Faculty of Resource Science and Technology

Biofilm Forming Ability and Genes Involved in Biofilm Formation of Locally Isolated Intermediate and Saprophytic *Leptospira*

Jennifer Anak Jalan

Master of Science
2020

Biofilm Forming Ability and Genes Involved in Biofilm Formation of Locally Isolated Intermediate and Saprophytic *Leptospira*

Jennifer Anak Jalan

A thesis submitted

In fulfillment of the requirements for the degree of Master of Science

(Microbiology)

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2020

DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Jennifer

.....

Signature

Name : Jennifer Ak Jalan

Student ID No : 15020848

Faculty Resource Science and Technology

Universiti Malaysia Sarawak

Date: 3 June 2020

ACKNOWLEDGEMENT

First and foremost, I want to thank God for His powerful grace and blessing. I would like to express my deepest gratitude to my main supervisor, Professor Dr. Kasing Apun for her endless support and encouragement throughout my research study in UNIMAS. Despite all the difficulties faced, she always assisted and motivated me during this journey. My appreciation also goes to my co-supervisor, Associate Professor Dr. Lesley Maurice Bilung and Dr. Hashimatul Fatma binti Hashim for lending their hands and sharing their knowledge for this project. In term of financial support, I was fortunately funded by Fundamental Research Grant Scheme FRGS/ST03(02)/1209/2014(10) and MyBrain15 from the Ministry of Higher Education Malaysia.

My special appreciation goes to Associate Professor Dr. Hairul Azman Roslan for his advice and encouragement in this research study. To all my fellow labmates, Dr. Pui Chai Fung, Syatir Ahmad, Dr. Anes Mohamed and Victoria for their kindness in sharing knowledge and beautiful friendship along this journey.

Finally, my earnest appreciation to both of my beloved parent, Jalan Tinggi and Christina Buli and my siblings, Maysilda and Vinniter for their boundless support and love toward me. Without them, I would never achieve this remarkable and delightful journey of mine. Thank you so much!

ABSTRACT

Leptospirosis or widely known as "rat urine disease" is a very common disease in Malaysia. One of the key factors that caused this chronic infection is the ability of the microorganism to produce biofilm formation. Despite its widely known, there is a lack of study on biofilm formation associated with intermediate *Leptospira* and saprophytic *Leptospira*. Therefore, the objectives of this study are to quantify the biofilm mass intermediate *Leptospira* (n=15) and saprophytic *Leptospira* (n=15) of locally isolated using time course study, to detect the genes involved in biofilm formation by intermediate and saprophytic *Leptospira*. Finally, to evaluate virulence of *Leptospira* isolates using *Artemia salina* in brine shrimp assay. A pathogenic *Leptospira* spp. was also included as positive control in brine shrimp assay. A full cycle of biofilm formation begins with the attachment on the surface, formation of microcolonies, biofilm mature and back to planktonic cell or cell death. The time course study for intermediate and saprophytic *Leptospira* was performed for 11 days in accordance to their general biofilm formation process. All of 30 isolates of intermediate (n=15) and saprophytic (n=15) *Leptospira* formed biofilms on abiotic surface which were represented by microtitre plates and biotic surfaces represented by *Dyera costula* or *Jelutong* wood. At day 5, intermediate *Leptospira* (G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2) was formed stronger biofilm on biotic surface 93.99% than on abiotic surface with 53.33%. While in saprophytic (S19, *Leptospira meyeri* strain 19CAP), it formed stronger biofilm on biotic surface with 86.67% and abiotic surface with 40%. A significance difference ($p<0.05$) occurred in biofilm produced between day 1 to day 11 when compared to the negative control (OD). A total of 20 selected strongest biofilm producers of intermediate (n=10) and saprophytic (n=10) were determined and further analysed for identification of biofilm genes. A total of eight genes;

icaA, icaB, icaC, icaD, bap, ompL1, flaB and *galK* genes were studied. Only *icaC* gene out of eight genes was identified 100% presence of *icaC* gene (192 bp) using polymerase chain reaction. In brine shrimp assay, pathogenic *Leptospira* showed the strongest virulence compared to intermediate *Leptospira* followed by saprophytic *Leptospira.* Different CFUs of the *Leptospira* cells were used to treat brine shrimp nauplii and its survival rate was measured 24h, 48h and 72h. It was repeated at least three times and data were statistically analysed using t-test where $p<0.05$ compared to the negative control. In conclusion, this study contributes additional information on the biofilm formation cycle of *Leptospira* which may be related to their ability to cause infection. It can be further used under clinical practice guidelines to provide impacts in healthcare and public health interventions.

