UNIVERSITI PUTRA MALAYSIA

EFFECTS OF RECOMBINANT *LACTOCOCCUS LACTIS* AND BACTERIOCIN UL4 IN THE PROTECTION OF TILAPIA *(OREOCHROMIS NILOTICUS)* AGAINST *AEROMONAS HYDROPHILA*

ANURADHA KARUNAKARAMOORTHY

FBSB 2009 6

EFFECTS OF RECOMBINANT *LACTOCOCCUS LACTIS* AND BACTERIOCIN UL4 IN THE PROTECTION OF TILAPIA *(OREOCHROMIS NILOTICUS)* AGAINST *AEROMONAS HYDROPHILA*

By

ANURADHA KARUNAKARAMOORTHY

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,

In Fulfilment of the Requirements for the Degree of Master of Science

April 2009

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

EFFECTS OF RECOMBINANT *LACTOCOCCUS LACTIS* AND BACTERIOCIN UL4 IN THE PROTECTION OF TILAPIA *(OREOCHROMIS NILOTICUS)* AGAINST *AEROMONAS HYDROPHILA*

By

ANURADHA KARUNAKARAMOORTHY

April 2009

Chairman: Foo Hooi Ling, PhD

Faculty: Faculty of Biotechnology and Biomolecular Sciences

The study was conducted to determine the effects of constructed recombinant *Lactococcus lactis* and bacteriocin UL4 for the protection of tilapia against *Aeromonas hydrophila*. For the constructions of recombinant *L. lactis*, a 250 bp domain 1 and 750 bp domain 4 of aerolysin produced by *A. hydrophila* were amplified by PCR and individually cloned into pNZ8048. The constructed plasmids, designated as pNHD1 and pNHD4, were then electrotransformed into *Lactococcus lactis*. Total RNA was then extracted and subjected to reverse transcriptase PCR. The agarose gel electrophoresis results showed the expected bands of pNHD1 and pNHD4 with 268 bp and 768 bp respectively. Subsequently, whole cell protein of recombinant *L. lactis* was extracted and separated by SDS-PAGE prior to Western blot analysis. The results of immunoblots using specific polyclonal antibodies showed that both domains 1 (~9 kDa) and 4 (~30 kDa) were successfully expressed in *L. lactis*.

On the first fish trial, tilapia was injected intraperitoneally using recombinant *L. lactis*. Growth performance of tilapia with recombinant *L. lactis* was more profound and ELISA results showed a significantly higher antibody level ($P<0.05$) compared to control groups. The survival rate after challenge was more than 80 % for recombinant *L. lactis* groups, whereas only 60 % was observed for control group. Lactic acid bacteria (LAB) count of intestine digesta of fish that survived was maintained at high count (> 6 log cfu/ml) compared to control. On the other hand, the Enterobacteriaceae and *A. hydrophila* count were maintained at low count (< 6 log cfu/ml) after the trial. For the second trial, tilapia was orally immunized using recombinant *L. lactis* for four weeks. The growth performance of fish with recombinant *L. lactis* was more profound than control fish, even after challenged with *A. hydrophila*. Moreover, the antibody level increased significantly in week 2 in fish serum fed with recombinant *L. lactis* compared to control. The survival rate of tilapia after challenge was 100 % for recombinant *L. lactis*. The Enterobacteriaceae and *A. hydrophila* count of intestine digesta of survived fish was maintained at low count (< 5 log cfu/ml) compared to control, whereas the LAB count were maintained at more than 4 log cfu/ml.

The best bacteriocin producer from six strains of *L. plantarum* isolated from local foods was identified and bacteriocin UL4 was selected based on antimicrobial activity determined by the diameter of the inhibition zone of *A. hydrophila*. Oral feeding was carried out and better growth performance was observed in bacteriocin UL4 fed tilapia compared to control. ELISA results showed the antibody level increased significantly in week 3 in fish serum fed with bacteriocin compared to

control. The survival rate after challenge was 100 % and only 45 % for bacteriocin fed fish and control fish respectively. Enterobacteriaceae and *A. hydrophila* count of intestine digesta of survived fish maintained at low count (< 5 log cfu/ml) compared to control, whereas LAB count were maintained at high count (> 6 log cfu/ml).

