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Assessment of possible human risk of probiotic application in shrimp farming

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Abstract: Aquaculture is one of the fastest growing industries. This impressive industry is incorporated with prophylactic use of antibiotic for disease prevention. Probiotics seems to be appropriate substitute for the antibiotic, but because those are live bacteria and residual of the probiotics in the aquaculture product may cause health problem for the consumers and labors in the aquaculture processing plant and aquaculture farms. In this study we used LD_{50} in a mammalian model animal to assess the safety of probiotics used in shrimp culture for human consumption. After assessment for an approximate range of lethality in a preliminary experiment, treatment groups were fed via a gavage with certain dosage range of candidate probiotic, *Shewanella algae*. The LD_{50} value was approximately 10^{36} cfu/animal with 95% fiducial limitation of 34.95 (lower band) and 37.07 (upper band), which is fairly high and most likely safe to use as probiotic. Furthermore, this study may aid to onset of thinking about an evaluation technique for safe using of probiotic in aquaculture.

Keywords: Shrimp industries, probiotics, food safety

Introduction

Industrial aquaculture is a rapidly growing industry in many developed and developing countries. It is expected that this growth will increase at an even faster rate in the future, stimulated by the depletion of fisheries and the market forces that globalize the sources of food supply (Goldburg, and Naylor, 2005). This attractive industrial development has been joined with some methods that potentially harmful to human and animal health (Goldburg and Naylor, 2005; Naylor and Burke, 2005) that consist of releasing vast amounts of chemical into the environment (Haya *et al*., 2000; Boxall *et al*., 2004). For instance, the aquaculture of shrimp and salmon has been linked with considerable utilization of prophylactic antibiotics which is accumulated in the aquatic environment. (Le and Munekage, 2004; Le *et al*., 2005). The prophylactic use of antibiotics caused an increment in antibiotic resistance in aquatic environment (Rhodes *et al*., 2000; Miranda and Zemelman, 2002; Alcaide *et al*., 2005). Furthermore, this prophylactic application of antibiotic has been reasoned for an increase of antibiotic resistance in fish pathogens, as well (Rhodes *et al*., 2000; L'Abee-Lund *et al*., 2001; Schmidt *et al*., 2001; Sørum, 2006). The appearance of antibiotic resistance within fish pathogens weakens the efficiency of the antibiotic use in aquaculture (L'Abee-Lund *et al*., 2001; Sørum, 2006) and increases the opportunities for transferring of these antibiotic-resistant bacteria and their antibiotic resistance determinants to bacteria of terrestrial animals, human beings and pathogens, as well (Cabello, 2006). The prophylactic utilization of antibiotics in industrial aquaculture and presence of residual antibiotics in commercialized fish and shellfish products has been considered (Goldburg *et al*., 2001).

Nowadays, the safety of consumed food is of great importance. Shrimp coming from Southeast Asia have become common at dinner tables in many European and American households, and its safety attracts enormous consideration (Oosterveer, 2006). Utilization of probiotic helps to eliminate this problem, which has led to undetected consumption of antibiotics by consumers of fish and shellfish with the added potential alteration of their normal flora that increases their susceptibility to bacterial infections and also selects for antibiotic-resistant bacteria (Greenlees, 2003; Salyers *et al*., 2004). Moreover, undetected consumption of antibiotics in food can generate problems of allergy and toxicity, which are difficult to diagnose because of a lack of previous information on antibiotic ingestion (Cabello, 2004).

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Although, there is not any report about harmful effect of probiotic incorporated with the aquatic products, but development of aquaculture industries and limitation of antibiotic use in aquaculture has forced to this alternate technique. Since some aquaculture products are used uncooked or half-cooked, there is a possibility that probiotics residual cause infection in final consumer. Moreover, handling and utilization of probiotic products in aquaculture farms and hatcheries might be accompanied with some risk for the workers, too. It seems necessary to be sure about safety of the residual probiotics in the aquaculture products specially when undergoes in the processing plant which might cause infection for the processing labors. There are enormous reports and protocols for screening of probiotic in aquaculture, wherein the beneficial of the selected probiotic for the host was considered. Although , Vijayan and his co-workers (Vijayan *et al*., 2006) performed LD_{50} potential of a brackish water isolate (*Pseudomonas PS-102*) on mice as mammalian model. Still, there is scarce information about residual probiotic in the host and its possible risk for final consumer, human. However it seems essential to develop a technique for evaluating human risk as final consumer of shrimp and aquatic products which are harbor of probiotic bacteria.

The current study is a recommendatory technique for reducing of possible risk for utilization of candidate probiotic, using LD_{50} in a mammalian model animal. *S. algae* exhibited fairly good probiotic properties in in vitro (Shakibazadeh, 2008) and in vivo. Therefore, the possible risk of its utilization was evaluated.

