UNIVERSITI PUTRA MALAYSIA

BIOCHEMICAL AND MOLECULAR CHARACTERISATION OF SELECTED MICROORGANISMS ISOLATED FROM BEEF, CHICKEN, MUTTON AND PORK MEAT PRODUCTS

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FBSB 2017 12

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By

CEESAY AMIE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2017

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DEDICATION

I dedicate this research work to Allah, my creator, strong pillar, sources of inspiration, wisdom, knowledge, and understanding. He has been my source of strength throughout this research. I also dedicated this research to my beloved mother, whose prayers and encouragement has made sure that I give it all it take to finish that which I have started. To my cousin (Sama Jawneh), whose support has paved the way for this program in the first place.

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

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SELECTED MICROORGANISMS ISOLATED FROM BEEF, CHICKEN, MUTTON AND PORK MEATS PRODUCTS

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May 2017

Chairman : Professor Shuhaimi Bin Mustafa, PhD
Faculty : Biotechnology and Biomolecular Sciences

Tracing and identification of meat products is one of the great concerns of consumers and meat products regulators. This is because consumers are more demanding and sensitive to the safety of the meat products they consume. Several methods have been employed in the characterization of microorganisms isolated from meat products such as phenotypic analysis, protein, biochemical, and molecular based techniques. The characterization based on protein and physiological techniques in meat profiling and classification by using microorganisms had been reported to be problematic since they share numerous characteristics. There have been limited reported studies on the characterization and profiling of microorganisms for meat classifications.

This study was based on the biochemical and molecular fingerprint in the characterisation and profiling of selected microorganisms isolated from beef, chicken, pork and mutton samples that may be linked statistically to meat sources. In order to determine a specific difference between bacteria genera isolated from different meat sources, 39 *Escherichia coli*, 66 *Lactobacillus*, and 54 *Pseudomonas* isolates identified by using API 20E, 50CHL, and 20NE test kits. The isolates were then analysed using 18 antibiotics for antibiotic susceptibility assay. Thirty four *E. coli*, was examined by molecular markers such as BOXAIR, Enterobacterial repetitive intergenic consensus (ERIC), polytrinucleotide $(GTG)_5$ and random amplified polymorphic DNA polymerase chain reaction ((RAPD-PCR) to generate genetic fingerprints, while 56 *Lactobacillu*s and 42 *Pseudomonas* species were typed using RAPD and (GTG)-PCR to generate fingerprint data. The fingerprints were resolved on 1.5% (w/v) agarose gels. The resistance or sensitivity of isolates to antibiotics was score as binary data and a similar procedure was carried out, for the fingerprinting data where absence or presences of bands were scored on excel and used to generate a data matrix. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and complete linkage arithmetic were used to analyse percentage of similarity. The similarities level

of *E. coli*, *Lactobacillus*, and *Pseudomonas* isolates from different sources were expressed as a dendrogram.

Bacteria colony counts (ranging from 2.2 to 6.5-log CFU/mL) showed a significant difference among the meat types ($p \leq 0.05$). Further analysis using API Kit revealed *Lactobacillus fermentum1* (12), *Lb plantarum*, *Lc brevis* 1 (8), *Lc lactis* spp lactis (5), *E. coli* 1 (29), *Pseudomonas luteola* (24) and *Aeromonas hydrophila/caviae* (7) as major groups identified. The dendrograms generated from antibiotic biogram showed a clear distinction between different meat products at the similarity coefficient of 0.60 to 1.0. The relationship of these isolates from each cluster was compiled and reported on tables. The fingerprints generated band sizes from 0.10 kb to 5.50 kb with the majority of isolates having 15 bands. The UPGMA and Dice coefficient clusters showed dendrograms based on 0.7 similarities of *E. coli* isolates of four techniques used. The dendrograms of all markers classified *E. coli*, *Lactobacillus* spp. and *Pseudomonas* spp. into five major clusters (I-IV) within the similarity coefficient of 1.0 (100%) to 0.65 (65%). All the markers except $(GTG)_5$ accurately classified grocery pork (TP2), beef (GB1), and wet market beef (PMB1) samples in their respective cluster. Similarly, the $(GTG)_5$ marker showed the same clustering pattern as ERIC marker for E. coli spp. The Principal component analyses (PCA) for BOXA1R and RAPD showed the clear distinction of sample classification. However, ERIC and $(GTG)_5$ showed a weaker correlation between isolates of the same source. RAPD, ERIC, BOXA1R and $(GTG)_5$ fingerprinting markers showed the highest discriminatory index at the following cut-off percentages: 0.80 at 80%, 0.81 at 90% and 0.87 at 100%. These results suggested that RAPD, $(GTG)_5$ and BOXA1R markers could be an effective tool for the characterization of bacteria from different sources. The similarity matrices confirmed the clustering pattern and genetic relationship between and among isolates.

