UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA FROM GASTROINTESTINAL TRACT OF SNAKEHEAD (Channa striatus, Bloch) AS PROBIOTIC FOR FRESHWATER FISH

SEYED KAMALEDIN ALLAMEH

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BY
SEYED KAMALEDDIN ALLAMEH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

June 2012
In the name of God

“From water everything is alive”
(Quran, Sourah: Al-Anbiya, Vers: 30)
DEDICATION

To all teachers and researchers
Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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June 2012

Chairman: Associate Professor Hassan bin Hj. Mohd. Daud, PhD

Faculty/Institute: Institute of Bioscience

The intensive application of antibiotics to prevent and control the bacterial diseases in aquaculture has resulted in development of antibiotic-resistant bacteria. Therefore, probiotics as an alternative strategy have been suggested to be used as replacement for antimicrobial drugs and growth promoters. Lactic acid bacteria (LAB) constitute a group of G+ve bacteria with a high ability to produce antibacterial compounds and improve fish performance which makes them excellent probiotics. In the present study, snakehead (Channa striatus), an indigenous commercial freshwater fish in Southeast Asia countries was chosen for the isolation of LAB. Isolates obtained on de Man Rogosa and Sharp (MRS) agar and broth showed 27 and 9 pure isolates from the fingerling and adults fishes, respectively. According to the antagonistic plate test against Aeromonas hydrophila as a main freshwater fish pathogen, five LABs i.e. three isolates from the fingerlings and two from the adults that showed the greatest inhibition zone were selected. The selected LABs were identified as Aerococcus
viridans, Lactobacillus delbreuckii sp. delbreuckii and Enterococcus faecalis from fingerlings and Lactobacillus fermentum and Leuconostoc mesenteroides sp. mesenteroides from adult fish. Probiotic property evaluation of LAB candidates showed that they could survive and grow at pH 3 up to 8 (P < 0.05). They could also tolerate bile salt concentrations from 0.0 to 0.3% (P < 0.05). The LABs were active at different levels of NaCl (0.0 to 4%) and also, at various temperatures ranging from 15 °C to 45 °C, but showed no growth at 10 °C and 50 °C. Antagonistic effects against three tested fish pathogens i.e. A. hydrophila, Pseudomonas aeruginosa and Shewanella putrefaciens indicated that En. faecalis, L. fermentum and Leu. mesenteroides sp. mesenteroides had the highest inhibition activities. The antibiotic sensitivity test showed that En. faecalis had more antibiotic’s resistance property against some antibiotics as compared to other LAB (P < 0.05). According to probiotic characterization as screening, En. faecalis, L. fermentum and Leu. mesenteroides were selected for in vivo experiments. The viability of LABs in feed stored at 4 °C was higher than 25 °C during four weeks storage. Significant increased of the LAB proportion was observed in the fish intestine fed LAB-fortified diets as compared to the control group (P < 0.05). In addition, the lower G-ve bacteria population in the fish intestine was observed for experimental groups as compared to the control group. Effects of mono and multi-species of LAB candidates on the body composition of Javanese carp (Puntius gonionotus) indicated that LAB-fortified diets could not seriously affect the chemical composition of experimental fish carcasses. The survivability was the same for all experimental groups. The concentration of 10⁷ cfu/g diets was overall more effective than 10⁵ and 10⁹ cfu/g diets. The use of En.
faecalis as probiotic had more positive effects than L. fermentum and Leu. mesenteroides on growth, feed conversion ratio, specific growth rate, and protein efficiency ratio (P < 0.05). Moreover, the use of En. faecalis resulted in an increase in the production of protease and lipase enzymes in the digestive tract of Javanese carp. Results obtained from the short chain fatty acid determination which included acetate, propionate and butyrate showed that treated group with En. faecalis could significantly (P < 0.05) enhance the propionate and butyrate production as compared to the control. An in vivo challenge test of A. hydrophila as a fish pathogen with En. faecalis as a probiotic on Javanese carp indicated that this probiotic could protect the fish against A. hydrophila and showed a higher survivability compared to the control. The presence of En. faecalis in the diet could affect immune response to enhance the antibody level as a humoral response. The fish treated with a LAB-fortified diet (En. faecalis) and added pathogen (A. hydrophila) into the water showed the highest antibody level as compared to the control group (P > 0.05). In conclusion, LABs can be normal microbiota in the gastrointestinal tract of the snakehead. In addition, the use of En. faecalis as a probiotic had positive effects on overall fish performance, digestive enzymes and short chain fatty acid production, survivability in challenge with pathogen and increased humoral immune response in Javanese carp. This LAB as an environmental friendly agent can be suggested as an alternative to antibiotics in freshwater fish culture.
PEMENCILAN DAN PENCIRIAN BAKTERIA ASID LAHTIK DARI SALURAN GASTROUSUS IKAN HARUAN (*Channa striatus*, Bloch 1793) SEBAGAI PROBIOTIK UNTUK IKAN AIR TAWAR