Materials and Methods

A total number of 208, eight weeks old male BALB/c mice were purchased from a local supplier. All mice were weighted before experimental procedure; there was no significant difference between body weight of the experimental groups or within different experiments (20 ± 2 g). A preliminary experiment was conducted for obtaining possible mortality range for the candidate probiotic. Forty mice were arranged in 5 cages, each containing 8 pieces. The mice in each group were fed with suspended bacteria in normal saline including 10^7, 10^9, 10^{11}, 10^{13} and 10^{15} cfu/mice. The mice were monitored for one week for sign of sickness or mortality. The experiment was continued because of no mortality observed. Then bacterial concentration of 10^{17}, 10^{19}, …, 10^{33}, 10^{35} was applied to 2 independent mice groups each contains 40 pieces, alternatively, until approximate mortality range was revealed. The mice were feed with mice commercial pellet. Animals were starved 3 hours before and 1 hour after administer of bacterial suspension but water *ad libitum*. They were kept at controlled temperature (30°C), light regime was cycles of 12 hours light and 12 hours dark.

Bacteria preparation

The candidate probiotic, *S.* algae, was stocked in a LB Broth, Lysogeny Broth or Luria Bertani, containing 20% glycerol at – 80°C. The bacteria was revived in Mueller Hinton broth, then 4 Erlenmeyer flask (1litre capacity) each was contained 750 ml of Mueller Hinton broth were inoculated with the revived candidate probiotic and incubated for 24 h in a shaker incubator at 30°C and 250 rpm. The cultured bacteria were harvested using a centrifuge (Thermospectronic, Genesys 20, USA) for 10 min at 3500 rpm. Then, those were washed 3 times with normal saline and suspended again in the same volume of normal saline and stock in 4°C until used. Bacterial enumeration was assessed using spectrophotometry according to the standard curve which was obtained following the method documented by Shi and Xia (Shi and Xia 2003). The accuracy of the bacterial count was confirmed by standard plate count. While the population density was achieved, the required density for the experiment was prepared via serial dilution or centrifugation.

LD_{50} of S. algae *in a Mammalian model*

While the approximate range of the mice mortality due to oral administration of candidate probiotic, *S. algae*, was revealed, the main experiment was designed. Total number of 88, eight weeks old male BALB/c mice were purchased from local supplier and arranged in 10 treatment groups and a negative control, each group consists of 8 mice. Treatment groups orally received 10^{31}, 10^{32}, 10^{33}, 10^{34}, 10^{35}, 10^{36}, 10^{37}, 10^{38}, 10^{39}, 10^{40} cfu/animal of candidate probiotic, *S. algae*, and negative control which received same volume of the normal saline. The bacterial suspension were prepared not more than two hours before application and kept in 4°C during experiment. Each mouse was fed with 0.1 ml of the appropriate bacterial concentration using an 1 ml syringe which was equipped with a gavage needle. Treatment groups were monitored for two weeks, behavioral abnormalities, behavioral batteries, dead or dying animals and obvious abnormalities checked and recorded within this period.

Statistical analysis

The median lethal oral dose, LD_{50}, was obtained via probit analysis. Goodness of fit, likelihood ratio and confidence limit was obtained. The statistical

analysis was carried out using SAS statistical software (SAS Institute Inc.).

Results and Discussion

Prophylactic uses of antibiotic in aquaculture force to undesired environmental problems resulting to antibiotic resistance, which can affect even terrestrial bacteria, animals and human. The residual of prophylactic use of antibiotic in aquaculture product is an important case of food safety, as well. However, problems contributed to prophylactic use of antibiotic were to such an extent that an alternative method was required. Probiotics can be suitable substitution, but because probiotics are live bacteria which can be colonized in the aquaculture products the risk of food safety, aquaculture processing labors and aquaculture farm workers is of great importance.

The treatment groups were fed with different levels of candidate probiotic. Possible mortality within experimental groups was recorded. The results showed the p-value in the goodness of fit of 0.6921 for the Pearson chi-square and 0.5842 for the likelihood ratio chi-squrae which indicate an adequate fit for the model fit with the normal distribution. The natural response threshold or the proportion of individuals responding at zero percentage was estimated to be 0.000. Both the intercept and the slope coefficient have significant p-values (<0.0001). The LD_{50} value for the candidate probiotic, *S. algae*, was 36.0947 log cell with 95% fiducial limitation of 34.9533 (lower band) and 37.0710 (upper band) (Table 1 and Fig. 1).