Keywords: Biofilm formation, intermediate, saprophytic, *Leptospira*, *Artemia salina*

Kebolehan Penghasilan Biofilm dan Gen yang Terlibat dalam Penghasilan Biofilem yang Dipencilkan daripada Intermediate dan Saprofitik* Leptospira *Tempatan

ABSTRAK

Leptospirosis atau lebih dikenali sebagai penyakit kencing tikus ialah wabak penyakit yang kerap berlaku di Malaysia. Salah satu punca utama penyakit kronik ini ialah kebolehan mikroorganisma yang dapat menghasilkan biofilem. Namun, tidak banyak penyelidikan yang dijalankan mengenai penghasilan biofilem iaitu Leptospira *intermediate dan* Leptospira *saprofitik. Oleh itu, objektif utama dalam kajian ini ialah mengira kuantiti biofilem mass yang terhasil dalam jangka masa yang diberikan pada* Leptospira *intermediate dan saprofitik yang dipencil secara lokal, mengenal pasti gen yang bertanggungjawab dalam penghasilan biofilem dalam* Leptospira *intermediate dan saprofitik. Akhir sekali menilai tahap keracunan yang terhasil dalam* Leptospira *patogen, intermediate dan saprofitik di dalam ujian brine shrimp. Patogen juga dikaji sebagai kawalan positif dalam ujian brine shrimp. Satu kitaran penuh biofiilem terdiri daripada gabungan biofilem di atas permukaan, penghasilan lapisan mikrokoloni, kemudian, biofilem menjadi matang dan akhirnya biofilem disebarkan dalam bentuk sel planktonik atau menjadi sel mati. Dalam kajian ini, satu kitaran penghasilan biofilem untuk 11 hari sudah memadai. Keseluruhan 30 isolat iaitu* Leptospira *intermediate (n=15) dan saprofitik (n=15) yang menghasilkan biofilem di atas permukaan abiotik yang diwakili plat polistirena dan permukaan biotik diwakili dengan* Dyera costula *atau kayu jelutong. Pada hari ke-5, penghasilan biofilem yang dihasilkan oleh intermediate* Leptospira (G7, Leptospira wolffii *serovar Khorat strain Khorat-H2) di atas permukaan biotik 93.99% berbanding dengan abiotik 53.33%. Manakala saprofitik (S19,* Leptospira meyeri *strain 19CAP) menghasilkan 86.67% di atas permukaan biotik berbanding dengan permukaan abiotik dengan hanya 40%. sahaja. Perbezaan signifikan (p<0.05) dalam penghasilan*

biofilem berlaku dari hari ke-1 sehingga ke hari ke-11 jika dibandingkan dengan kawalan negatif (OD). Sebanyak 20 dari intermediate (n=10) dan saprofitik (n=10) Leptospira *yang dipilih atas penghasilan biofilem yang terkuat dan seterusnya dikaji untuk pencarian gen biofilem. Kajian ini dijalankan menggunakan lapan gen iaitu icaA, icaB, icaC, icaD, bap, ompL1, flaB dan galK gen. Daripada lapan gen dikaji, hanya satu gen, icaC gen yang ditemui 100% hadir dan telah menunjukkan kehadiran icaC gen (192bp) yang dikaji menggunakan tindak balas berantai polymerase ke atas* Leptospira *intermediate dan saprofitik. Bagi ujian brine shrimp, kesemua* Leptospira *mempunyai tahap keracunan bakteria yang berbeza terutamanya dalam patogen* Leptospira *yang mempunyai tahap keracunan yang tinggi berbanding dengan intermediate dan kemudian diikuti* Leptospira *saprofitik. Perbezaan CFUs pada* Leptospira *bakteria telah dijalankan ke atas brine shrimp nauplii dan kadar kematian direkodkan mengikut masa yang telah ditetapkan iaitu 24 jam, 48 jam dan 72 jam. Kemudian diikuti dengan ulangan tidak kurang dari 3 kali dan data diambil dianalisa secara statistik menggunakan t-test di mana $p<0.05$ berbanding dengan kawalan negatif. Kesimpulannya, kajian ini berhasil menambahkan data kajian penyelidikan ke atas penghasilan biofilem dalam kitaran masa yang diperlukan yang juga mungkin boleh digunakan untuk mengetahui punca masa penyakit ini bertindak. Ia juga boleh digunakan secara klinikal untuk memberi panduan dan memberi impak dalam bidang kesihatan serta kesejahteraan umum.*

Kata kunci: *Penghasilan biofilm, intermediate, saprofitik,* Leptospira*, Artemia salina*

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
ABSTRAK	v
TABLE OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xvii
CHAPTER 1: INTRODUCTION	1
1.1 Overview of the study	1
1.2 Research questions	4
1.3 Problem Statement	5
1.4 Objectives	5
1.5 Hypothesis	6
1.6 Thesis organisation	6
CHAPTER 2: LITERATURE REVIEW	7
2.1 Discovery of *Leptospira* genus	7
2.2 General characteristic and taxonomy of pathogenic, intermediate and saprophytic *Leptospira*	8
2.3 Growth of *Leptospira*	10