Oleh

SEYED KAMALEDDIN ALLAMEH

Jun 2012

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Penggunaan antibiotik secara intensif bagi mengelak dan merawat penyakit bakteria dalam akuakultur telah menyebabkan terjadinya bakteria yang rentang antibiotik. Oleh itu probiotik adalah strategi alternatif yang telah disyorkan sebagai pengganti kepada dadah antimikrobe, penggalak pertumbuhan dan peransang imuniti. Bakteria asid laktik (BAL) terdiri dari kumpulan bakteria G+ve yang mempunyai ciri morfologikal, metabolik dan fisiologi yang menjadikan mereka probiotik yang amat baik. Di dalam kajian ini, ikan haruan (*Channa striatus*), sejenis ikan tempatan berkomersial di negara Asia Tenggara telah dipilih untuk pemencilan BAL. Percambahan koloni di atas agar de Man Rogosa and Sharp (MRS) dan kaldu memperolehi 27 isolat tulin dari ikan bersaiz jari dan sembilan dari ikan dewasa. Keputusan menunjukkan beberapa BAL yang lazim dalam perut dan saluran usus kedua-dua sais ikan. Mengikut ujian plat antagonis melawan *Aeromonas hydrophila*.
sebagai patogen ikan, lima BAL yakni tiga isolat dari ikan bersaiz jari dan dua dari ikan dewasa menunjukkan zon kesekatlakuan terbesar. Isolat tersebut dikenalpasti sebagai *Aerococcus viridans*, *Lactobacillus delbreuckii* sp. *delbreuckii* dan *Enterococcus faecalis* dari ikan bersaiz jari, dan *Lactobacillus fermentum* dan *Leuconostoc mesenteroides* sp. *mesenteroides* dari ikan dewasa. Penilaian sifat-sifat calon probiotik BAL menunjukkan bahawa mereka boleh hidup dan tumbuh pada pH 3 sehingga pH 8 (P < 0.05). Mereka juga boleh tahan dalam garam hampedu dari kepekatan 0.0 ke 0.3% (P < 0.05). BAL aktif pada beberapa paras NaCl berlainan (0.0 to 4%) dan juga pada suhu berjulat dari 15 °C to 45 °C, tetapi tiada pertumbuhan pada 10 and 50 °C. Kesan antagonis terhadap tiga patogen ikan iaitu *A. hydrophila*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens* menunjukkan *En. faecalis*, *L. fermentum* dan *Leu. mesenteroides* sp. *mesenteroides* mempunyai aktiviti kesekatlakuan tertinggi. Ujian kepekaan antibiotic menunjukkan *En. faecalis* mempunyai lebih banyak kerentangan terhadap beberapa antibiotik dibandingkan dengan LAB lain (keertian pada P < 0.05). Berdasarkan pencirian probiotik, *En. faecalis*, *L. fermentum* and *Leu. mesenteroides* telah dipilih untuk eksperimen in vivo. BAL terpilih dengan kesan keertian (P < 0.05) menunjukkan kebolehidupan tinggi dalam diet disimpan pada 4 °C and 25 °C. Tambahkan lagi, BAL juga dapat merendah percambahan populasi bakteria G-ve dalam saluran gastrousus. Pertambahan yang bererti BAL dapat dilihat dalam usus (P < 0.05). Kesan calon BAL secara tunggal dan pelbagai terhadap komposisi badan ikan lampam jawa (*Puntius gonionotus*) menunjukkan bahawa diet yang ditambah dengan BAL tidak memberi kesan jelas terhadap ke atas komposisi kimia karkas ikan ujian. Selain itu tiada kematian yang