Table 1. Probit analysis of LD_{50} of candidate probiotic, *S. algae* (Log_{10}), on mice

Probability	*S. algae* (cfu/ mouse)	95% Fiducial Limit	
		Lower band	Upper band
0.01	31.0521	27.4810	32.7813
0.02	31.6430	28.4075	33.2330
0.03	32.0179	28.9927	33.5223
0.04	32.2999	29.4313	33.7415
0.05	32.5293	29.7868	33.9210
0.06	32.7246	30.0885	34.0748
0.07	32.8958	30.3522	34.2105
0.08	33.0491	30.5876	34.3327
0.09	33.1885	30.8010	34.4444
0.10	33.3168	30.9968	34.5479
0.15	33.8481	31.8006	34.9834
0.20	34.2704	32.4293	35.3398
0.25	34.6327	32.9590	35.6551
0.30	34.9580	33.4253	35.9477
0.35	35.2595	33.8475	36.2286
0.40	35.5455	34.2379	36.5055
0.45	35.8223	34.6045	36.7845
0.50	36.0947	34.9533	37.0710
0.55	36.3671	35.2891	37.3706
0.60	36.6438	35.6163	37.6890
0.65	36.9299	35.9395	38.0330
0.70	37.2314	36.2641	38.4116
0.75	37.5567	36.5973	38.8372
0.80	37.9190	36.9501	39.3295
0.85	38.3413	37.3410	39.9236
0.90	38.8726	37.8087	40.6952
0.91	39.0009	37.9185	40.8847
0.92	39.1403	38.0365	41.0919
0.93	39.2936	38.1650	41.3209
0.94	39.4648	38.3071	41.5782
0.95	39.6601	38.4674	41.8733
0.96	39.8895	38.6537	42.2221
0.97	40.1715	38.8802	42.6535
0.98	40.5464	39.1774	43.2308
0.99	41.1372	39.6390	44.1475

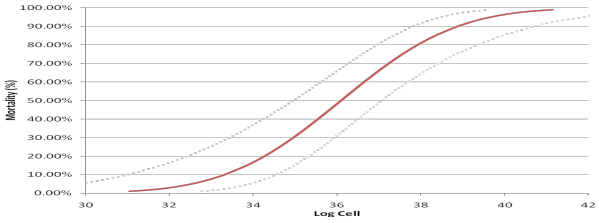

Figure 1. Dose response curve of mice administered with candidate probiotic, *S. algae*

The obtained LD_{50} value, 10^{36} cfu, is fairly higher than previous report which was documented by Vijayan and his co workers (Vijayan, 2006), 10^{9} cfu. They were evaluated LD_{50} value of a brackish water probiotic isolate, *pseudomonas PS-102*, on mammalian model (mice). Nasal route was the administration technique in this study. Similar study was carried out to assess Pathogenicity of other member of this bacterial Genus, *Shewanella marisflavi*. LD_{50} values of *Apostichopus japonicas* (sea cucumbers), *Xiphophorus helleri* (Swordtail fish) and mouse was determined and compared by applying *S. marisflavi*. The bacteria were inoculated by intraparitoneal and intramuscular injections. the LD_{50} values were 3.89×10^{6}, 4.85×10^{4} and 6.8×10^{4} cfu/g body weight, respectively (Li *et al*., 2008). The approximate bacteria cause mortality in 50% of mice was1.36×10^{6} per animal, if the average body weight of mice would be 20 g. In both similar experiments the reported LD_{50} values are fairly lower than what obtained in current study. Obviously, although LD_{50} provide a rather relative index of xenobiotic response for comparison of different bacteria, but it along with slope and confidence intervals are not absolute values. Because response of a test animal to a "chemical" is influenced by the choice of test species and strain (even individual genotype), test conditions (temperature, light regime, food, stress, experiment conductor, …), age, sex and body weight of the animals. Same condition should be considered for evaluating influences of a live bacterium on test animal. Since pathogenicity and growth of a microorganism quite related to the growth condition (pH, temperature, culture medium, stocked condition,…) and bacterial strain or serotype, the interaction of microorganism and host has a much more complicated procedure than applying of a chemical. Additionally, the route of administration of the bacteria in those researches was different. In the current study *S. algae* was administered via digestive tract which is fairly lower sensitive route for infections than intraparitoneal and intramuscular injection in *S. marisflavi* and nasal administration in *Pseudomonas PS-102*. Furthermore, the pathogenicitiy of members of different Genus as well as the species and even strains within a species

can be entirely varied. Therefore, the LD_{50} variations between *S. algae*, *S. marisflavi* and *Pseudomonas* may be due to the administration route and pathogenicity difference of various bacteria. The Obtained LD_{50} shows that using of the candidate probiotic, *S.algae*, can be safe for shrimp consumer, processing plant labors, hatchery and shrimp farms workers.

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