2.4 Outbreaks of leptospirosis cases 11

2.5 Microbial biofilm 16

2.6 Biofilm formation development 19

2.7 Genes associated with biofilm 22

2.8 Bacterial virulence study 24

CHAPTER 3: Materials and Methods 26

3.1 *Leptospira* growth condition and maintenance 26

3.2 Dark field microscopic analysis 30

3.3 Phase contrast microscopy 30

3.4 Confirmation of *Leptospira* strains 30

3.5 Assessment of biofilm formation on abiotic surface 31

3.6 Assessment of biofilm formation on biotic surface 33

3.7 Classification of biofilm ability 34

3.8 Determination of biofilm genes by PCR 34

3.9 Extraction of DNA and detection of biofilm gene 36

3.10 Agarose gel electrophoresis 36

3.11 Bacterial virulence analysis by using brine shrimp test 37

3.12 Statistical data analysis 38

CHAPTER 4: RESULTS AND DISCUSSION 39

4.1 Growth and morphology of *Leptospira* 39

4.2 Biofilm formation of abiotic surface 42

4.3 Biofilm formation on biotic surface 53

4.4 Phase contrast light microscopic 63

4.5 Detection of biofilm genes 67

4.6 Bacterial virulence study using brine shrimp test 69

CHAPTER 5: CONCLUSION 75

REFERENCES 78

APPENDICES 98

LIST OF TABLES

		Page
Table 2.1	The annual occurrence of leptospirosis worldwide cases	13
Table 2.2	Total of leptospirosis cases from 2011 to 2016 in Malaysia	14
Table 3.1	List of intermediate *Leptospira* microbial culture collection	28
Table 3.2	List of saprophytic *Leptospira* microbial culture collection	29
Table 3.3	The primers list in biofilm formation	35
Table 4.1	Number of tested intermediate *Leptospira* with different biofilm forming abilities on abiotic surface at different day	52
Table 4.2	Number of tested saprophytic *Leptospira* with different biofilm forming abilities on abiotic surface at different day	52
Table 4.3	Number of tested intermediate *Leptospira* with different biofilm forming abilities on biotic surface (wood chips) at different day	61
Table 4.4	Number of tested saprophytic *Leptospira* with different biofilm forming abilities on biotic surface (wood chips) at different day	61

LIST OF FIGURES

		Page
Figure 2.1	The schematic drawing represent the cycle process of biofilm formation	20
Figure 2.2	The life cycle of *Artemia salina*	24
Figure 2.3	The different cross section of male and female *Artemia salina* leach. Male *Artemia salina* composed of claspers while female *Artemia* salina has brood sac to store their cysts	25
Figure 4.1	A (*Leptospira wolffii* serovar Khorat strain Khorat-H2 (G7-intermediate)); B (*Leptospira inadai* serovar Aguaruna strain MW4 (G15-intermediate)); C (*Leptospira meyeri* strain 19CAP (S19-saprophytic)) and D (*Leptospira biflexa* strain Patoc strain Patoc 1 (S1-Saprophytic)) were showed hooked end and spiral leptospiral cells under dark field microscope under 400X magnification. All leptospiral cells were confirmed active viable at concentration of 10^6- 10^8 CFU/ml or OD_{420}= 0.3-0.4 after 30 days at 30 ^oC incubation at room temperature	40
Figure 4.2	Crystal violet stained cell for G7, intermediate *Leptospira* in 11 days. The highest biofilm production was observed from day 5 to day 8. From day 1 to day 4, the biofilm cells attached and formed microcolonies. From day 5 to day 8, the biofilm strongly formed and attached well at the bottom of well. The cells clumped and resist from washing step. At day 9, the cells started to disperse and detachment from bottom plates until day 1	45

Figure 4.3 The crystal violet stained cell for S19, saprophytic *Leptospira* in 11 days. The From day 1 to day 2, no biofilm cell was attached on the bottom wells. At day 3 onwards, the biofilm formed bottom wells intense with multiple colonies resisted until 5 from washing step. At day 6, the biofilm cells attached on bottom well plates started to detach from the well. From day 10 onwards, no biofilm cells were observed highest biofilm production was observed as the strongest from day 3 to day 5 45

Figure 4.4 Quantification of biofilm formation (OD_{600}) for 15 intermediate *Leptospira* comprises of 4 soil samples, 10 water samples, 1 rat liver sample tested on abiotic surface (24 well microtiter plates) compared with positive control from FRST, Unimas. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments 48

Figure 4.5 Quantification of biofilm formation (OD_{600}) for 15 saprophytic *Leptospira* comprises of 3 soil samples, 4 water samples, 8 rat liver samples tested on abiotic surface (24 well microtiter plates) compared with positive control from IMR, Malaysia. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments 49

Figure 4.6 OD_{600} of biofilm formation on wood surface in G7 (*L. wolffii* serovar Khorat strain Khorat-H2). The OD for strongest biofilm producer of intermediate strain was reported as 2.335 ± 0.052 on day 5 54

Figure 4.7 OD_{600} of biofilm formation on wood surface in S19 (*L. meyeri* strain

19CAP). The OD for saprophytic strain was recorded as 2.308 ± 0.043 on day 5 of biofilm assay 54

Figure 4.8 The stained cell of intermediate, G7 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) from water sample viewed under phase contrast microscope. On day 1, the leptospires do not stained well on the woodchip. However, on day 2 onwards the crystal violet had retained well on the wood chip surface. At this phase, the dye was resistant to the washing step. The dye started faded on day 11 due to biofilm detachment 56

Figure 4.9 The stained cell of saprophytic, S19 (Leptospira meyeri strain 19CAP) from water sample viewed under phase contrast microscope. On day 1, the leptospires do not stained well on the woodchip. However, on day 2 onwards the crystal violet had retained well on the wood chip surface. At this phase, the dye was resistant to the washing step. The dye started disappeared on day 10 due to biofilm detachment 56

Figure 4.10 Quantification of biofilm formation (OD_{600}) for 15 intermediate *Leptospira* comprises of 3 soil samples, 4 water samples, 8 rat liver samples tested on biotic surface (*Dyera Costulata*) compared with positive control from FRST, Unimas. The amount of biofilm was quantitatively measured at OD_{600}.Error bars are standard errors derived from triplicate experiments. 58

