ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM BLACKTIP SHARK (*Carcharhinus limbatus*) WITH PROBIOTIC POTENTIAL

BY

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ABSTRACT

A complex and diverse range of microorganism communities live within the gastrointestinal (GI) tract of living organisms. Amongst the bacterial communities in the GI tract, lactic acid bacteria (LAB) is generally regarded as safe, and probiotic due to their inherent benefits in improving the overall host GI tract, limiting intestinal infections, combatting pathogenic bacteria and controlling some types of cancer. In this study, 80 isolates were obtained from brain, epidermal organ, stomach and the end of intestinal tract of Blacktip shark (*Carcharhinus limbatus*). Preliminary inhibition test using spot-on-lawn assay was conducted to eliminate the isolate with no antagonistic activity against selected indicator strains. Out of these 80, only 4 isolates showed antagonistic activities against selected indicator strains in which they were selected for further biochemical tests such as Gram-staining, catalase, oxidase, and motility, the were found to be Gram positive coccoid shape, lactose fermentation positive, and negative to both oxidase and catalase tests. The isolates FA1, FA2, FA3 and FA4 demonstrated broad range of activity spectrum by inhibiting the Gram-positive indicator strains (*Staphylococcus aureus* and *Bacillus cereus*) and the Gram-negative indicator strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *salmonella typhimurium*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus*); with the highest inhibition zone recorded at 20 ± 0.1 mm on *V. parahaemolyticus* using agar well diffusion method as a confirmatory test. The isolates were chosen for further characterization using 16S rRNA PCR amplification. Each sequence was respectively deposited in GenBank (NCBI) with an accession number: MN975529 for FA1, MN982712 for FA2, MN982711 for FA3 and MN982710 for FA4. These isolates were identified to be belonged to member of the *Lactococcus lactis* group with 98% homology. The cell free supernatant (CFS) of the isolates was harvested for further tests. Treatment of the CFS with pH and catalase ruled out any inhibition caused by organic acids or hydrogen peroxide (H_2O_2) and was considered as crude bacteriocin. Complete inactivation of the isolates CFS antimicrobial activity was observed after treatment with proteinase K, thus proving its proteinaceous nature. This is one of a very few reports on LAB strains isolated from the Blacktip shark (*Carcharhinus limbatus*) producing antimicrobial proteins or bacteriocin. These isolated strains could prove to be a very useful probiotic strain in aquaculture or animal feed as a mean to fight the ever-growing problem of drug resistant pathogenic bacteria as well as, promoting growth and the overall host health.