The findings of this research revealed that the biochemical and genetic fingerprinting of *E. coli*, *Lactobacillus* spp. and *Pseudomonas* spp. isolated from meat and meat products could be used as a potential technique to characterise microorganism according to meat types.

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

CIRI-CIRI BIOKIMIA DAN MOLEKUL ORGANISMA-ORGANISMA TERPILIH YANG DIPENCILKAN DARIPADA PRODUK DAGING LEMBU, AYAM, KAMBING DAN BABI

Oleh

CEESAY AMIE

Mei 2017

Pengerusi : Profesor Shuhaimi Bin Mustafa, PhD
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Menjejaki dan menenalpasti produk daging merupakan perhatian utama para pengguna dan penyelia produk. Ini adalah kerana pengguna kini lebih ingin mengetahui serta sensitive terhadap produk makanan yang mereka ambil. Beberapa kaedah telah pun dijalankan bagi pencirian mikroorganisma yang dipencilkan daripada produk daging seperti analisis fenotip, teknik-teknik protin, biokimia, dan berasaskan molekul.

Pencirian berasaskan protin dan teknik fisiologi di dalam pemprofilan daging dan pengkelasan menggunalan mikroorganisma pernah dilaporkan agak sukar kerana terdapat banyak ciri-ciri yang bertindan. Hanya sedikit sahaja kajian yang dilaporkan berkenaan ciri-ciri dan profil mikroorganisma bagi pengkelasan daging. Kajian ini merupakan kajian berdasarkan 'fingerprint' biokimia dan molekular di dalam pengecaman dan pemprofilan mikroorgaanisma terpilih yang dipencilkan daripada daging lembu, ayam, babi dan kambing yang boleh dikaitkan secara statistik dengan sumber daging. Untuk mengetahui perbezaan spesifik di antara genera bakteria yang pencilkan daripada sumber daging berbeza, 39 isolat *Escherichia coli*, 66 isolat *Lactobacillus* spp., dan 54 isolat *Pseudomonas* spp. telah dikenalpasti melalui ujian menggunakan API 20E, 50CHL, and 20NE. Isolat-isolat tersebut seterusnya dianalisis menggunakan 18 jenis antibiotik untuk melihat tahap kesesuaian esei terhadap jenis antibiotik tersebut. Tiga puluh empat isolat *E. coli* telah diperiksa melalui penanda molekular seperti BOXAIR, 'Enterobacterial repetitive intergenic consensus' (ERIC), politrinukleotida $(GTG)_5$ dan 'random amplified polymorphic DNA polymerase chain reaction' (RAPD-PCR) untuk menjana 'fingerprint' genetik, manakala 56 isolat *Lactobacillu*s spp. dan 42 isolat *Pseudomonas* spp. telah dikelaskan menggunakan RAPD and $(GTG)_5$-PCR untuk menjana data 'fingerprint' genetik. Fingerprint' dijanakan dengan 1.5% (w/v) gel agaros. Ketahanan dan sensitivity isolat-isolat terhadap antibiotik-antibiotik diskor sebagai data binary. Untuk data 'fingerprint', prosedur serupa telah dijalankan di mana kewujudan band-band diskor di dalam Excel

dan seterusnya matrik data dijanakan. Kaedah 'Unweighted Pair Group' dengan 'Arithmetic Mean' (UPGMA) dan 'complete linkage arithmetic' telah digunakan untuk menganalisa peratus persamaan. Tahap persamaan isolat *E. coli*, *Lactobacillus* spp., dan *Pseudomonas spp.* daripada sumber berbeza telah diterjemahkan di dalam dendrogram.