Figure 4.11 Quantification of biofilm formation (OD_{600}) for 15 saprophytic *Leptospira* comprises of 3 soil samples, 4 water samples, 8 rat

liver samples tested on biotic surface (*Dyera Costulata*) compared with positive control from IMR, Malaysia. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments 59

Figure 4.12 Light micrographs of intermediate, G7 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) formed biofilms on glass slide (A to F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. The cells on (A) formed a matrix from cell to cell. The highest number of cell was observed on 24 h (C). The number of cells started to decrease in (E) and (F) 64

Figure 4.13 Light micrographs of intermediate, G15 (*Leptospira inadai* serovar Aguaruna strain MW4) formed biofilms on glass slides (A) to (F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. The cells appeared to be in a single cell, (B) to (D) appeared to clump and become a matrix. The highest number of cell was observed on 24 h (C). The number of cells became lesser as in (E) and (F) 64

Figure 4.14 Light micrographs of saprophytic, S11 (*Leptospira meyeri* strain Semaranga_DB49) formed biofilms on glass slides (A) to (F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. A does not show a single cell. The cells formed matrix from (C) onwards and started to decrease at (F). The highest number of cell was observed on 24 h (C) 65

Figure 4.15 Light micrographs of saprophytic, S19 (Leptospira meyeri

strain 19CAP) strain biofilms on glass slides (A) to (F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. (A) only show a few cells, (B) to (E) shows clump and (F) shows less number of cell. The highest number of cell was observed on 24 h (C) 65

Figure 4.16 The representative amplication of random sequence of *ica C* gene for identification of biofilm gene in 10 intermediate *Leptospira* isolates with strong biofilm forming ability. Lane M showed the 100bp DNA gene ruler (Transgen), lane 1 represented positive control (*Leptospira wolffii* serovar Khorat strain Khorat –H2). Lane 2 to 11 represented the representative biofilm isolates, lane 2-G7, lane 3-G15, lane 4- G5, lane 5-G3, lane 6-G13, lane7-G16, lane 8-G17, lane 9-G29, lane 10-G21, lane 11-G12. Lane 12 represented the negative control 67

Figure 4.17 The representative amplication of of *ica C* gene for identification of biofilm gene in 10 saprophytic *Leptospira* isolates with strong biofilm forming ability. Lane M showed the 1kb DNA gene ruler (Transgen), lane 1 represented positive control (*Leptospira biflexa* serovar Patoc strain patoc 1). Lane 2 to 11 represented the representative biofilm isolates, lane 2-S19, lane 3-S11, lane 4- S21, lane 5-S14, lane 6-S12,lane7-S20, lane 8-S34, lane 9-S16, lane 10-S5, lane 11-S3. Lane 12 represented the negative control 68

Figure 4.18 Comparison of survival analysis of brine shrimp treated with S19, *Leptospira meyeri* strain 19CAP, G7, *Leptospira wolffii*

serovar Khorat strain Khorat-H2, P18, *Leptospira noguchii* strain LT796 (positive control) at 10^3 cfu/ml in 3 days. At day 3, no survival of brine shrimp after treated with pathogenic *Leptospira*, 10% and 20% for intermediate and saprophytic *Leptospira* respectively 71

Figure 4.19 Comparison of survival analysis of brine shrimp after treated with S19, *Leptospira meyeri* strain 19CAP, G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2, P18, *Leptospira noguchii* strain LT796 (positive control) at 10^3 cfu/ml in 3 days. At day 3, no survival of brine shrimp after treated with pathogenic *Leptospira*, 15% and 25% for intermediate and saprophytic *Leptospira* respectively 71

Figure 4.20 Comparison of survival analysis of brine shrimp after treated with S19, *Leptospira meyeri* strain 19CAP, G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2, P18, *Leptospira noguchii* strain LT796 (positive control) at 10^3 cfu/ml in 3 days. At day 3, only 5% of survival rate of brine shrimp after treated with pathogenic *Leptospira*, 20% and 30% for intermediate and saprophytic *Leptospira* respectively 72

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Base pairs
cm	Centimeter
cfu	Colony forming unit
DNA	Deoxynucleotide triphosphate
dNTPs	Deoxynucleotide triphosphate
EtBr	Ethidium bromide
EMJH	Ellinghausen-McCullough-Johnson-Harris
g	Gram
kb	Kilobase pairs
MgCl2	Magnesium chloride
M	Molar
mg	Milligram
min	Minute
Mm	MilliMolar
mL	MilliLiter
µM	MicroMolar
µmL	MicromilliLiter
µg	Microgram
µm	Micrometer
µL	Microliter
mol	Mole

sec	Second (s)
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
rpm	Revolution per minute
Taq	Thermus aquaticus DNA polymerase
TBE	Tris-borate EDTA electrophoresis buffer
TE	Tri-EDTA buffer
USA	United State of America
V	Volts
%	Percent
^{O}C	Degree centigrade

CHAPTER 1

INTRODUCTION

1.1 Overview of the study

Leptospirosis, or commonly known as rat urine disease is caused by *Leptospira* that is transmitted by host animals to human through direct or indirect exposure from contaminated water and food. Rodents are the most well-known natural carrier that is associated with leptospirosis (Erickson, 2017). Other natural carriers that are associated with leptospirosis include cattle, sheep, swine, dogs and buffaloes (De Vries et al., 2014; Chadsuthi et al., 2017). The occurrence of leptospirosis is commonly associated to heavy rainfall and floods which increase the risk of leptospirosis by bringing the bacteria and their animal host into close contact with humans (Lau et al., 2010).

