PCR-DGGE METHOD FOR MICROFLORA ALTERATION IN SHRIMP DIGESTIVE ORGAN FOLLOWING *Lactobacillus* ADMINISTRATION

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ABSTRACT

Recently, bacteria as probiotic usage in aquaculture are deeply investigated. Probiotic application in aquaculture leads to alteration in bacteria community within environment and inside digestive system of the host. However, the evidence of treated bacteria presence is very limit. This study was aimed to reveal the effectiveness of PCR-DGGE method to bring some evidence of the applied probiotic existence within aquatic organism. Two species of *Lactobacillus* were applied in this experiment. *Litopenaeus vannamei* was used as host for applied bacteria, *L. vannamei* was reared using natural sea water in the 45 cm x 15 cm x 25 cm aerated glass tank. Twice daily of enriched *Artemia* were gived as nutrition during experiment. The result showed that *Lactobacillus* represent higher in treatment group compare with control after 20 days treatment. In the other hand there was no different of bacteria number between two treated *Lactobacillus*. PCR-DGGE is a rapid and reliable method for bacteria detection within aquatic organism.

KEYWORDS: **PCR-DGGE, probiotic, *Lactobacillus***

INTRODUCTION

Bacterial community in human and animal digestive organ is one of the most complexes, diverse, and volatile community. Billions of bacteria live inside the digestive organ of all organisms, and they play an important role for health or robustness of living organism.

In aquatic animal, bacterial community are transient and may change rapidly by influence both indigenous and extraneous factors, including; age, environmental condition (Kapetanovi□ *et al.*, 2005), the ingestion of the surrounding free-living bacterial community, physicochemical aspects of the gut, life history, diet, physiological condition of the host, and possibly even habitat type, and farming practices (Oxley, 2002).

Probiotic usage has been investigates for many years, and demonstrated improving human and animal health and welfare. Influence of probiotic on the organism intestine has various mechanisms, including; competition for substrates, direct antagonism by inhibitory substances, competitive exclusion, and potentially host-mediated effects such as improved barrier function and altered immune response. The basic concept behind probiotics is animal or human body is host to microorganisms or bacteria known as gut flora that are essential to the health (O'Toole, 2008).

Aquaculture frequently employs probiotic to confer health of cultured animal digestion, maintain water quality and enhance immune response of aquatic animal (Wang *et al.*, 2005; Wang, 2007; Balcázar, 2007). Probiotic was acknowledged as producer some essential substance and help the digestive tract to reduce pathogen bacteria colonization. The ability of probiotic to alter bacterial commu-

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nity is limited to the presented result with no evidence of the applied probiotic existence.

Bacteria identification using conventional biochemical method is time consuming, and did not have accuracy to identify a number of bacteria species. This method sometime presents an ambiguous result, e.g: one isolated bacteria is known as different species using standard biochemical method and commercial biochemical kit (Ma *et al.*, 2001). Instead of biochemical method, molecular method has been increasingly applied in bacterial detection. Molecular method shows the advantageous for the rapid and accurate result. PCR is the frequent method for specific species detection usage in molecular technique. Combination of PCR method and Denaturing Gradient Gel Electrophoresis (DGGE) demonstrate a satisfying result for microbial community fingerprinting (Simpson *et al.*, 1999; Fasoli *et al.*, 2003; Shulze *et al.*, 2006).

This study was aimed to reveal the effectiveness of PCR-DGGE method to bring some evidence of the applied probiotic existence within aquatic organism. In the future this method is reliable to be applied for bacterial fingerprint in aquaculture science.

MATERIAL AND METHOD

Bacteria and Growth Condition

Two species of *Lactobacillus* were used in this study, *L. plantarum* was provided by microbiology laboratory of National Kaohsiung Marine University of Taiwan and *L. casei* was provided by pathology laboratory of NKMU of Taiwan. Full loops of thawed *Lactobacillus* were cultured in 10 mL of de Man, Rogosa and Sharpe (MRS) broth and incubated overnight at 35°C. Afterwards, broth was centrifuged at 12,000 rpm for 5 minutes; and followed by three times washing with 1x PBS solution.

