ISOLATION AND CHARACTERIZATION OF BACTERIOCINS FROM ENTEROCOCCUS SPECIES FROM NON-BROILER CHICKEN.

BY

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ABSTRACT

Bacteriocins are small proteins or peptides produced by varieties of microbes with antimicrobial activity against closely related species. These antimicrobial agents are gaining more attention not only as alternative therapeutics, but also as an important bio-preservative in food. The aim of this study was to isolate, identify and characterize bacteriocin and the bacteriocin producing lactic acid bacteria from Malaysian non-broiler chicken and explore on their potential biotechnological properties. Using MRS agar medium, a total number of 56 bacterial strains were isolated from several internal organs such as bile, cecum, gizzard and intestine of a local non-broiler chicken from Kuantan area. Bile was the best source for the isolation of Enterococci followed by gizzard, intestine and cecum. Standard biochemical, morphological and molecular biology analyses were carried out on 7 selected isolates. By using polymerase chain reaction (PCR), amplification of 16S rRNA gene in the presence of 16S rRNA universal primers, 1.5 kb fragments were amplified and through the nucleotide sequencing and homology searches, confirmed the identification of these isolates as strain B3L3 to be *Ent. faecium*, strains B4L4 and G5L5 as *Ent. hirae*, strains B5L6, B10L7 and I1L8 as *Ent. faecalis* and strain C4L10 as *Ent. mundtii*. These sequences were subsequently submitted to gene bank, accession number was then allocated to each strain, (KC731419 for B3L3; KC731420 for B4L4; KC731421 for B5L6; KC731422 for B10L7; KC731423 for C4L10; and KC731424 for I1L8) respectively. Antimicrobial screening revealed that strain B3L3 showed good potential as producer of bacteriocin. The bacteriocin of the 7 selected strains showed good antimicrobial activity against Methicillin resistance *Staphylococcus aureus* (MRSA). Extracted bacteriocins from the bacterial pellets of the selected strains gave a molecular weight of approximately 10 kDa based on SDS-PAGE analysis. The purified bacteriocin were highly thermostable, retaining their activity even after treatment at 121°C for 15min, and were stable in a pH range of 4-9 with enhanced activity in acidic pH. There was a loss of activity upon protease treatment; while catalase and lysozyme has no effect on the antimicrobial activity. PCR amplification of the genomic DNA of the selected strains using several Enterocin gene primers revealed that Enterocin_A, Enterocin_L and Enterocin_P genes were present in the genome of *E. faecalis* I1L8; *E. hirae* B4L4; while *E. mundtii* C4L10 has Enterocin_B and Enterocin_P. The structural genes of the bacteriocin were all chromosomally encoded. The PCR product of the enterocin genes was then sequenced, translated and subsequently checked for enterocin homology using a specialized bacteriocin database, Bactibase. Enterocin_P was found to dominate the encoding genes of the bacteriocin among these Enterococcus strains. Based on the database search, the *Enterococcus sp.* strains I1L8, B4L4, and C4L10 were found to have high homology to Bioticin (47% identity), Bacteriocin L-1077 (63% identity) and BacteriocinL-1077 (83% identity), respectively. In addition, novel double-glycine signals in two of these strains, B4L4 and C4L10 were detected. Furthermore, a novel YGNGP characteristic of class IIa bacteriocins was also detected in bacteriocin from *E. mundtii* strain C4L10. Bacteriocin sequence from strain C4L10 contained an enzyme glycotransferases conserved domain, which is implicated in anti-proliferative action. Subsequent studies to check for the possibility for an anti-proliferative effect against human cell-lines, futher tests were carried out on four human neoplastic cell lines: breast cancer (MCF7); lung cancer H1299; colon cancer HCT116; and oral cancer HSC3. Oral cancer cell lines (HSC3) was found to be the most sensitive cell line to the bacteriocin of C4L10 with cytotoxic index of IC_{50} of 9.009µg/ml, followed by breast cancer (IC_{50} of 11.51µg/ml) then lung cancer (15.25 µg/mL), colon cancer cell line (HCT116) was the least sensitive (IC_{50} of 20.57µg/ml). The different antimicrobial regions within the bacteriocin amino acid sequences were also predicted. Sequence analysis showed that B4L4 enterocin contained one putative bactericidal region, while strain C4L10 and I1L8 contained two bactericidal regions each. In conclusion, the isolated enterococci are promising candidate for further investigation of their biotechnological potential in relation to their use in dairy food products and antitumor potentials.