Leptospirosis affects 900,000 people annually worldwide and it has been gazetted as a notifiable disease in many sub-tropical and tropical countries including Malaysia under the Prevention and Control of Infectious Diseases Act 1998 (Bharti et al., 2003). In 2017, the leptospirosis cases reported increases drastically 68.8% from 2011 to 2016 with a total of 3,143 cases and Sarawak reported as the second highest state with 844 leptospirosis cases (Ministry Health Malaysia, 2017). Severe cases of leptospirosis can cause jaundice, liver failure, renal failure and even fatal in human.

Biofilm is known as a layer of cells embedded underneath exopolysaccharides. Most bacteria species can form biofilm that helps them to survive in extreme environment. Like any other bacteria, *Leptospira* spp. can form biofilm and further mature to disperse and become planktonic cells. In general, the term biofilm refers to the layer of bacterial cells that co-operate and become colonies of microorganism for cell attachment. When the

infected hosts urinate, the bacteria contaminate the surface of water and allow them to survive in extreme environment for a long period of time. Therefore, it helps *Leptospira* spp. to build a protective casing (biofilm) around themselves for protection (Picardeau, 2008). According to Donlan and Costerton (2002), the biofilm community is defined as the irreversible cells that are attached to the substratum or interface to each other and embedded in polysaccharide matrix that can alter the bacteria growth rate and gene transcription. By adapting to external threats, biofilm helps the microbial communities to increase the resistance against antibiotics or host immune system (Hoffman et al., 2005). A study also mentioned that biofilm can increase the antibiotic resistance up to a thousand fold which causes insensitivity against the immune system (Potera, 2010).

World Health Organization (WHO, 1999) stated that microorganism will grow as biofilm on surfaces in contact with water. Once the microbial cell has irreversibly attached to the surface, the process of biofilm maturation on the surface starts. By integrating the time course study, biofilm forming ability can be observed continuously on different type of surfaces; abiotic and biotic. Abiotic surface was used to represent *in vitro* environment while biotic surface is to mimic *in vivo* environment. To the best of our knowledge, this is the first reported study in Malaysia using biotic surface to analyse time course study of biofilm formation in *Leptospira.* Moreover, the quantity of biofilm mass produced by the bacterial cell can be measured to identity strong biofilm producers. Numerous studies demonstrated the important findings of strong biofilm producers that provide potential therapeutic applications particularly in multidrug resistant bacteria on medical devices, chronic infections and food products (Hoyle & Costerton, 1991; Sahal & Bilkay, 2014; Hashem et al., 2017).

A previous study has reported that the transition from planktonic cells to mature biofilm requires the regulation of genes that are involved during biofilm formation. To date, there is no study on the characterisation of biofilm gene being reported on intermediate *Leptospira*. For specific gene characterisation, *LipL32* gene is highly conserved in pathogenic *Leptospira,* 16S rRNA gene targeted on intermediate *Leptospira* and *rrs* gene used to detect saprophytic *Leptospira* (Cetinkaya et al., 2000; Vein et al., 2012; Pui et al., 2015). In gene expression, the biofilm genes are primarily involved in leptospires motility, sugar or lipid metabolism, outer membrane-encoding gene and DNA replication (Iraola et al., 2016). This study aims to evaluate the biofilm producers of intermediate and saprophytic *Leptospira* that are able to express *icaA, icaB, icaC, icaD, bap, ompL1, flaB* and *galK* genes. The *icaADCB* operon encodes for the synthesis of intercellular polysaccharide adhesion (PGA) and for cell-to-cell adhesion in *Staphylococcus aureus* (Mirzaee et al., 2014). Meanwhile transcriptome sequencing found that overexpression of *ompL1* encodes for leptospiral extracellular matrix (ECM)-binding protein and plasminogen receptor. As for *flaB* encodes for leptospiral of the flagellar filament for motility and *galK* genes encodes for UDP-glucose 4-epimerase in *Leptospira biflexa* (Iraola et al., 2016).

The potential of biofilm forming ability in *Leptospira* on surfaces is of importance, not only for their survival strategy but also can contribute to disease transmission and pathogenicity (Iraola et al., 2016). To further understand the bacterial virulence of biofilm producers, our study incorporated a model organism in our virulence assay. A simple, fast and short-life span of brine shrimp model organism was selected to study the bacterial virulence in *Leptospira* spp. Other pathogenic bacteria such as *Pseudomonas aeruginosa, S. aureus* and *Vibrio vulnificus* were widely used for bacterial virulence assay (Lee et al.,

2014). Hence, the aim of the study was to investigate motility of strong biofilm producers of *Leptospira* spp.

Different approaches have been used to elucidate the structure of biofilm in *Leptospira* spp. under the microscope. Phase contrast light microscope and dark field microscope were employed to view the amount of biofilm matrix and cell-to-cell interaction under microscopic view (Ristow et al., 2008). Under dark field microscope, the leptospires cell demonstrates active movement and morphologically seen as long, thin and spiral shapes. Under phase contrast light microscope, the amount of bacterial cell attached on the glass slide can be seen clearly. Thus, the biofilm forming ability in this study can add new value to the existing biofilm data analysis in *Leptospira* spp. specifically for intermediate strains.

