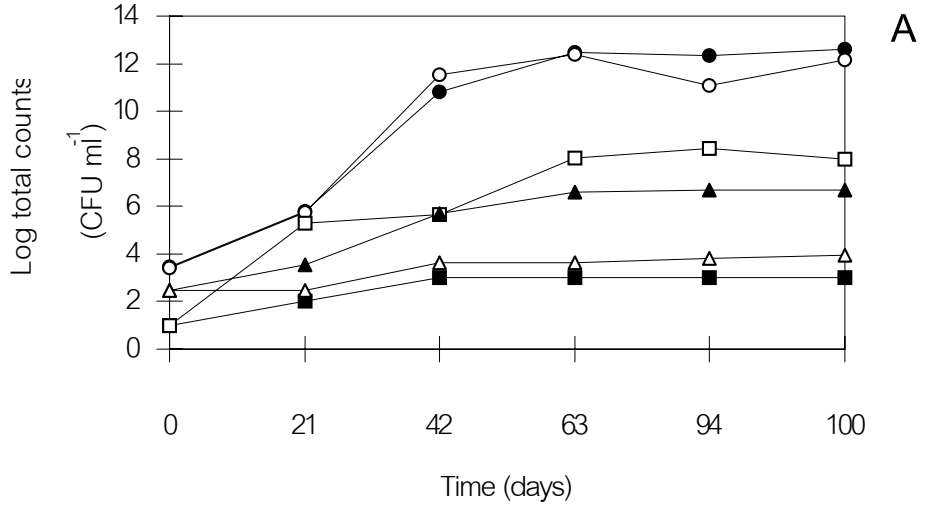
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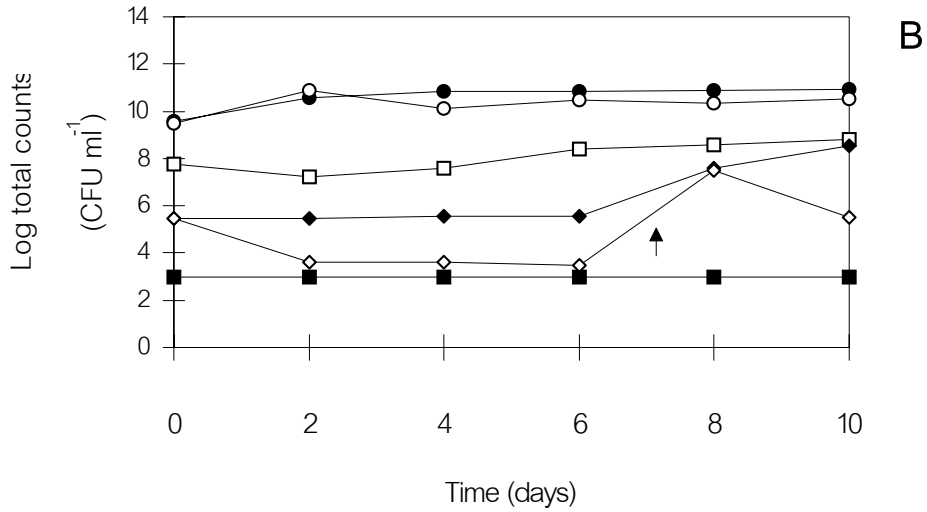
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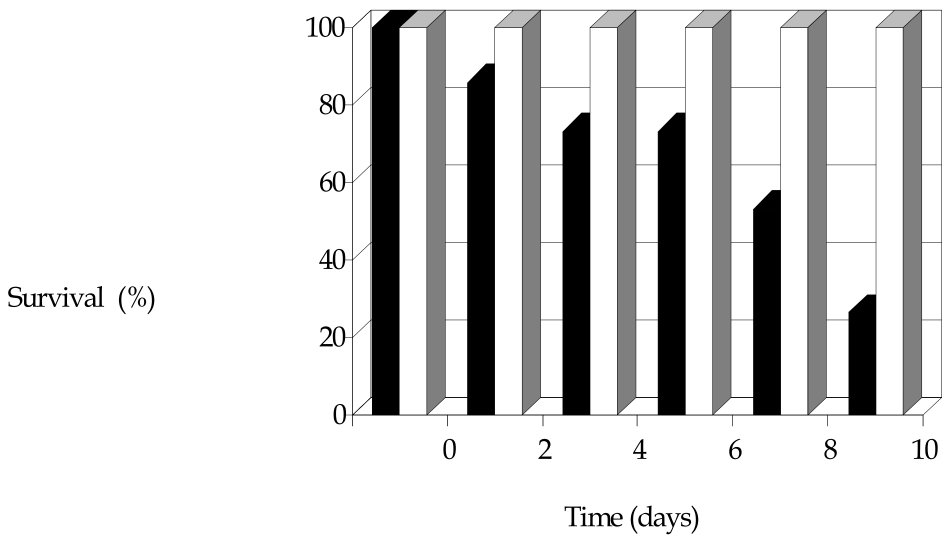
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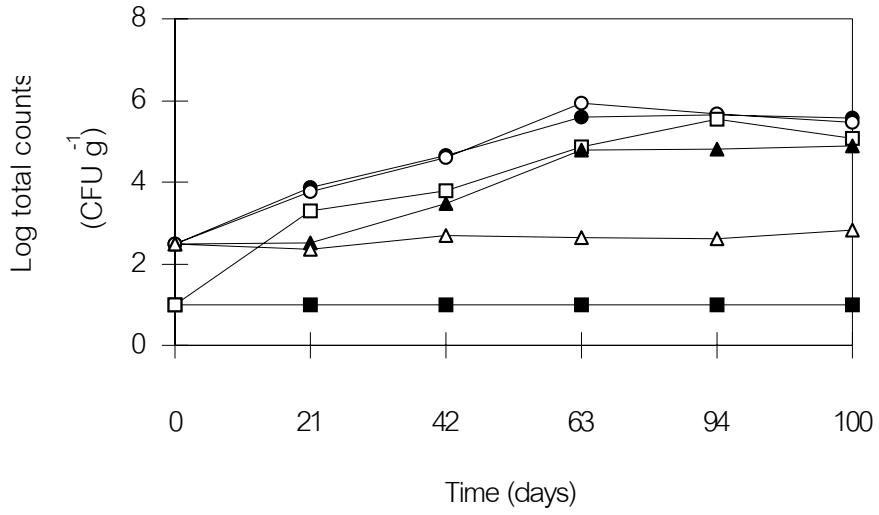
10
Time (days)


Log total counts
(CFU g-1)
8
6
4
2
0
0
21
42
63
94
100
Time (days)