The results obtained in this study indicate the vast potential of recombinant *L. lactis* as a promising vaccine to prevent the infection of *A. hydrophila* particularly and generally to reduce the extensive use of antibiotics in controlling diseases and for the overall improvement of the health of fishes. The bacteriocin from LAB also showed good effects on the health improvement of fish and could be an ideal alternative to be used as a supplement for a general protection and prevention of diseases.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN REKOMBINAN *LACTOCOCCUS LACTIS* DAN BAKTERIOSIN UL4 UNTUK PERLINDUNGAN BAGI IKAN TILAPIA, *OREOCHROMIS NILOTICUS* TERHADAP *AEROMONAS HYDROPHILA*

Oleh

ANURADHA KARUNAKARAMOORTHY

April 2009

Pengerusi: Foo Hooi Ling, PhD

Fakulti: Fakulti Bioteknologi dan Sains Biomolekul

Kajian ini dijalankan untuk menentukan kesan rekombinan *Lactococcus lactis* dan bakteriosin UL4 untuk perlindungan ikan tilapia terhadap *Aeromonas hydrophila*. Dalam kajian ini, 250 bp domain 1 dan 750 bp domain 4 aerolysin telah diamplifikasi dengan PCR dan diklon dalam pNZ8048 secara berasingan. Plasmid yang telah dihasilkan, iaitu pNHD1 dan pNHD4 dielektrotransformasikan ke dalam *Lactococcus lactis*. Selepas itu, RNA diekstrak dan digunakan untuk transkrip terbalik PCR dan keputusan gel agaros menunjukkan jalur pada saiz yang dijangka bagi pNHD1 dan pNHD4 dengan 268 bp dan 768 bp masing-masing. Sejurus itu, protein sel rekombinan *L. lactis* diekstrak dan dipisahkan menggunakan SDS-PAGE sebelum kajian Western Blot dilakukan. Western Blot dengan mengunakan spesifik antibodi poliklonal menunjukkan kedua-dua domain 1 (~ 9 kDa) dan 4 (~30 kDa) telah berjaya diekspres dalam *L. lactis*.

Bagi experimen yang pertama, ikan tilapia telah disuntik secara intraperitoneal dengan mengunakan rekombinan bakteria *L. lactis*. Kadar pertumbuhan tilapia dengan mengunakan rekombinan *L. lactis* lebih tinggi dan keputusan ELISA menunjukkan tahap antibodi meningkat dengan signifikan ($P<0.05$) bagi rekombinan *L. lactis*. Kadar hidup tilapia selepas dicabar, adalah melebihi 80 % bagi kumpulan rekombinan *L. lactis*, manakala hanya 60 % untuk rawatan kawalan. Kiraan LAB dalam digesta ikan dikekalkan pada tahap tinggi (> 6 log cfu/ml) berbanding rawatan kawalan dan kiraan Enterobacteriaceae dan *A. hydrophila* dikekalkan pada tahap rendah (< 6 log cfu/ml). Bagi eksperimen kedua, ikan tilapia diberi vaksin secara oral untuk 4 minggu. Kadar pertumbuhan tilapia dengan mengunakan rekombinan *L. lactis* lebih tinggi daripada rawatan kawalan, walaupun selepas dicabar dengan *A. hydrophila*. Keputusan ELISA menunjukkan tahap antibodi meningkat dengan signifikan ($P<0.05$) bagi rekombinan *L. lactis* pada minggu kedua berbanding dengan rawatan kawalan. Kadar hidup selepas dicabar dengan *A. hydrophila* adalah 100 % bagi rekombinan *L. lactis*. Kiraan Enterobacteriaceae dan *A. hydrophila* dalam usus ikan dikekalkan pada tahap rendah (< 5 log cfu/ml) berbanding kontrol. Manakala, kiraan LAB dikekalkan pada tahap tinggi (> 4 log cfu/ml).