1.2 Research Questions

This research project is designed to address the following problem statements:

i. Does biofilm contribute to the pathogenicity of *Leptospira* strains isolated locally from the environment of Sarawak?
ii. What are the genes responsible in the formation of biofilm among the selected *Leptospira* strains?
iii. Is there any difference among the level of virulence in pathogenic, intermediate and saprophytic strains?

1.3 Problem Statement

Each year approximately 300,000 to 500,000 severe cases reported annually and up to 30% mortality (Tilahun et al., 2013). About 5 to 10% mortality rate reported annually in developing countries which assumed to carry huge burden. Leptospirosis is recognized as a globally re-emerging disease with a marked increase in the number of cases in Latin America and Southeast Asia included Malaysia (Hartskeerl, 2006). Besides, leptospirosis has been gazetted as notifiable disease in Malaysia under the Prevention and Control of Infectious Diseases Act 1998. While according to Sarawak Health Department (2013), there are total of 271 severe cases and 10 deaths in which resulted from leptospirosis notified in 2012 carry 404 severe cases and 24 deaths which reported in 2013. However there is not much known about factors such as the genes that involved could contribute to the pathogenesis of these strains, in particular those isolated from the habitats in Sarawak. Through this study, we will have better understanding on the survival of *Leptospira* in the diverse environment conditions which may relate to transmission route and hence help in the prevention of leptospirosis outbreak in Sarawak and Malaysia.

1.4 Objectives

The main objectives of this study are:

i. To quantify the biofilm mass in intermediate and saprophytic *Leptospira* using time course study.
ii. To detect the genes involved in biofilm formation by intermediate and saprophytic *Leptospira*.
iii. To determine bacterial virulence analysis in selected biofilm producer of *Leptospira* spp. in *Artemia salina.*

1.5 Hypothesis

The biofilm forming ability of intermediate strains is stronger compared to saprophytic strains. Therefore, the biofilm genes can be detected among the strong biofilm producers. By using the model organism, the bacterial virulence analysis can be used to determine the infectious doses among biofilm producers.

1.6 Thesis Organisation

This thesis is organised into five chapters. Chapter 1 comprises general introduction of the study, research problem statements, objectives and research questions. Chapter 2 includes the literature review, while Chapter 3 comprises of materials and methods. Next, Chapter 4 consists of result and discussion and Chapter 5 includes the general conclusion and recommendation for future studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Discovery of *Leptospira* genus

In 1886, *Leptospira* was first discovered and known as the causative agent for severe human syndrome of Weil's disease. The first symptom was described as a type of jaundice with splenomegaly, renal dysfunction, conjunctivitis and skin rashes (Weil, 1886). General symptoms that are cause by *Leptospira* include jaundice, conjunctivitis, inappetence, anemia, hemorrhages and albuminuria. The first sample was isolated from patient died due to yellow fever and the spirochetes were isolated from kidney by Stimson in 1907. In Japan, the symptoms were identified as "autumn fever" or "seven day fever" while in Europe and Australia, the symptoms were recognized as "cane-cutter's diseases", "swine-herd's disease" and "Schlammfieber or mud fever" (Kitamura & Hara, 1918; van Thiel, 1948; Alston & Broom, 1958). As the research progressed, the bacterium was further known as *Spirochete interrogans* (*Leptospira interrogans*). The transmissibility including routes of infection, pathological changes, tissue distribution, urinary excretion, leptospiral filterability morphology and their motility were further discovered. The microorganism was named as *Spirochaeta icterohaemorrhagiae* and it was one of the first survival *Leptospira* strains (Ictero No. 1). Subsequently, it was officially accepted by the Subcommittee on the Taxonomy of *Leptospira* in 1990 as the Type Strain of *Leptospira interrogans* (Marshall, 1992).

Rat is known as the famous zoonotic carrier of *Leptospira.* In Malaysia, it causes disease commonly known as "Rat Urine Disease". Malaysia is well known to have tropical climate and it has conducive environment for leptospirosis outbreak. In 1917, a study

conducted by Ido and his team (Ido et al., 1917; Levett, 2006) reported that rats were the main renal carriers of *Leptospira*. Their findings were supported by other group who worked on spirochetes in the kidneys of field mice. These researchers made the key observation based on rats' kidney, the condition of the mice (healthy or unhealthy) and rat appeared to as asymptomatic carrier state. Other studies suggested that rodents are major animal species that can shed leptospires throughout their lifespan without clinical manifestation or prolonged carrier state (Cosson et al., 2014). World Health Organisation stated the leptospiral seropositivity has been observed in many wild animal species including opposums and sea lions (WHO, 2011). Nonetheless, their role in epidemiology of human leptospirosis remains unclear. Other than rodents, natural carriers for *Leptospira* are also often associated with dogs, pigs, cattles (Priya et al., 2007; Adler & de la Pena Moctezuma, 2010). Currently, leptospirosis has emerged as the most widespread zoonosis and notifiable disease worldwide in many domestic animal species such as rat, swine and etc.