Bacaan koloni bakteria (julat di antara 2.2 ke 6.5-log CFU/mL) menunjukkan perbezaan yang siknifikan di antara jenis daging ($p \leq 0.05$). Kajian lanjutan menggunakan Kit API menunjukkan bahawa *Lactobacillus fermentum1* (12), *Lb plantarum*, *Lc brevis* 1 (8), *Lactococcus lactis* spp lactis (5), *E. coli* 1 (29), *Pseudomonas luteola* (24) dan *Aeromonas hydrophila/caviae* (7) adalah merupakan kumpulan utama. Dendrogram yang dijana daripada biogram antibiotik menunjukkan perbezaan yang nyata pada persamaan koefisyen 0.60 sehingga 1.0 di antara produk daging. Hubungan di antara isolat-isolat daripada setiap kluster telah dikumpulkan dan ditabulasikan. Band-band 'fingerprint' yang terjana bersaiz di antara 0.10 kb ke 5.50 kb dengan kebanyakan isolat mempunyai 15 band. UPGMA dan cluster koefisyen Dice menunjukkan dendrogram berdasarkan kepada 0.7 persamaan isolate-isolate *E. coli* daripada empat teknik yang digunakan. Dendrogram-dendrogram bagi kesemua penanda mampu mengkelas yang dikelaskan *E. coli*, *Lactobacillus* spp. dan *Pseudomonas* spp. kepada lima kluster utama (I-IV) pada koefisyen 1.0 (100%) ke 0.65 (65%). Kesemua penanda kecuali $(GTG)_5$ mengkelaskan dengan tepat sampel-sampel daging babi (TP2) dan daging lembu (GB1) daripada pasaraya, serta sampel daging lembu daripada pasar basah (PMB1) di dalam cluster tertentu. Demikian juga, penanda $(GTG)_5$ menunjukkan corak cluster yang sama seperti ERIC untuk *E. coli* spp. 'Principal component analyses (PCA) untuk BOXA1R dan RAPD menunjukkan perbezaan pengkelasan sampel yang ketara. Penanda-penanda fingerprin RAPD, ERIC, BOXA1R dan $(GTG)_5$ menunjukkan indeks diskriminasi pada peratusan 'following cut-off': 0.80 at 80%, 0.81 at 90% and 0.87 at 100%. Matrik persamaan mengesahkan bahawa corak cluster dan hubungan genetik di antara serta di kalangan isolat, menunjukkan persamaan dan juga variasi. Keputusan ini membuktikan bahawa penanda-penanda RAPD, $(GTG)_5$ dan BOXA1R boleh menjadi kaedah yang efektif untuk pencirian bakteria daripada kelas-kelas daging yang berbeza.

Penemuan-penemuan daripada kajian ini menunjukkan bahawa 'fingerprint' biokimia dan fingerprint genetik *E. coli*, *Lactobacillus* spp. dan *Pseudomonas* spp. daripada daging dan produk daging boleh digunakan sebagai teknik yang berpotensi untuk mencirikan organisma mengikut jenis-jenis daging.

ACKNOWLEDGEMENTS

I will first thank The Almighty Allah SWT for giving me the strength and good health to accomplish this challenging task. I would like to express my deepest appreciation and heartfelt acknowledgement to my supervisor Professor Dr Shuhaimi Bin Mustafa for his moral, selflessness, and passionate supervision. Your encouragement and foresightedness have indeed trailed me through the rough path of my research. I am humble and feel grateful to my co-supervisors professor Dhilia Udie Lamasudin and Dr Nurulfiza Binti Mat Isa and for their contributions into my work.