dilihat. Oleh itu BAL yang dipilih adalah tidak berbahaya dan tidak memberi kesan sampingan terhadap ikan ujian. Diet berkepekatan $10^7$ cfu/g pada keseluruhannya adalah lebih efektif dari kepekatan $10^5$ dan $10^9$ cfu/g. Walaubagaimana pun, data yang diperolehi menunjukkan penggunaan En. faecalis sebagai probiotik memberi lebih kesan positif dari L. fermentum and Leu. mesenteroides terhadap pertumbuhan, nisbah pertukaran makanan, kadar pertumbuhan tentu dan nisbah kecekapan protein (keertian pada P < 0.05). Tambahan lagi, penggunaan En. faecalis sebagai probiotik menyebabkan peningkatan pengeluaran enzim protease dan lipase dalam saluran penghadaman ikan lampam jawa (P. gonionotus). Juga bakteria ini lebih efektif merembeskan enzim protease dari lipase. Keputusan dari penentuan asid lemak berantai pendek termasuk asetat, propionat dan butirat menunjukkan kumpulan yang dirawat dengan En. faecalis boleh, secara bererti (P < 0.05) meningkatkan pengeluaran propionat and butirat berbanding dengan kawalan. Ujian cabaran in vivo A. hydrophila sebagai patogen ikan dengan En. faecalis sebagai probiotik ke atas lampam jawa menunjukkan probiotik ini boleh melindungi dari jangkitan dan menunjukkan kesan penghalang yang efektif terhadap patogen ini. Keputusan dari gerak balas sistem imun menunjukkan kehadiran En. faecalis dalam diet boleh merangsang gerak balas peningkatan paras antibodi sebagai keimunun humoral. Kumpulan ikan dengan diet yang ditambah dengan BAL dan bersama patogen (A. hydrophila) dalam air menunjukkan paras antibodi tertinggi berbanding dengan kawalan (P > 0.05). Pada kesimpulannya, BAL boleh menjadi mikrobiota normal dalam saluran gastrousus ikan haruan. Tambahan lagi, penggunaan En. faecalis sebagai probiotik memberi kesan positif ke atas keseluruhan perkembangan ikan.
enzim pencernaan dan pengeluaran asid lemak berantai pendek, kemandirian dalam cabaran dengan patogen dan peningkatan ransangan imun humoral di lampam jawa. Bakteria asid laktik ini sebagai satu agen yang mesra alam boleh disyorkan sebagai alternatif kepada penggunaan antibiotik dalam kultur ikan air tawar.
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Lastly, even though there are many hardships on our way to scientific career, it feels great to Learn, Know and Understand.
I certify that a Thesis Examination Committee has met on 20 June 2012 to conduct the final examination of Seyed Kamaleddin Allameh on his thesis entitled “Isolation and Characterization of Lactic Acid Bacteria from Gastrointestinal Tract of Snakehead (Channa striatus, Bloch) as Probiotic for Freshwater Fish” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

SEYED KAMALEDIN LLAMEH

Date: 20 June 2012
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