خلاصة البحث

تعيش مجموعة معقدة ومتنوعة من مجتمعات الكائنات الحية الدقيقة داخل الجهاز الهضمي للكائنات الحية، وتبقى هناك ضمن هذه المجتمعات التي تستعمرالجهاز الهضمي. تعتبر بكتيريا حمض اللاكتيك (LAB) وبروبيوتيك آمنة بشكل عام بسبب فوائدها في تحسين الجهاز الهضمي والحد من الالتهابات المعوية، ومكافحة البكتيريا المسببة للأمراض داخل العائلة والسيطرة على بعض أنواع السرطان. في هذه الدراسة تم الحصول على 80 عينة من الدماغ والمعدة ونهاية المسالك المعوية لقرش.(*Carcharhinus limbatus*) Blacktip تم إجراء اختبار التثبيط الأولي باستخدام spot-on-lawn assayللقضاء على العينة مع وجود نشاط ضد البكتيريا المختارة كمؤشر. من بين 80 عينة فقط أظهرت 4 أنشطة معاكسة ضد البكتيريا المختارة كمؤشر. تم اختيارها لمزيد من الاختبارات الكيميائية مثل صبغة الجرام ،اختبار الكاتالاز ، اختبار أوكسيديز، واختبار الحركة. تم العثور على شكل كروي الإيجابي، وتخمر اللاكتوز إيجابي، وسالب لكل من اختبارات الأكسدة والكاتالاز. أظهرت العينات FA1 و FA2 و FA3 و FA4 نطاقًا واسعًا من طيف النشاط عن طريق تثبيط البكتيريا المأخوذة كمؤشرات إيجابية الجرام *Staphylococcus aureus* و *Bacillus cereus*و سلبية الجرام و *Pseudomonas aeruginosa* و *Salmonella typhimurium*و *Vibrio alginolyticus* ، حيث سجلت أعلى منطقة تثبيط عند 20 سم على *V. parhaemolyticus*باستخدام طريقة agar well diffusion method كاختبار تأكيدي. تم اختيارالعينات لتوصيفها باستخدام 16 s rRNA PCR. تم وضع التسلسل الجيني في GenBank (NCBI)مع أرقام الانضمام FA1 MN975529 :و FA2 MN982712و FA3 MN982711 و FA4 MN982710. وبالتالي، تم تحديد العينات بشكل إيجابي كأعضاء في مجموعة *Lactococcus lactis* بنسبة 98 ٪ من التماثل. تم جمع (CFS) لمزيد من الاختبارات. استبعد علاج CFS مع (H2O2) والكاتالاز الناجم عن الأحماض العضوية أو بيروكسيد الهيدروجين (H2O2) واعتبر بكتيريوسين خاماً . لوحظ إبطال نشاط عينات CFS المضادة للميكروبات بعد المعالجة بالبروتيناز k ، مما يثبت طبيعتها البروتينية. أنتجت هذه السلالات من الكائنات الحية المجهرية المأخوذة من سمك القرش الأسود (*Carcharhinus limbatus*) بروتينًا مضادًا للميكروبات أو بكتيريوسين. يمكن أن تكون هذه السلالات المعزولة سلالة بروبيوتيك مفيدة للغاية في تربية الأحياء المائية أو الأعلاف الحيوانية كوسيلة لمكافحة المشكلة المتزايدة باستمرار للبكتيريا المسببة للأمراض المقاومة للأدوية، وكذلك لتعزيز النمو والصحة العامة للعائل.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology).

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I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

LAB	Lactic Acid Bacteria
AMP	Antimicrobial Peptide
ATCC	American Type Collection
GRAS	Generally Recognized as safe
MRS	Man Ragosa and Sharpe
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
rRNA	Ribosomal RNA
EtBr	Ethidium Bromide
NA	Nutrient Agar
CFS	Cell Free Supernatant
BLIS	Bacteriocin-Like Inhibitory Substance
UV/Vis	Ultraviolet/Visible
GIT	Gastrointestinal Tract
DNA	Deoxyribonucleic Acid

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Lactic Acid Bacteria (LAB) are a diverse group of bacteria that belongs to the phylum Firmicutes and consists of 11 genera. LAB are characterized as Gram-positive, non-motile, non-sporulating and either rods or coccus shaped (Khalid, 2011). During carbohydrates fermentation LAB produce energy and lactic acids (Jay, 2000). LAB are also known to produce diacetyl, hydrogen peroxide, organic acids and antimicrobial peptides or bacteriocins as a by-product from their fermentation process (Oyetayo et al., 2003). LAB are widespread in almost any environment and can commonly be found in milk, fermented dairy products, fermented beverages, fermented vegetables and the intestinal tracts of humans and animals (Shivsharan and Bhitre 2013). LAB is generally regarded as safe and probiotic (Hegarty et al., 2016). “Pro Bios” is a Greek word that means “in support of life“, the World Health Organization (WHO) define Probiotics as “Live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host” Many probiotics are used in our daily life such as the Lactic Acid Bacteria (LAB) and yeast (Hegarty et al., 2016).