Shrimp Cultivation and Treatment

The experiment was conducted to examine the effect of *L. plantarum* and *L. casei* administration for *Litopenaus vannamei* as the host. Treatments were arranged triplicate. L. vannamei were carried from Yong-An earthen pond and transfer to NKMU using plastic bag provided with O_2 to avoid mortality. Fifteen shrimps, 0.1 g in weight, were cultured in 45 cm x 15 cm x 25 cm aerated glass tanks filled with natural sea water (salinity : 30±1 ppt) and maintain under ambient temperature. Shrimps were fed twice daily with enriched *Lactobacillus Artemia nauplii*. Three shrimps were collected from each treatment tank, control was taken from day 0 tanks, and no dissected organs due to small size of shrimps. After 20 days culture; stomach, hepatopancreas, and intestine were dissected before homogenized in 100 µL of 0.85% sterilized saline water, prior homogenization sample was rinsed with sterilized ddH_2O to minimize the contamination.

Bacteria Plate Count

The edigestive organ (stomach, intestine and hepatopancreas) were dissected prior homogenized in 100 µL sterilized saline water. Total bacteria were counted by incubate 100 µL of 10^6 serial dilution on TSA. Total bacteria and total *Lactobacillus* were determinated by incubation of 40 µL 10^2 serial dilutions on TCBS and MRS. All plates were incubate at 35°C for 24 hours.

DNA Isolation

The digestive organs were dissected prior three times wash in sterilized 1x PBS solution and homogenized in TSB and MRS broth, followed by incubation for 48 hours. 10 mL of cultured broth were centrifuged at 12,000 rpm for 10 min., supernatant were decanted and washed with 5 mL of 1x SSC solution. Bacterial DNA was isolated using Phenol-Chloroform method. Briefly, clean bacteria pellet were suspended to 1 mL tris-HCl buffer (10 mM Tris, 20% Sucrose, 2.5 mg/mL Lysozyme, pH 8.0) for 45 min. at 37°C, 9 mL of lysis buffer buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 500 µg/mL Protenase K, pH 8.0) were added and incubated for 30 min. at 37°C. Extract with same volume of phenol-chloroform-isoamyl alcohol (25:24:1), followed by centrifugation at 12,000 rpm for 10 min. at 4°C, this step was repeated if necessary. Precipitate by adding 0.1 volume of sodium acetate solution and double volume of 95% ethanol, followed by centrifugation at 12,000 rpm for 10 min. at 4°C. Finally pellet was washed with 80% alcohol and air dry, followed by dissolving DNA pellet in sterile H_2O. Estimate the DNA number with ND-1000 nanodrop technologies spectrophotometer, store at -20°C until assay.

PCR Condition

All samples were run in nested PCR method to bring an obvious result. First step PCR is

amplifying 16SrRNA region using FD (5'- AGA GTT TGA TCC TGG CTC AG-3') and RD (5'- AAG GAG GTG ATC GAC CC-3') primer pair (Weisburg *et al.*, 1991). PCR reaction was performed in 25 µL of total volume; contain 10 µM of each primer, 5 µM dNTP, 1x PCR buffer, 0.5 µL of 5U taq enzyme and 30-50 ng DNA template. PCR reaction use following stages; initial denaturation at 95°C for 5 minutes followed by 34 cycle of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, elongation at 72°C for 1 minute, and final elongation at 72°C or 10 minutes. Second step of PCR is amplifying V2-V3 region of 16SrRNA using HDA1 and HDA2 (Walter *et al.*, 2000). A 40 nucleotide of GC clamp (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) attached to the 5' end of HDA1 primer (5'ACT CCT ACG GGA GGC AGC AG 3') and HDA2 (5' GTA TTA CCG CGG CTG CTG GCA C 3'). PCR reaction was performed in 40 µL each tube reaction; contain 10 µM of each primer, 5 µM dNTP, 1x PCR buffer, and 0.5 µL of 5 U taq enzyme and 2 µL PCR product from first step PCR reaction. PCR reaction was generated using initial denaturation step at 94°C for 4 minutes followed by 30 cycle of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, elongation at 72°C for 1 minutes and final elongation at 72°C for 8 minutes.