2.2 General Characteristics and Taxonomy of Pathogenic, Intermediate and Saprophytic *Leptospira*

Generally, spirochete has two families which include as spirochaetaceae and leptospiraceae. In Leptospiraceae family, it includes *Leptonema* genus, Turneriella genus and *Leptospira* genus-the agent of leptospirosis (Kososey-Vrain, 2004). Initially, *Leptospira* spp. was clustered into two species namely, pathogenic and saprophytic *Leptospira* (Levett, 2015). Due to the discovery of several novel species, the genus of *Leptospira* was amended based on 16S rRNA gene sequence comparison and DNA-DNA re-association studies. The genus was further classified into three classes; pathogenic, intermediate and saprophytic *Leptospira* (Zuerner, 2011). In pathogenic strains, *Leptospira*

genus comprises of 8 species known as *L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. alexanderi* and *L. alstonie* (Levett, 2001).

Intermediate *Leptospira,* also known as opportunistic pathogens consist of 6 species and different serovars. The six species reported are *Leptospira wolffii, Leptospira inadai, Leptospira fainei, Leptospira broomii* and *Leptospira licerasiae*. To our best knowledge, the intermediate species have not been well studied. Some studies suggested that the intermediate *Leptospires* can evolve and further become pathogenic strains. Intermediate species include *Leptospira broomii* (Levett et al., 2006), *Leptospira licerasiae* (Matthias et al., 2008), *Leptospira wolffii* (Slack et al., 2008) and *Leptospia kmetyi* (Slack et al., 2009). In a recent study by Pui et al. (2015), the intermediate *Leptospira* strains were characterised using 16S rRNA gene. Another study also showed the presence of intermediate leptospiral DNA and the absence of serious symptoms of leptospirosis that reported mild disease and linked to intermediate *Leprospira* species- *Leptospira broomii* (Chiriboga et al., 2015).

Saprophytes strains also known as the free living microorganism do not generally cause any disease. Saprophytic *Leptospira* has similar morphological characteristics as pathogenic *Leptospira* but they differ in biological characteristics. The genus of saprophytic Leptospires includes *L. biflexa, L. meyeri, L. yanagawae, L. kmetyi, L. vanthielii* and *L. wolbachii*. The first saprophytic strain isolated was reported as *Leptospira biflexa* (Wolbach & Binger, 1914).

Leptospira spp. are a long, spiral shaped, thin and motile bacteria with an average diameter of approximately 0.1 µm length between 6 to 20 µm, with helic amplitude of 0.1 to 0.15 µm and wavelength of 0.5 µm (Goldstein & Charon, 1990). *Leptospira* can be

observed and are best viewed under dark- field microscope and phase contrast light microscope. The structural membrane of *Leptospira* is located on the outer membrane components of lipopolysaccharide (LPS), the porin, OmpL1 and the lipoproteins LipL41 and LipL36. The ultrastructure of *Leptospira* one similar to that of gram negative bacteria which include an outer membrane associated with lipoposaccharides. Besides, Levett and Haake (2015) also stated that leptospires consisted of two axial flagella lying under the membrane sheath. According to Bharti (2003), *Leptospira* exhibit features of both gram-positive and gram-negative bacteria in which their morphology is indistinguishable and only can be differentiated by mode of living. During incubation, *Leptospira* are motile, obligate aerobes, and known as slow-growing bacteria that have an optimal growth temperature of 30 ^{o}C (Bharti, 2003; Trueba et al., 2004).

2.3 Growth of *Leptospira*

Generally, leptospires cell is known as slow-growing bacterium compared to other common bacteria. Leptospires cell grows conducively at optimum temperature between 28 ^{o}C and 30 ^{o}C. The first media used to isolate *Leptospira* was Fletcher or Vernoort semi-solid media. Nowadays, the common media for culturing and maintaining *Leptospira* strain is Ellinghausen-McCullough-Johnson-Harris (EMJH) media. The media include the supplements such as long chain fatty acids, ammonium salts and vitamin B1 and B12 which are essential for the growth of leptospires. EMJH media was first discovered by Ellinghausen and McCullough in 1965, followed by a few modifications (addition of tween) by Johnson and Harris in 1967. There are a few studies of growing *Leptospira* using sterile water, synthetic media and broth but the attempt was unsuccessful (Wolbach & Binger, 1914). In addition, the contamination issues in the nutrient rich media were

serious in *Leptospira* strains. Studies have shown that cross-contamination generally occurs and subsequently causes the outgrowth of saprophytic strains in a mixed culture with pathogenic strains (Wilson & Fujioka, 1995; Ganoza et al., 2006). This situation usually happens during isolation of pathogenic strains from environmental sources such as contaminated water and soil. Therefore, it is recommended to check all the cultures regularly using dark field microscope for evidence of growth or potential contamination.

Saprophytic strains required 30 days to reach stationary phase in EMJH media (Saito et al., 2013). For solid media cultivation, leptospires form transparent heterogeneous colonies inside the agar. The leptospires may need different replication cycle or incubation period for pathogenic and non-pathogenic strains. For saprophytes, they can replicate within 8 to 16 hours while 16 to 24 hours for pathogenic strains. The colonies are visible after one week for saprophytic and one month for pathogenic strains.