According to Klaenhammer (1988), 99% of all bacteria may produce at least one anti-microbial peptide (AMP) or bacteriocin and the only reason we have not identified them fully is the lack of researchers finding and isolating all of them. AMP produced by LAB was first discovered in 1925 by Gratia while he was searching for methods to kill bacteria, his research has led to the isolation of the first AMP from LAB, which he named Colicine because it killed *Escherichia coli* (*E. coli*) (A. Gratia, 1925). AMP

produced by LAB are defined as ribosomally synthesized peptides which exert bacteriostatic or bactericidal effects on related and non-related bacteria (Cotter, Hill, & Ross, 2005). LAB probiotic traits have several advantages over antibiotics such as a broad spectrum of antimicrobial activity, combating multidrug-resistant bacteria, do not easily induce resistance and is significantly less toxic against the host (Matsuzaki, 2009). Hence, making LAB a promising alternative to antibiotics. As well as combating pathogenic bacteria, LAB is also used to control harmful microbes in the food industry (Joo et al., 2012). LAB can also change the microbiota found in animal intestine and reduce the number of pathogens (Ringø et al., 2018), This make them a great supplement in animal feed and aquaculture.

1.2 PROBLEM STATEMENT

According to the World Economic Forum, the greatest risk to humankind is the emergence antibiotic resistant bacteria (super bug) (O'Niell, 2014). This distressing threat poses a major health risk not only to humans but as well as animals. It has been suggested that the death caused by drug resistant bacteria will rise from the current estimates of 700,000 lives per year to ten million lives annually by 2050 (O'Niell, 2014). Antibiotic resistant bacteria can kill, even with the world's most advanced medical settings (Snitkin et al., 2012). Additionally, the lack of drug development is apparent from the fact that there have been no new classes of antibiotics being discovered since 1984 (Sun, 2016). Pathogenic bacteria are becoming increasingly resistant even to our most clinically available antibiotics and a fatality rate of up to 50% (Borer et al., 2009). Not only bacteria can form resistance to antibiotics, but antibiotics are known to cause side effects such as yeast infection, allergic reaction due to the

sulphate contained in the antibiotics as well as other common side effects including indigestion, nausea, diarrhea, and sensitivity to light.

Thus, the need to develop an alternative to the classical antibiotics which is non-toxic to humans, easily metabolized and does not develop a resistance in the host. LAB are generally recognized as safe (GRAS), due to their prevalent appearances in food products and their probiotic contribution to the healthy microbiota of animals and humans (Sharma et al., 2016). LAB is also known to produce proteinaceous compounds known as bacteriocin which are antimicrobial agents that can potentially be used in treating pathogenic infections as well as overcome the drug resistant bacteria problem (Pingitore et al., 2007, Jiang et al., 2017). LAB plays an important role in the food industry as they are used as a starting culture, co-culture, and bio protective agent against pathogens. LAB also give significant advantages in food quality and safety (Silva et al., 2018). They are also used to inhibit the growth of microbial contaminants in the food industry (Silva et al., 2018).

Diverse marine microorganisms isolated from marine life are considered as an untapped treasure (Leal et al, 2012). In marine ecosystem, cartilaginous fishes (*Chondrichthyes*) are predators/scavengers by nature of which their feeding habits vary by species. Sharks and rays are ancient marine predators belonging to Cartilaginous fishes or *Chondrichthyes*. Sharks are considered apex predators whereas rays are bottom-dwelling feeders. Bigger cartilaginous fish, such as whale sharks, and manta rays feed on tiny plankton. This dietary habit exposes these fishes to pathogenic bacteria that can invade their internal organs and intestinal system. In addition, Ringø et al. (2018) reported that LAB isolated from the fish intestine are antagonistic towards fish pathogens. A study by Ahmad et al., (2013) reported a successful isolation a LAB from the skin of broad headed shark (*Chiloscyllium griseum*) that demonstrated a broad range

of antibacterial activities. Similarly, a study by Jiang et al., (2017) have uncovered a novel bacteriocin produced by LAB isolated from a grey carpet shark intestine active against Multiple Drug-Resistant *Staphylococcus aureus (*MRSA).