PCR-DGGE Analysis

Separation of PCR fragments were employs denaturing gradient gel electrophoresis (DGGE) from Bio-Rad DCodeTM Universal Mutation Detection System. The sequence specific separation amplicon was obtained in 8% of (w/v) polyacrylamide (acrylamide : Bis = 37.5 :1) gels with 7 litre of 0.5x TAE buffer.

Linear gradient of 30%-70% urea was prepared by mixing between 0% and 100% denaturant solution. 30 µL of second step PCR product was mixed with 10 µL of 2x loading dye and loaded onto assembled gel. Gel running performed at 130 volt for 5 hours or 80 volt for 12 hours. After achieved time, gel was stained in staining solution for 1 hour and examined under UV illumination (Theunissen, 2005).

Statistical Analysis

All assays were arranged in triplicate and analyzed with one way ANOVA. The significance different test was determined with Tukey HSD analysis at $P<0.05$. The statistical analysis was brought in SPSS version 10.0 software.

Relationship between treatment and control groups were analyzed using Quantity One analysis software.

RESULTS AND DISCUSSION

Bacteria Plate Count

Lactobacillus was undetected in all samples assayed on pre-treatment samples. After 20 days treatment, *Lactobacillus* within treated sample organs represents significantly higher compare with control. In the other hand there was no different of *Lactobacillus* number between two treatments (Table 1).

Result was presented as means ± S.E of triplicate observation. Different letter represent significantly different ($P<0.05$) between treatment on day 0; different symbol represent significantly different ($P<0.05$) between day 0 and day 20 in same treatment.

Total of bacteria did not show any different between treatment and control groups, exclude the intestine of control group and within hepatopancreas of *L. plantarum* group (Table 2). Intestine of the control group represent higher significantly, and the opposite was represented by hepatopancreas of *L. plantarum* which shows lowest number of total bacteria number.

Table 1. Bacteria count result before receiving *Lactobacillus*

Treatments	Total bacteria counts±SE 10^7 CFU/mL	*Lactobacillus*±SE 10^5 CFU/mL
L. casei	1.7±0.2^{a*}	Not determined
L. plantarum	2.8±1.0^{a*}	Not determined
Control	1.4±0.3^{a*}	Not determined

Table 2. Bacteria count result after receiving *Lactobacillus*

Treatment	Organ	Total bacteria counts±SE 10^8 CFU/mL	*Lactobacillus*±SE 10^4 CFU/mL
L. casei	Stomach	$14.3\pm9.0^{a,a}$	$4.1\pm2.3^{a,a}$
	Hepatopancreas	$11.4\pm5.3^{a,a}$	$1.3\pm0.6^{a,a}$
	Intestine	$7.8\pm4.4^{a,a}$	$7.4\pm5.5^{a,a}$
L. plantarum	Stomach	$0.8\pm0.4^{a,a}$	$11.7\pm8.5^{a,a}$
	Hepatopancreas	$0.5\pm0.2^{b,a}$	$10.3\pm5.7^{a,a}$
	Intestine	$1.0\pm0.5^{a,a}$	$10.2\pm2.9^{a,a}$
Control	Stomach	$16.9\pm2.0^{a,a}$	$0.7\pm0.5^{b,a}$
	Hepatopancreas	$20.6\pm1.0^{a,a}$	$0.2\pm0.1^{b,a}$
	Intestine	$18.6\pm2.7^{b,a}$	$0.1\pm0.1^{b,a}$

Note: Result was presented as means ± S.E of triplicate observation. Different superscript are means significantly different ($P<0.05$); first letter was comparing same organ in different treatmenst, second letter was comparing different organs in one treatment

PCR DGGE Analysis

Culturing of bacteria content within organ assayed prior PCR-DGGE were purposed to increase lactobacteria number, since the limitation of PCR-DGGE is greater than 10^4 CFU/mL (Plant *et al.*, 2003).