Bakteriosin yang terbaik daripada enam strain *Lactobacillus plantarum* di kenalpasti dan bakteriosin UL4, dipilih berdasarkan aktiviti bakteriosin dan diameter zon perencatan dengan *A. hyrophila*. Pemakanan oral diberikan dengan mengunakan bakteriosin dan kadar pertumbuhan tilapia dengan mengunakan bakteriosin lebih tinggi daripada kawalan, walaupun selepas dicabar dengan *A. hydrophila*. Keputusan ELISA menunjukkan tahap antibodi meningkat dengan signifikan ($P<0.05$) bagi bakteriosin pada minggu ketiga berbanding dengan rawatan kawalan. Kadar hidup

tilapia selepas dicabar dengan *A. hydrophila* adalah 100 % bagi bakteriosin manakala hanya 45 % bagi kawalan. Kiraan Enterobacteriaceae dan *A. hydrophila* dalam digesta ikan dikekalkan pada tahap rendah (< 5 log cfu/ml) dan kiraan LAB dikekalkan pada tahap tinggi (> 6 log cfu/ml) berbanding kawalan.

Keputusan yang diperolehi menunjukan potensi rekombinan *L. lactis* sebagai vaksin bagi mencegah jangkitan daripada *A. hydrophila* khususnya dan juga mengurangkan pengunaan antibiotik bagi mengawal penyakit. Manakala, bakteriosin dari LAB menunjukan kesan yang baik bagi memperbaiki kesihatan ikan dan boleh menjadi alternatif sebagai makanan tambahan untuk mencegah jangkitan secara umum.

ACKNOWLEDGEMENTS

I would like to express my most sincere appreciation to my supervisor, Assoc. Prof. Dr. Foo Hooi Ling for her invaluable guidance, patience and continuous encouragement throughout my study. I'm also immensely grateful for the advice and support given by my supervisory committee members, Prof. Dr. Raha Abdul Rahim and Assoc. Prof. Dr. Mariana Nor Shamsudin. I would like also to thank Assoc. Prof. Dr. Loh Teck Chwen for his helpful comments and guidance.

I would like to convey warm thanks to my labmates especially Yin Sze, Tze Young, Sabrina, Kak Yan, Yaya, Deela, Shahrul, Boon Hooi and Hossein for their help. I would also wish to express my gratitude to Mr. Perumal from MARSLAB for his advice and technical assistance.

Last but not least, thanks to my family and my fiancé, Mr. Nageswaran for their love and care.

DECLARATION

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

_____

ANURADHA KARUNAKARAMOORTHY

Date:

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percentage
Ω	ohm
A	absorbance
AU	arbitrary unit
bp	base pair
BSA	bovine serum albumin
CFU	colony forming unit
D1	domain 1
D4	domain 4
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EDTA	ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Eosin Methylene Blue Agar
EtBr	ethidium bromide
FCR	feed conversion ratio
GSP	Glutamate Starch Phenol Red Agar
h	hour
kDa	kiloDalton
LAB	lactic acid bacteria
LB	Luria Bertani
M	molarity

min	minute
mM	milimolar
MRS	de Man, Rogosa and Sharp agar
N	normality
ng	nanogram
nm	nanometer
° C	degree Celcius
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RE	restriction enzyme
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SGR	specific growth rate
T	treatment
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus*
TEMED	tetramethyl-ethylene diamine
U	unit
µl	microlitre
µg	microgram
UV	ultra-violet
v	volt
v/v	volume per volume
w/v	weight per volume

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Foo Hooi Ling, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mariana Nor Shamsudin, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____
HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 June 2009

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Lactic acid bacteria (LAB) are Gram positive and nonspore forming cocci or rods, which produce lactic acid as their main metabolic product. The genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* are important members of this group. LAB are commonly found in foods, including fermented meat, vegetables, fruits, beverages and dairy products, but also in the respiratory, intestinal and genital tracts of humans and animals, in sewage and in plant materials (de Vuyst and Vandamme, 1994). The importance of LAB is increasing due to its classification as “generally regarded as safe” organism (GRAS) (Gatesoupe, 1999).