Furthermore, there have been few studies on the isolation of LAB from marine life intestinal system especially from shark species. Therefore, it is interesting to understand and characterise the LAB group dwelling in these fishes. The gastrointestinal tract of fish is one of the most important environments to study due to its constant exposure to pathogens. Researching the GI tract of fish could highlight the presence of beneficial probiotic LAB that colonize the host GI tract since early larval stages (Ringø et al., 2018). Those LAB strains could prove to harbour probiotic traits and antagonistic activity against pathogenic bacteria. The aims of this study were to isolate LAB from the cartilaginous fish Blacktip Shark (*Carchahinus limbatus),* biochemically and morphologically characterize the isolates and conduct inhibitory studies against selected pathogenic strains for their antimicrobial activities. A final version describing the outcomes of the work was reported and published (Hamid et al., 2020). These isolates could potentially be used in aquaculture and in animal feed as a probiotic supplement in combating pathogenic bacteria and to improve the overall health of the fish or shrimp host.

1.3 RESEARCH OBJECTIVES

The major aims of this work are to achieve the following:

1. To isolate and identify Lactic Acid Bacteria (LAB) from the intestinal organs of Blacktip shark (*Carcharhinus limbatus*) with antimicrobial properties.
2. To characterize biochemically, morphologically and genotypically the isolated LAB from Blacktip shark (*Carcharhinus limbatus*).
3. To characterize the effect of bacteriocin-like inhibitory substances (BLIS) or antimicrobial peptide (AMP) produced by LAB against the growth of selected pathogenic bacteria strains.

1.4 HYPOTHESIS

This research seeks to conduct further research on the following:

Cartilaginous fish such as Blacktip shark (*Carcharhinus limbatus*) should harbour useful probiotic strains. These strains can serve as a potential probiotic strain in aquaculture and animal feed. LAB that colonize the cartilaginous fishes (*Chondrichthyes*) will be expected to be of LAB producers. The isolates, like many LAB are expected to have the ability to demonstrate broad spectrum of inhibition against both Gram-positive and Gram-negative bacterium. These strains are expected to produce bacteriocin or antimicrobial peptides which can be used in supressing pathogenic bacteria.

CHAPTER TWO

LITERATURE REVIEW

2.1 LACTIC ACID BACTERIA

Lactic Acid Bacteria (LAB) are defined as Gram-positive either Coccus or rods, aerotolerant but non-aerobic with the ability to produce energy and lactic acid as the byproduct of the carbohydrate's fermentation. Lactic acid bacteria are ubiquitous and heterogeneous organisms with the shared factor of producing lactic acid (Mokoena, 2017). LAB can be found in two specific phyla, mainly firmicutes and actinobacteria. In firmicutes phylum, the lactic acid bacteria (LAB) belong to the *Lactobacillales* order and includes the following group: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Carnobacterium*, *Weissella* (Kocková, et al.2011). LAB exists within the actinobacteria phylum, and belong to the Atopobium and Bifidobacterium groups, with a guanine-cytosine content of 36-46% and 58-61%. In addition, the term Lactic Acid Bacteria or LAB does not reflect the phylum class of these bacteria but rather the metabolic speciality of this heterogeneous bacteria group, the most important speciality is the ability to ferment sugars into lactic acid. LAB is also characterized by being Gram positive, catalase negative, non-sporulating organisms that are devoid of cytochromes and of nonaerobic habit but are aero tolerant, fastidious, non-motile, acid tolerant (Ruiz et al., 2019).

2.2 LACTIC ACID BACTERIA PROBIOTIC TRAIT

In 1908, Elie Metchnikoff a Nobel prize winner while working at the Pasteur Institute, observed that a surprising number of Bulgarian peasants lived for more than 100 years (Schepper et al., 2017). He further observed that those peasants consumed large quantities of fermented yogurt, which subsequently led him to isolate beneficial bacteria from the yogurt that conferred health promoting benefits. For thousands of years LAB have been used to ferment foods (Ruiz et al., 2019). Fermented products are the main source of delivering probiotics. Popularly used dairy products includes fermented yogurt and cheese (Giraffa, 2012). Probiotic organisms include LAB belonging to the species of *Lactobacillus acidophilus*, *L. gasseri*, *L. fermentum*, *L. coccus*, *L. plantarum* and *L. lactis* (Castro et al., 2015).