In order to bring an obvious result, nested PCR were applied to amplify V2-V3 region of 16SrRNA. PCR result was loaded to gradient acrylamide gel, and the result represents similarity of the band as the presence of applied lactobacteria (Figure 1).

For further assay, TFPGA (Tools for Population Genetic *Analyses*) software was employed to examine the alteration of all organs assayed after receiving Lactobacillus. Detected microflora presented that *Lactobacillus* application able to alter the microbial community within shrimp's digestive organ. For the stomach or-

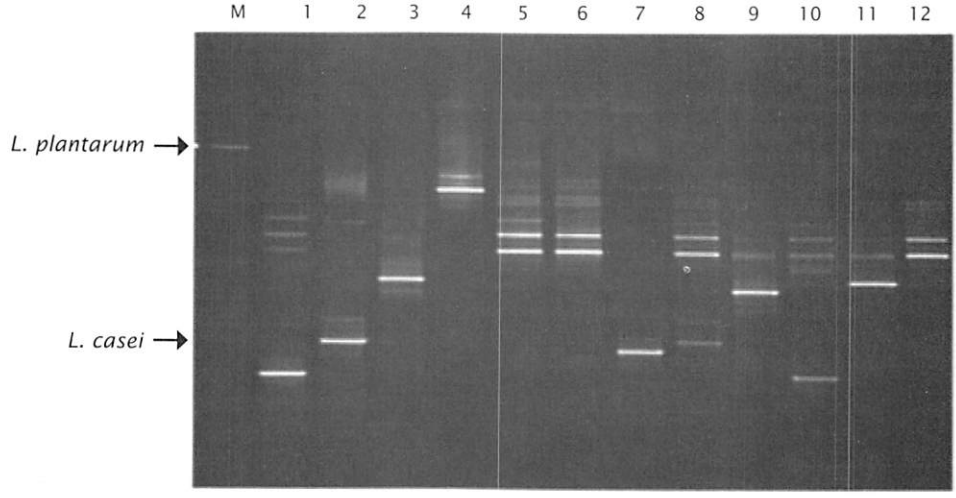

Figure 1. PCR DGGE result of pre treatment and day 1. M: Marker; lane 1-3: stomach, hepatopancreas, and intestine of control samples; lane 4-6: stomach, hepatopancreas, and intestine of *L. casei* samples; lane 7-9: stomach, hepatopancreas, and intestine of *L. plantarum* samples

gan, the alteration of the microflora diversity is not significant (Figure 2).

DISCUSSION

Lactobacillus is widely used as probiotic and applied for both human and animal consumption. Large number of experiments in this field led to the conclusion that probiotic addition in nutrition has a good impact for the host (Chythanya *et al.*, 2002; Suzer *et al.*, 2008; Ma *et al.*, 2009).

The application of probiotic together with aquatic animal nutrition will be affected to the growth and immune response of the host. But unfortunately it is nearly impossible to distinguish between enzyme synthesized by the host and enzyme synthesized by probiotic (Solano & Soto, 2006; Nejad, 2006; Wang, 2007).

After receiving *Lactobacillus* in the diet of *L. vannamei*, *Lactobacillus* was detected on the digestive organ, although it is not a dominant species, since *Lactobacillus* does not occur naturally in crustacean. Application of *Lactobacillus* represent was able to alter the microbial community, even it did not change the whole community. The possibility of microbial community alteration in this study was due to the host that was reared in glass tanks for 20 days without environmental interruption. Phylogenetic analysis also represent no significant different between pre and post receiving *Lactobacillus*. This result was similar with the previous study that was found the application of *Lactobacillus* affected the intestinal *lactobacilli* community of the host but it did not alter the overall microbial structure (Moriarty *et al.*, 2005; Fuentes *et al.*, 2008; Qi *et al.*, 2009).