خلاصة البحث

تُعتبر الباكتيريوسيناتBacteriocins بروتينات صغيرة أو ببتيدات يتم إنتاجها عبر عدّة جراثيم وتتمتّع بفعاليّة مُضادّة للجراثيم ضدّ الأنواع وثيقة الصلة بها. لفتت مُضادّات الجراثيم هذه انتباه الباحثين ليس لمجرّد أنّها علاجات بديلة، وإنما لكونها تُعد بمثابة عوامل حافظة حيويّة هامّةBio-preservatives للأطعمة. هدفت هذه الدراسة إلى عزل وتحديد وتوصيف باكتيريوسينات، والجراثيم المُنتجة لحمض اللبن المفرزة لتلك الباكتيريوسينات انطلاقاً من الدجاج المالیزي غير اللاحم، وفحص خواصها التكنولوجيّة الحيويّة Biotechnological. تمّ عزل 56 سلالة جرثوميّة من عدّة أعضاء حشويّة Internal organs؛ كالصفراء، والأعور، والحواصل، والأمعاء باستخدام وسط آغار من نوع MRS انطلاقاً من دجاج محلي غير لاحم تمّ أخذه من منطقة كوانتان. اعتُبرت الصفراء أفضل مصدر لعزل جراثيم المكورات المعويّة Enterococci، تلاها الحواصل (القوانص) والأمعاء والأعور على الترتيب. تمّ إجراء تحاليل جزيئيّة حيويّة، ومورفولوجيّة، وكيمياحيويّة قياسيّة على 7 سلالات معزولة. تبيّن عبراستخدام تقنيّة تفاعل سلسلة البوليميراز Polymerase chain reaction (PCR)، وتضخيم مورثة 16S rRNA بوجود 16S rRNA universal primers، و عبر1.5 Kb من الأجزاء المُضخمة، وتتالي النوكليوتيدات Nucleotide sequencing، وبحوث التناظر Homology searches أنّ هويّة هذه السلالات المعزولةكمايلي:سلالة B3L3 هي لجراثيم *Ent.faecium*،وسلالتي B4L4 وG5L5 هي لجراثيم *Ent.hirae*،وسلالات B5L6 و B10L7 و I1L8لجراثيم *Ent.faecalis*، وسلالة C4L10لجراثيم *Ent.mundtti*. تمّ تقديم هذه المتتاليات في وقت لاحق لبنك الجينات، ثم تمّ فيما بعد تخصيص رقم الوصول لكل سلالة (KC731419 لسلالة B3L3،KC731420 لسلالة B4L4، KC731421 لسلالة B5L6، KC731422 لسلالة B10L7، KC731423 لسلالة C4L10، KC731424 لسلالة I1L8) على الترتيب. كشف المسح المُضاد للجراثيم أنّ السلالة B3L3أبدت إمكانات جيّدة كمُنتجة للباكتيريوسين. أظهر الباكتيريوسين الخاص بالسلالات السبع الجرثوميّة خواص مضادّة للجراثيم جيّدة ضد جراثيم العنقوديات المذهّبة المقاومة للميثيسيللين Methicillin resistance staphylococcus aureus (MRSA). بلغ الوزن الجزيئي للباكتيريوسين المُستخلص من الكريّات الجرثوميّة التابعة للسلالات المُختارة حوالي 10 كيلو دالتون 10kDa بالاعتماد على تحليل SDS-PAGE. كانت الباكتيريوسينات المُنقّاةثابتة حراريّاًومُحتفظة بفعاليتها حتى بعد تعريضها لحرارة 121 درجة سيليزيوس ولمدّة 15 دقيقة، وبدرجات حموضة تراوحت بين 4 – 9 مع زيادة في فعاليتها في وسط حمضي. زالت فعاليتها عند مُعالجتها بأنزيمات البروتياز، في حين لم يكن للكتالاز والأنزيمات الحالّة أي تأثير على فعاليتها المُضادّة للجراثيم.