Probiotics has many benefits including balancing the gastrointestinal tract microbiota. Thus, it regulates the intestinal function and effectively preventing infections as well as, treating several cases of gastrointestinal diseases such as, antibiotic-related diarrhea, irritable bowel syndrome, travelers' diarrhea and Crohn's disease (Mandal and Sahi, 2017). Other benefits of probiotic supplemented in food includes, immunomodulatory effect of *L. casei*, *L. reuteri* benefits in reducing cholesterol level (Jones et al., 2012). LAB can also effectively increase vitamin levels in dairy products (Laiño et al., 2013). During diary fermentation process, *Lactobacillus* strains can produce B vitamins which increases the products nutrient value as well as preventing liver and skin disorders due to deficiencies in Vitamin B2 (Gu et al., 2015). Vegetarians who are prone to B12 deficiency because of their lack of animal proteins can alternatively receive it from LAB, as demonstrated by *L. reuteri* that synthesized B12 in soy-yogurt (Gu et al., 2015).

2.3 SOURCES OF LACTIC ACID BACTERIA

Lactic Acid Bacteria (LAB) are generally found in decomposing plants, dairy products, fermented fish, vegetables, fermented beverages, and in cavities of humans and animals (Liu et al., 2014), in humans they inhabit the oral cavity, colon, and vagina (Todorov, 2009). LAB have also been detected in soil (Lamont et al., 2017), the *Lactobacillus* species can be isolated from carbohydrates in rich environment vegetables, and fermented vegetables products such as Kimchi (Djadouni and Kihal, 2012). The *Leuconostoc* genus are mainly isolated from chilled meat, dairy products and wine (Goldstein, Tyrrell & Citron, 2015). Whereas, the *Pediococcus* group is normally associated with spoilage of fermented beverages, as well as some sub species of *Lactococcus* have been isolated from plants and dairy products (Parada et al., 2007). LAB has also been isolated from fish gastrointestinal tracts (Ahmad et al., 2013, Jiang et al., 2017).

It is also well investigated that LAB are a part of the native microbiome of aquatic animals (Ringø , 2018). According to Merrifield et al., (2014) members belonging to *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Streptococcus, Carnobacterium, Pediococcus,* and *Weissella* genera are indigenous species in fish. It is also suggested by Ringø et al., (2018) that fish gut is a natural harbor of LAB because it is consistently found in the gastrointestinal tract of fishes from different locations.

2.4 BIOCHEMISTERY OF LACTIC ACID BACTERIA

Lactic Acid Bacteria are chemotrophic in their nutritional mode, they find the energy needs for their metabolism from the oxidation of chemical compounds. LAB are classified by the fermentation pathway use to ferment glucose. The two major pathways are firstly, homofermentative (Figure 1) ferments glucose to two moles of lactic acid,

generating 2 ATP per mole of glucose with lactic acid as the main by-product, Homofermentative LAB consume nearly all the sugars they use, especially glucose into lactic acid. The homofermentative LAB include yogurt strains consisting of (*Lactobacillus delbruckii subsp. bulgaricus*, *Lactobacillus acidophilus*) and cocci (*Streptococcus salivarius subsp. thermophilus*), they are used in dairy starter culture where development of lactic acid and low pH. conditions are desirable.

Secondly, heterofermentative (Figure 1) fermentation produces 1 mole of glucose to ever mole of lactic acid, 1 mole of ethanol, and 1 mole of CO^2. One mole of ATP is generated per mole of glucose. Heterofermentative LAB include *Leuconostoc* spp. (Gram-positive cocci) and Gram-positive rods such as *Lactobacillus brevis, Lactobacillus. fermentum*, and *Lactobacillus. reuteri*. They are rarely used in dairy starter culture due to their ability to cause defects in product such as slits in hard cheese and bloated packaging because of the CO_2 production. The major difference in these two-fermentation processes is their enzyme product which includes the production of fructose 1,6-diphosphate of the EMP and phosphoketolase of the PK pathway (König and Fröhlich, 2009).