The PCR-DGGE as one method of detection the microbial community is able to cover the most of the bacterial presence in samples. It is powerful to replace of the bacterial plating count method. The PCR-DGGE method allows a rapid screening of multiple samples and provides information about community alteration in samples by distinguishing from

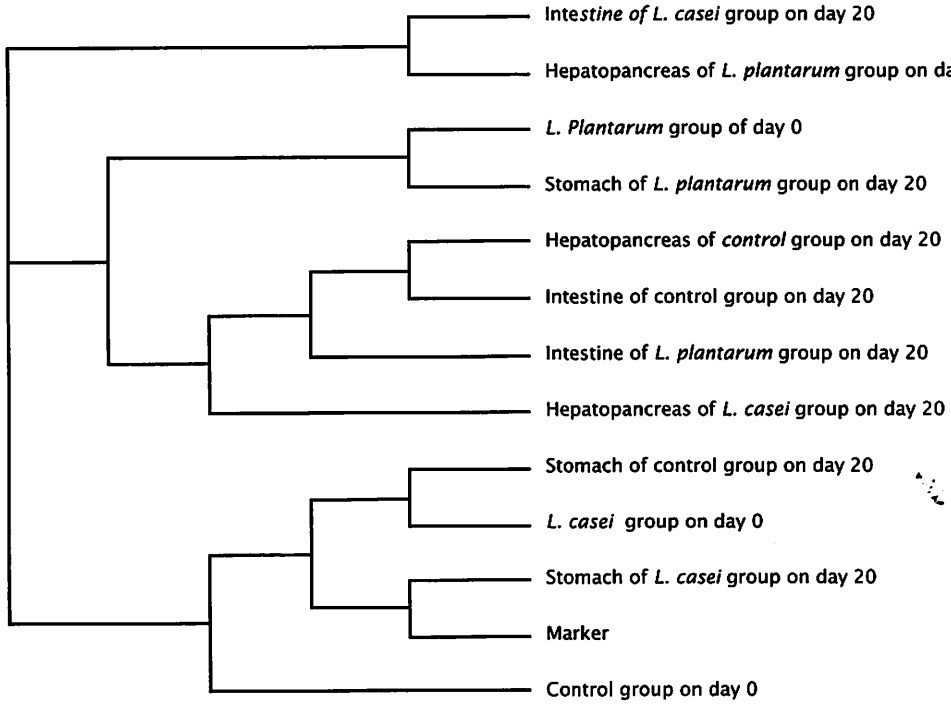

Figure 2. Phylogenetic tree of *L. casei*, *L. plantarum* on day 0 and day 20 in the intestine, stomach, and hepatopancreas of *L. vannamei*

the molecular structure (Torsvik *et al.*, 1998; Riemann *et al.*, 1999).

In present study, PCR-DGGE method were used to identify the bacterial community structure by amplify the V2-V3 region of 16S rDNA and compared the target band with the identification marker. This method is capable to identify the alteration of microbial community within digestive system of *L. vannamei*. Previous study presented that PCR-DGGE method was able to reach the species level, to detect certain species and to examine the phylogenety in sample (Montesi *et al.*, 2005; Donskey *et al.*, 2003; Liu *et al.*, 2010). Although some evidence reveal the sensitivity of PCR-DGGE, but some study present that this method cannot distinguish some species that differed by one to three nucleotides (Plant, 2003).

PCR-DGGE method also cannot figure out the number of the bacteria presence as Colony Forming Unit in one mililiter (CFU mL^{-1}) but it is able to present the result as percentage of the bacterial number. The important of the PCR-DGGE application is detection limit of this method, PCR-DGGE noted the minimum concentration of the species is 10^5 CFU mL^{-1} for the concentration (Theunissen *et al.*, 2004).

CONCLUSION

- *Lactobacillus* administration were able to alter microflora ecosystem within digestive organ of *L. vannamei*.
- PCR-DGGE is one of the reliable method for microflora alteration detection in aquaculture science.

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