كشف تضخيم genomic DNA عبر تقنيّة PCR للسلالات المُختارة باستخدام عدّة بوادئ إنتيروسين جينيّة Enterocin gene primers، أنّجينات Enterocin_A و Enterocin_L و Enterocin_P كانت موجودة في جينوم E.faecalis I1L8 وE.hirae B4L4، في حين أنّ E.mundtii C4L10 كانت مسؤولة عن Enterocin_B و Enterocin_P.تمّ ترميز جميع الجينات البنيويّة للباكتيريوسين صبغيّاً. بعد تضخيم جينات الباكتيريوسين عبر تقنيّة PCR، تمّ عمل تتالي وترجمة ومن ثمّ فحص تناظر الإنتروسين باستخدام قاعدة بيانات خاصّة بالباكتيروسين Bactibase. تبيّن أنّ Enterocin_P يُسيطر على ترميز جينات الباكتيريوسين ضمن سلالات المكورات المعويّة. تبيّن عبر البحث في قاعدة البيانات، أنّ سلالات أنواع المكورات المعويّة I1L8 و B4L4 و C4L10 لها تناظر عالي للبيوتيسين Bioticin (بنسبة 47%) والباكتيريوسين L-1077 (بنسبة 63%) والباكتيريوسين L-1077 (بنسبة 83%) على الترتيب. بالإضافة إلى ذلك، فقد تمّ تحديد إشارات مُضاعفة الغلايسين حديثة في إثنين من هذه السلالات هما:B4L4 و C4L10. والأكثر من هذا، فقد تمّ تحديدصفاتYGNGP حديث ينتمي للباكتيريوسينات من النمط IIa ضمن الباكتيريوسين المُفرز من السلالة C4L10 لجراثيم E.mundtii.احتوى تتالي باكتيريوسين السلالة C410 على نطاق مُحتفظ لأنزيمات Glycotransferases والذي يملك خواص مُضادّة لتكاثر الخلايا. أُجريت دراسات سابقة للتحرّي عن إمكانيّة التأثيرات المُضادّة لتكاثر الخلايا ضد خطوط الخلايا البشريّة؛ حيث جرت الاختبارات على أربعة خطوط خلويّة بشريّة ورميّة هي MCF7 لسرطان الثدي، H1299 لسرطان الرئة، HCT116 لسرطان القولون وHSC3 للسرطانات الفمويّة. تبيّن أنّ الخطوط الخلويّة للسرطانات الفمويّة HSC3 هي أكثر الخطوط الخلويّة حساسيّةً للباكتيريوسين الخاص بسلالة C4L10، وتمتّعت بمنسب سُمّي خلويIC_{50}=9.009 مكغ/مل، أتى بعدها خط سرطان الثدي IC_{50}=11.51 مكغ/مل،ثمّ خط سرطان الرئة IC_{50}=15.25 مكغ/مل، ثمّ الخط الخلوي لسرطان القولون HCT116 وكان الأقل حساسيّةًIC_{50}=20.57 مكغ/مل. تمّ أيضاً توقّع عدّة مواقع مُضادّة للجراثيم ضمن تتاليات الحموض الأمينيّة للباكتيريوسين. أظهر تحليل التتالي، أنّ الإنتروسين التابع لسلالة B4L4 احتوى على موقع وهمي قاتل للجراثيم، في حين أنّ السلالتين C4L10 وI1l8 احتوت كل واحدة منهما على موقعين قاتلين للجراثيم. خُلاصة القول هي أنّ المكورات المعويّة المعزولة هي جراثيم مُرشّحة واعدة ليتم استقصاءخواصها الحيويّة التكنولوجيّة الكامنة فيما يتعلّق باستخدامها في مشتقات الألبان ولخواصها المُضادّة للأورام.