LAB and their biotechnological exploitations have received vast attention for the last two decades. One significant example is the application of protein engineering techniques in LAB, notable examples being the lactococcal proteinase (Vos *et al*., 1990), the lactose repressor gene of *Lactococcus lactis* (van Rooijen *et al*., 1993) and the lantibiotic nisin (Kuipers *et al*, 1992). Bacteriocins in general have been characterized in considerable detail, for instances the additional lantibiotics such as lactocin S from *Lactobacillus sake* (Nes *et al*., 1993) and the emergence of a group of related 'small heat stable bacteriocins' which produces by several species of LAB (Marugg *et al*., 1992). Bacteriocins can be used as a ‘target drugs’ towards specific pathogens without disturbing the beneficial microbiota. Hence, they could then limit the use of antibiotics to some extent and thus delay the evolution of antibiotic resistance. Bacteriocins are naturally produced, so they are more easily accepted by

consumers. Interest in bacteriocins is to some extent fuelled by their potential as novel biopreservatives and several have been shown vast potential in this regard.

LAB that use as live organisms in food fermentations may be the suitable microoganisms for live vaccine delivery vectors. For example, *L. lactis* is a food-grade, non-pathogenic, non-invasive and non-colonizing bacterium that has the potential to deliver vaccine antigen effectively (van Rooijen *et al*., 1993). Recently, recombinant strains of *L. lactis* have been developed to deliver cytokines and specific antigens across mucosal surfaces to the immune system of animals (Nga, 2005). Protein secretion by this GRAS bacterium would allow production directly in a food product and interaction between the secreted protein (enzyme or antigen) and the environment (the food product itself or the digestive tract). High level production of heterologous proteins in *L. lactis* has been obtained using lactococcal constitutive or inducible promoters (Kuipers *et al.*, 1997; de Vos, 1999).

To develop efficient vaccines, two components are essential: the bacterial vector strain and a well-adapted antigen presentation system. Ideal mucosal vaccines should promote an effective contact between the antigen and the immune system, stimulate humoral and cellular immune responses, produce long term protection after a single dose, stable and non-toxic (Jennings *et al.*, 1998). To circumvent some of the safety and environmental issues inherent to the wide-scale dissemination of engineered pathogens, non-pathogenic Gram-positive LAB vectors have been developed (Ribeiro *et al.*, 2002). In addition to their GRAS status, some LAB are able to stimulate the immune system of the hosts as adjuvants due to their probiotic properties and their immunomodulation capacity (Shu and Gill, 2002). The

combinations of these properties makes LAB to be very advantageous live vaccines and many studies are under way to express antigens in LAB and to evaluate the effect of this antigen presentation system on the immune system.

The research of LAB for aquatic animals is increasing with the demand for evolution of aquaculture approach to “environment-friendly” or “Green agriculture”. Aquaculture plays an important role in global food supply, food security and the development of national economies. In Malaysia, fish production is expected to increase from 1.48 million metric tonnes in 2000 to 1.93 million metric tonnes by 2010 (Liaw and Fung, 2000). The fact that national marine capture fisheries already have an upper limit of production. It means that the demand must be met by the aquaculture industry. The main drawbacks identified in aquaculture industry are the nutrition and disease problems, which are the main problems of the unsatisfactorily production of aquaculture.