I acknowledged, with sincere gratitude to my sponsors, the Islamic Development Bank (IDB) for giving me this great opportunity of sponsoring my Master's Degree program. My sincere appreciation also goes to the Research Grant Scheme of Malaysia Higher Education for funding my research work.

My special thanks to my colleagues in both Biotechnology and Halal Institute and staffs of the Halal Food Product Research institute, who were always there throughout my journey. My special thanks to Dr Ummi Kalthum Hannap for her support, patient and sisterly love, and guidance. I would do no justice if I do not thank Dr Aliyu Mahmuda, Dr. Ibrahim Wasiu Arolu and Dr Akeem Babatubde and Daude and Isatou Hydara for helping me with data analysis and formatting of this thesis.

Last but not the least, my special and heartfelt thanks go to my Mother and my siblings, for their valuable encouragement and inspiration throughout my study that had kept me going. Aside from this, many people have knowingly and unknowingly helped me in the successful completion of this research.

Words cannot express my appreciation, thank you all.

I certify that a Thesis Examination Committee has met on 2 May 2017 to conduct the final examination of Ceesay Amie on her thesis entitled "Biochemical and Molecular Characterisation of Selected Microorganisms Isolated from Beef, Chicken, Mutton and Pork Meat Products" 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 Master of Science.

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LIST OF ABBREVIATIONS

MHA	Mueller-Hinton Agar
CFU	Colony Forming Unit
ATCC	America Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase chain Reaction
°C	Degree Celsius
µL	Micro Litre
mL	Milli litre
rpm	Revolution per minute
R^2	Regression Square
BLAST	The Basic Local Alignment Search Tool
NCBI	National Centre of Biotechnology Information
UPGMA	Unweighted Pair-Group Method Arithmetic Averaged
TSA	Trypticase soy Agar
DNA	Deoxyribonucleic acid
µM	Microlitre
MRS	de-Man Rogosa and Sharpe
RAPD	Random Amplified Polymorphic DNA
rRNA	Ribosomal Ribonucleic acid
SPSS	Statistics package for Social Sciences
PMB1	Pasar Malam Beef sample
PMC1	Pasar Malam Chiccken sample
GB1	Giant Beef sample
GC1	Giant Chicken samples
LP1	Wet market pork sample Market Pork
TP2	Tesco Pork samples
E. coli	*Escherichia coli*
LAB	Lactic Acid Bacteria
PCA	Principal Component Analysis
AM1	Mutton at First collection
BM2	Mutton at Second collection
TSGA	Trypticase soy Glucose Agar
EMB	Eosin Methylene Blue

TMPD	Tetramethyl-p-Phenylenediamine
CEC	Cofactor
MTZ	Metronidazole
CN	Gentamicin
AMP	Ampicillin
C	Chloramphenicol
PB	Polymyxin B
VA	Vancomycin
CL	Cephalexin
S	Streptomycin
TE	Tetracycline
F	Nitrofurantoin
E	Erythromycin
OX	Oxacillin
B	Bacitracin
CTX	Cefortaxime
CIP	Ciprofloxacin
SF	Sulphalfurazole
CXM	Cefuroxime
C1	Chicken from wet market (PMC1)
C2	Chicken from grocery store (GC1)
C3	Chicken from grocery store (AC1)
C4	Chicken from grocery store (AC2)
Pork (L)	Pork from wet market (LP1)
Pork (C1)	Pork from grocery store (TP2)
Pork (C2)	Pork from grocery store (AP1)
Beef (L)	Beef from wet market (PMB1)
Beef (C1)	Beef from grocery store (GB1)
Beef (C2)	Beef from grocery store (AB1)
Beef (C3)	Beef from grocery store (AB2a)
Lc.	*Lactococcus*
W	*Weissella*
Br.	*Brochothrix*
Lb.	*Lactobacillus*
Leu	*Leuconostoc*