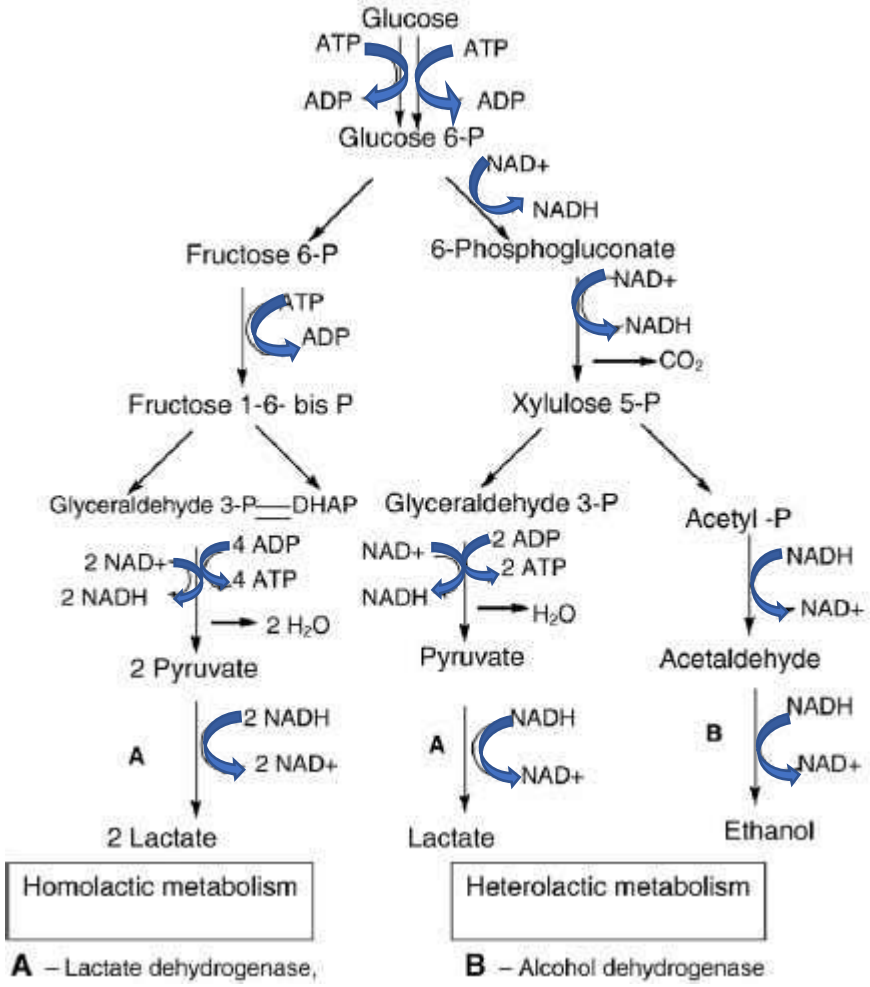

Figure 1 The Metabolic Pathway of Lactic Acid Bacteria (Reddy et al., 2008)

2.5 MARINE ORGANISMS AS A POTENTIAL SOURCE OF LAB PRODUCING ANTIMICROBIAL PEPTIDES (BACTERIOCIN)

The marine environment differs greatly from the terrestrial and freshwater habitats for the reasons of its burdensome, competitive and aggressive nature. It is estimated that the density of bacteria in seawater ranges from 10^5 to 10^7 /mL and 10^8 to 10^{10}/g (Austin, 1988). In the meantime, little is known about the diversity of marine microorganisms. Its estimated that the number of species ranges from as low as 10^4 - 10^5 to as high as 10^6 - 10^7 (Glöckner, 2012). Marine bacteria producing bacteriocin are of prime interest to researcher due to their applications as probiotics as well as, antibiotics in the seafood