Specific bacterial pathogens can be an important cause of mortalities in fish hatcheries, as intensive husbandry practice often result in breakdown of the natural host barriers. One of the difficulties of intensive fish culture is the control of diseases caused by pathogens such as *Aeromonas hydrophila*, one of the most common bacteria in freshwater. Possible consequences of *A. hydrophila* infection to fish are skin lesions, which can result in haemorrhagic septicaemia and followed by high mortalities (Rahman and Kawai, 1999). However, the indiscriminate use of antibiotics in disease control in many sections of the aquaculture industry has led to selective pressure of antibiotic resistance in bacteria, a property that may be readily transferred to other bacteria (Sorum, 1999). It also poses a significant risk to

consumer's health through the potential transfer of resistance to human pathogens, antibiotic residues or chemical contaminants in marketed aquaculture products. Further, widespread use of antibiotics also places the production environment at risk (Sahoo and Mukherjee, 1999). Hence, the use of live microbial feed supplement which benefit the host by modifying the host-associated or ambient microbial community, by enhancing the host response towards disease, by ensuring improved use of feed or enhancing its nutritional value or by improving the quality of its ambient environment (Vershuere *et al.*, 2000) in aquaculture is being encouraged.

The exact mode of action of the probiotic bacteria has not been fully elucidated, nevertheless it is thought to be mediated through the production of inhibitory compounds, competition for chemicals or available energy or for adhesion sites besides enhancing immune responses. Very little is known about the relative importance of these mechanisms. In addition, only Villamil *et al.*, (2002) and Rengpipat *et al*., (2000) reported the immune responses in aquatic animals after probiotic supplementation. On the other hand, during the last decade the application of probiotics taking advantage of its pathogen control potential has been increasing in aquaculture. According to Gudding *et al.*, (1999), stimulation of the specific and non-specific immunity is the basis for developing aquaculture into sustainable bioproduction in aquatic ecosystems.

A need to overcome this problem has arisen and possible solutions can be found by using LAB as feed supplement or as vaccine delivery vehicle. Although the promising prospects of LAB have been extensively reported, but considerable research on recombinant LAB carrying specific antigen epitopes of pathogen as live

vaccines and the potential of LAB metabolites on aquaculture have not been conducted. Therefore, this study was conducted to investigate the use of recombinant *Lactococcus lactis* and bacteriocin UL4 for the protection of tilapia, *Oreochromis niloticus*, against *Aeromonas hydrophila* and the specific objectives of this study were:

i) To construct recombinant *L. lactis* harboring aerolysin domains 1 and 4 of *A. hydrophila*.

ii) To determine the efficacy of *L. lactis* recombinants harbouring the constructs of aerolysin domains 1 and 4 of *A. hydrophila* as vaccine for tilapia via intraperitoneal injection.

iii) To determine the efficacy of *L. lactis* recombinants harbouring the constructs of aerolysin domains 1 and 4 of *A. hydrophila* as oral vaccine for tilapia.

iv) To determine the effect of bacteriocin UL4 as feed supplement for tilapia.

CHAPTER 2

LITERATURE REVIEW

2.1 Aeromonads

Aeromonads are ubiquitous, oxidase-positive, facultatively anaerobic, glucose-fermenting, Gram-negative bacteria that are native to aquatic environments (Hazen *et al.,* 1978). They have been found in brackish, fresh, estuarine, marine, chlorinated and unchlorinated water supplies worldwide, with the highest numbers obtained in the warmer months (Van der Kooj *et al.,* 1988; Kaper *et al.,* 1981 and Hazen *et al.,* 1978). Aeromonads have been isolated from diseased cold and warm blooded animals for over 100 years and from humans since the early 1950s (Mathewson and Dupont, 1992).

The motile aeromonads, as the group appellation suggests, are characterized by active motility, achieved by means of a single polar flagellum, and production of gas, as well as acid from carbohydrates. They are bacilli or cocci-bacilli measuring 0.5 µm × 1.0 - 1.5 µm. The optimum growth temperature for motile aeromonads is 28 °C but depending upon the species, it varies with a very wide temperature growth range (< 4 °C to 45 °C) and optimal pH value around 6.5 - 7.5 with a range of 5.2 to 9.8 pH tolerability (Anjana *et al.,* 2005).

The genus *Aeromonas* has undergone a number of taxonomic and nomenclature revisions (Chacon *et al.,* 2002). Although originally placed in the family which also