Ps	*Pseudomonas*
A	*Aeromonas*
V	*Vibrio*
Bu	*Burkholderia*
Ra	*Raothultella*
Ent	*Enterobacter*
Kl	*Kebsiella*
$MgCl_2$	Magnesium chloride
$(GTG)_5$	Polytrinucleotide
ERIC	Estragenic Repetitive Intragenic Consensus

CHAPTER 1

INTRODUCTION

1.1 Background

Meat is a nutritious substrate suitable for growth of most microorganisms. Meat is part of muscle of many animals such as sheep, cattle, swine, goat, and poultry (Gauje *et al*., 2013). Meat and its products have high quality content of protein, vitamins, minerals, and micronutrients as a source for human food (Lawrie and Ledward, 2006). Meat consumption is an essential area of human diet with strong implications, health, economic and culture around the world (Miszczycha *et al*., 2013). The consumption and demand of red and white meat products continued to escalate in all regions of the globe which was said to be as a result of increase in world population (Leite *et al*., 2015).

The animal bodies provide favourable environment for the growth of microorganisms, which enter into different degrees of symbiotic relations with the host. Various skin surfaces and mucosal membranes in animals such as the content of the digestive tract have a microbial flora with characteristics composition, which could contaminate meat products (Cohen *et al*., 2007; Mertz *et al*., 2014). The microbial flora present at any part of the healthy animal could be anaerobes or aerobes. This had been reported by numerous studies that established the prevalence of various groups of Enterobacteria such as *Escherichia coli* (intestines), *Pseudomonas* species, from skin of the host (Lerma *et al*., 2014), and *Lactobacillus* species, gut flora on meat products (Holko *et al*., 2013). *Escherichia coli* have been used as an indicator bacterium because it can easily acquire antimicrobial resistance faster than most bacteria. Similarly, *Pseudomonas* is the pre-dominant microorganisms limiting the self-life of processed fluid milk and meat products, which caused significant economic lost for the industry (Dogan *et al.*, 2003). In addition, *Lactobacillus* bacteria have been known as a preservative for foods such as fermented meat, where it is added as a starter (Zhang *et al*., 2010). Numerous literatures revealed *Lactobacillus* species utilization in sausage fermentation, which pave a way for research topic like identification of sources of *lactobacillus* used in cheese, milk, and sausages (Arief *et al*., 2014).

Recently, meat safety got the attention of most consumers and food regulators (Montet, *et al*., 2008). The determination of meat sources can be achieved, especially for fresh or unprocessed products, by determining food microbial profile diversity using phenotypic and genetic fingerprinting methods. Since it has been reported that the microbial profile of food products from different sources are unique and this uniqueness could be used as specific biological marker to differentiate the sources of food products such as meat (Peres *et al*., 2007; Mills, 2007; El Sheikha *et al*., 2011; Arcuri *et al*., 2013). Similarly, the Beef Industry and Food Safety Council (BIFSCO, 2014) was formed to allow industry leaders, beef companies, and food safety researchers to find solutions for meat identification (Wheeler *et al*., 2014). Thus, there

is limited study on classification of sources of meat products using its microbial profile.

The identification of bacterial species can also be done by combining two or more methods of biochemical and molecular techniques. (Peres *et al*., 2007; Mills, 2007). As it was suggested that biochemical identification should be supported by molecular techniques to obtained meaning results for identification of bacterial species. The API gallery 20E, 20 NE and 50CHL (Merieux, France) are traditional multiple biochemical test that can be used to determine the microbial flora present in meat products, as well as the bacteria antibiotic resistance and multi-resistance profiles. The genetic fingerprinting markers (techniques) such as random amplified polymorphic DNA (RAPD), evolutionarily conserved repetitive sequences which include BOX, ERIC and $(GTG)_5$ (Versalovic *et al*., 1994). The fingerprinting techniques have been used for microbial diversity or variability and their ecological distribution. They are highly convenient and cost effective, commonly employed for bacterial identification and verification estimation. These are also considered as valuable tool for classifying and typing of large range of Gram-negative and Gram-positive bacteria (Versalovic *et al*., 1994). The techniques amplifies variable regions of DNA flanked by the repetitive sequences, leading to amplicons patterns specific for an individual bacterial species (Braem *et al*., 2011). In addition, gene fingerprint based on genomic polymorphism (RAPD) is a recent approach widely used for the assessment of inter and intraspecific genetic variation (Saxena *et al*., 2014; Versalovic *et al*., 1994).