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, 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.

Moshood Alhaji Yusuf

Signature …… Date ……..

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ISOLATION AND CHARACTERIZATION OF BACTERIOCINS FROM ENTEROCOCCUS SPECIES FROM NON-BROILER CHICKEN

Affirmed by Moshood Alhaji Yusuf.

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Signature Date

Dedicated to my late Mother Alhaja Fatima Yusuf & all of my Family Members

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

LAB belongs to the phylum Firmicutes.Lactic acid bacteria (LAB) are a diverse bacterial group consisting of 11 genera. They are bacteria with the following characteristics: gram-positive, non-spore-formers, rods or coccus and survive in varying oxygen level (Michaela et al., 2009; Khalid, 2011), and produce energy and lactic acid during carbohydrate fermentation (Jay, 2000). Organic acids, diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins are some of the materials resulting from the fermentation of lactic acid (Oyetayo, Adetuyi, and Akinyosoye, 2003). LAB is a diverse group of microorganisms that occur naturally in many foods. LAB find increasing acceptance as probiotics, which aid in stimulating immune responses, preventing infection by entero-pathogenic bacteria, treating and preventing diarrhea (Reid, 2001).

LAB is present in almost any environment such as milk, meat, fermented products, fermented vegetables and beverages (Atrih et al., 2001). Milk was the first habitat where LAB was isolated (Metschnikoff, 1907; Sandine, Radich, and Elliker, 1972;Carr, Hill, and Maida, 2002). It was also isolated from soil, water, manure and sewage (Holzapfel, et al., 2000).

Enterococci belong to group of the lactic acid bacteria (LAB) and have obscure importance in foods with both advantages and harmful aspects. They can prolong shelf-life resulting from the production of antimicrobial agents, synthesize flavor compounds, and contribute to health promotion as probiotic cultures. However, they possess different detrimental attributes which possess large number of virulence

factors, antibiotic resistance genes and act as an indicator of fecal contamination (Ross, Morgan, and Hill, 2002). Many Enterococci are known to produce bacteriocins used as a defense mechanism against closely related bacteria (Oscariz and Pisabarro, 2001). Bacteriocins-producing Enterococci have varied ecological niche and many may produce class IIabacteriocins that are known to be heat stable, cationic, hydrophobic, and low molecular weight peptides (Galvéz, López, and Abriouel, 2008). Among Enterococci strains, there are some that produce lantibiotic (class I), cyclic (class III), and large bacteriocins (class IV) that remain stable at varying pH values with a broad spectrum of antimicrobial activity (Strompfová and Lauková, 2007).

Many species of LAB from the genera of Lactobacillus, Lactococcus, Leuconostoc, Streptococcus and Carnobacterium, among others are, capable of producing small peptides that can inhibit a broad range of gram positive bacteria (Cleveland, et al., 2001).. Many different types of LAB bacteriocins have been studied and characterized, but the most widely known are nisin, lactacin, enterocin, pediocin, and plantaracin(Ray, 2003). These have been extensively studied for their application in foods, but just a few of them have been used in livestock. They have the potential to be used in the food and feed industry as substitute for chemical preservative (Gao et al., 2010).

Bacteriocins are ribosomallytranscribed, and are known to have bactericidal activity against closely related species of the producer cell. Bacteriocins are heterogeneous compounds with varying molecular weights, biochemical properties as well as inhibitory spectra (O'Sullivan, Ross, and Hill, 2002). The mechanisms of action of bacteriocins are diverse, but the bacterial membrane is the target for most bacteriocins(Klaenhammer, 1993).Most LAB bacteriocins inhibit bacteria by forming

pores in the cell membrane by dissipating the proton motive force, cell lyses, and interference with degradation and metabolism of macromolecules are some of the mechanisms by which the bacteriocins inhibit the target bacteria (Martinis, Alves, and Franco, 2002). Gram-negative bacteria are protected from the lethal effect of LAB bacteriocins by the outer membrane.

One of the many advantages of bacteriocins as compared to the conventional antibiotics is that they are naturally produced which leads to their enormous use as preservatives and therapeutic agents (Rittenberger, et al., 2011). The prevalence of Enterococcus bacteriocins encoding traits cannot be ascertained due to the limited data available since only one Enterococcus genus was successfully sequenced completely. It is a known fact that the frequency of occurrence of bacteriocins in Enterococcus and Streptococcus is more as compared to many other lactic acid bacteria, e.g. Lactococcus and Lactobacillus (Ingolf, et al., 2007).

Staphylococcus aureus is known to be the causative agent of many infectious agents prominent among them is bone abscesses intrasurgical infections of the tissues sepsis and invasive endocarditis (Rittenberger, et al., 2011). The indiscriminate use of antibiotics has led to drug resistance in *Staphylococcus aureus*. The most prominent is the Methicillin resistance *Staphylococcus aureus* (MRSA) that has developed resistance to many of the common antibiotics such as penicillin, methicillin and tetracycline(Andonian et al., 2011).

The extensive use of the conventional antibiotics in the treatment of diseases of animals and human in recent years, generated a lot of concern following the development of antibiotic resistance (Jarvis, 1967; Ming and Daeschel, 1993; Mantovani and Russell, 2001; Diez-Gonzalez, 2007). Based on this setback, the use of antibiotics as growth promoters was restricted. This paves the way for the search and

production of alternative antimicrobial agent that has gain increase medicinal prominence (Callewaert and De Vuyst, 2000; Ekinci and Barefoot, 2006).

The use of bacteriocins as food preservative offers many advantages based on studies that were conducted recently (Thomas et al., 2000; Gálvez, et al., 2008). These include (1)Bacteriocin helps in prolonging the keeping time of foods. (2) It protects the food against unfavorable conditions of temperature. (3), It reduces the problem of transmission of food borne pathogens. (4), It reduces economic loss when food spoiled. (5) It lowers the use of chemical compounds for preservation. (6), It allow the use of less heat during processing therefore prevent damages to the nutrients in the final food product, and (7), It allows for novel foods with less acid and salt composition to be marketed.

1.2 STATEMENT OF PROBLEMS

The expenses involved in producing suitable producer strains, the hard task needed to produce purified bacteriocins and high expectation in the investments in research and development of bactriocin are some of the setback in the use of bacteriocin as an antimicrobial agent. Another problem is the emergence of resistance bacteria strain previously susceptible to conventional antibiotics.

So far, antibiotics routinely applied in order to prevent infection and the excessiveor improper dosage of the antibiotics used had caused the development of resistant bacterial strains in both humans and animals, they are also known to affect the immune system and add toxins to the body. They are also known to cause complications, like yeast infections. In addition, the overuse of antibiotics may give rise to the incidence of bacterial infections from the previously non resistance strains. Furthermore, most antibiotics contain sulfa, which may cause allergic reactions.

Common side effects of antibiotics include sensitivity to light, indigestion, nausea, diarrhea and discomfort. Therefore there is a need to obtain an antimicrobial agent that is non-toxic, easily metabolized and does not produce resistance in target organisms. Probiotic organism were shown to produce wide array of antimicrobial compounds of which some are potentially be used in the treatment of certain pathogens and even food preservation. In this work, in order to avoid a source of antibiotic resistant LAB, Kuantan indigenous chickens not fed any commercial feed or antibiotics, were used as a source of bacteriocin-producing LAB and probiotic strains. These LAB strains, and the bacteriocin they produced were subsequently subjected to further characterization and analysis.

1.3 OBJECTIVES OF THE STUDY

The aims of this study can be summarized as follows;

- To isolate and identify the positive producing bacteriocinfrom local non-broiler chicken (*Ayam Kampong*).
- To extract and purify antimicrobial peptides (bacteriocins) from the isolates.
- To carry out bioassay of the bacteriocin on the indicator organism.
- To characterize the bacteriocins and determine anti-proliferative activity of selected bacteriocins on cancer cell lines.

1.4 SCOPE OF WORK

The major experimental works involved in this study can be summarized based on following:

i). General microbiology works involving isolation, characterization and identification of isolates. The use of antagonistic assay to detect bacteriocin using indicator organism.

ii). Molecular work (DNA extraction, rRNAand bacteriocin genes amplification and sequencing for strain identification. This include sequence analysis of rRNA genes and baceriocin genes using bioinformatics tools.

iii) Bacteriocin purification and characterization. Further characterization on antineoplastic properties of purified bacteriocin on human cell lines.

1.5 HYPOTHESIS

The bacteriocin isolated from non-broiler chicken, showing good antimicrobial and anti-proliferative activity may be used as an alternative to antibiotics.