This study aimed to use selected microorganisms isolated from beef, chicken, mutton and pork meats collected from different sources to evaluate the potential use of biochemical and DNA fingerprinting markers for determination of unique molecular biology profile for *Escherichia coli*, *Lactobacillus* spp. and *Pseudomonas* spp. for classification of meat to their sources.

1.2 Problem statement

The meat identification systems usually revolved around devices that were attached to an animal such as ear tags and labels, which are mainly paper-based documentations. The documentary system is very difficult to verify the sources of animal products.

During processing in the slaughterhouse, at all times, there is a division of the animal carcass generating various cuts of meat, the aggregation of different cuts of meat products in the same package can lead to difficulties in maintaining the animal's identity.

The increase concern of consumers over meat safety issues such as identification of animal sources are the requirement for reliable analytical methods.

The DNA based techniques have the ability to identify uniqueness to an individual animal, it have been used as a tool to proof the identity or audit in meat identification system. In addition, most of these researches have not used the microbial flora of meat products as a tool for identification and classification.

1.3 Significance of the study

This study could be used as a quality control tool for identification and characterization of meat products using meat microbial profile. This would be achieved using the antibiotic resistant pattern and DNA fingerprinting data of bacteria isolated from different meat products collected from various sources. Similarly, safety and quality is showed to be the consequence of control (Fernandes *et al*., 2014). Thus, the analysis of microbial profile is based on the principle that the environment has an effect on the bacterial ecology of food. Therefore, bacteria can vary by their number and especially by their species' characteristics and genetic makeup (Peres *et al*., 2007; Mills, 2007).

1.4 Study Hypothesis

It is hypothesized that *E. coli*, *Pseudomonas* spp. and *lactobacillus* spp. profile could identify and profile the sources of the meat products.

1.5 General objective

To evaluate the potential use of biochemical and DNA fingerprinting markers for determination of unique molecular biology profile for *Escherichia coli*, *Lactobacillus* spp. and *Pseudomonas* spp. isolated from beef, chicken, mutton and pork meats collected from different sources.

1.6 Specific Objectives

- To characterize bacterial species isolated from different meat products and to use their antibiotic susceptibility profile for tracing the meat sources.
- To isolate *Escherichia coli* (*E. coli*), *Pseudomonas*, and *Lactobacillus* species using eosin methylene blue agar (EMBA), Pseudomonas selective agar base (PSAB), De Man-Rogosa and Sharpe agar (MRSA) respectively.
- To identify the isolates using analytical profile Index kit (API) of 20E, 20NE and 50CHL for *E. coli*, *Pseudomonas* and *Lactobacillus* isolates.
- To carry out antibiotic susceptibility assay on these isolates using Muller-Hinton agar plates
- To determine the genetic diversity of selected microorganism isolated from different meat species using molecular techniques and use the diversity to classify the meat into their sources.
- To genotype *E. coli*, *Pseudomonas* and *Lactobacillus* species using Random amplified polymorphic DNA (RAPD), Enterobacteria intergenic repetitive sequences (ERIC), BOX elements (BOX) and polytrinucleotides $(GTG)_5$ fingerprinting markers.
- To determine the genetic diversity of these bacterial species by using the data generated by the genetic fingerprinting markers after analysis with Bionumeric and NTSYS-pc software.

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