2.4 Outbreaks of Leptospirosis Cases

The first few isolated *Leptospira* spp. included *Leptospira interrogans* serovars Icterohaemorrhagiae*, L. interrogans* serovar Hebdomadis and *L. interrogans* serovar Pyrogenes (Fletcher et al., 1928). Since then, more than 500, 000 cases of severe leptospirosis occur each year, with a mortality rate of 5-20% (WHO, 1999). The first case of leptospirosis in Malaysia was reported in April 1925 (Bahaman & Ibrahim, 1988). After that, a drastic number of leptospirosis cases were reported in Malaysia especially between 2010 and 2015. Recently, the health ministry of Malaysia revealed that the leptospirosis cases have increased from 2,268 cases (55 deaths) in 2011 to 8,291 cases (52 death) in 2015. Leptospirosis is known as a bacterial infection which commonly transmitted by animals.

Leptospirosis diseases are commonly known as Weil's disease, Weil-Vasilyev disease, Swineherd's disease, rice-field disease, waterborne disease, nanukayami fever, cane-cutter fever, swamp fever, mud fever, Stuttgart disease and Canicola fever. This infection poses risk mostly due to the occupational exposure, recreational activities and household exposure. Over the years, approximately 0.10 to 975 per 100,000 populations infected by leptospirosis globally with 6.85% of fatal cases worldwide reported. India reported 282 deaths due to leptospirosis outbreak that caused involved the pulmonary system and central nervous system failures (Garba et al., 2017).

Many factors contribute to leptospirosis epidemiology which remains unknown because there is only limited knowledge on leptospiral population genetic and the role of environmental factors such as environmental persistence of leptospires during disease occurrence. Due to their complex interaction between humans, animals and the environment, leptospirosis occurs more in the tropical regions compared to temperate regions such as South Asia, South East Asia, Africa, and Central Latin America (Pappas et al., 2008). The epidemicity of the disease is mainly located in the Caribbean, South America, Southeast Asia and Oceania (Costa et al., 2015). According to Costa et al. (2015), they identified high cases in rural population and tropical regions compared to urban settings. Table 2.1 shows the annual occurrence of leptospirosis in humans in the selected high risk countries.

Table 2.1: The annual occurrence of Leptospirosis Worldwide Cases

Country	Cases/ per 100,000 population	Deaths
South-East Asia	266,000	14,200
Carribean	22,300	1,300
East Asia	142,000	6,900
South Asia	289,000	16,500
Central Latin America	36,000	1,600
North Africa	33,300	1,600
Oceania	16,700	1,100

However, leptospirosis disease is well known as environmentally transmitted disease and therefore varies in different environmental settings (Lau et al., 2010). The factors can be contributed to the transmission of leptospirosis disease such as rainfall and flooding, temperature, exposure to animals, poor sanitation and inadequate waste disposal. In Malaysia, leptospirosis cases indicated a progressive trend in incidence and mortality. Early study in Malaysia with total of 30 pathogenic leptospiral serovars identified by Alexander et al. (1957) showed a high sero-prevalence in humans throughout West Malaysia (Thayaparan et al., 2013). The highest distribution of West Malaysia was found in labourers working in rubber estate, sewage, drainage, forestry and town cleaning industries. Another comprehensive investigation of leptospirosis conducted in 1950s reported the tests of various mammals from a range of environments (Alexander et al., 1957). Majority studies found that leptospirosis is endemic in Malaysia. Leptospirosis cases reported 68.6% increases drastically from 2011 to 2016 with a total of 3,143 cases. Different environment may circulate and produce different *Leptospira* serovars. The statistical data collected from Ministry of Health (MOH) is show in Table 2.2.

Table 2.2: Total of leptospirosis cases from 2011 to 2016 in Malaysia

States	2011	2012	2013	2014	2015	2016	Total
Perlis	19 (1)	27 (2)	11	20 (1)	16	11	104 (4)
Kedah	111 (1)	267 (5)	294 (11)	699 (7)	760 (3)	410 (4)	2,541 (31)
P.Pinang	33	128 (2)	98 (3)	191 (4)	140 (4)	42 (2)	712 (17)
Perak	242 (7)	280 (4)	429 (1)	621 (8)	502 (10)	340 (1)	2,414 (31)
Selangor	442 (13)	853 (7)	1,352 (14)	1,832 (8)	1,233 (12)	847 (8)	6,559 (62)
K. Lumpur	246 (2)	282	410	350 (1)	49 (6)	179	1,516 (9)
Negeri Sembilan	155 (3)	152 (2)	177(3)	302(9)	296(7)	169(2)	1,251 (26)
Melaka	191(3)	441 (2)	108(1)	141	231(1)	227 (6)	1497 (12)
Johor	55(2)	69 (1)	62	387 (1)	306(4)	207 (7)	1524 (32)
Pahang	133(2)	163	277(5)	288(3)	378(1)	239(3)	1,478 (14)
Terengganu	137(9)	126(3)	168(4)	374(1)	789(5)	691(2)	2,285 (24)
Kelantan	276(1)	168(4)	264(11)	1,030 (16)	1,956(14)	863(5)	4,557 (51)
Sabah	68	410(8)	367(10)	930 (15)	470(3)	174(9)	2,419 (45)
Labuan	3(2)	7(2)	6	25(1)	296	42	379 (5)
Sarawak	157(9)	292(7)	434(8)	616 (17)	800(8)	844(3)	3,143 (52)
Total	2,268 (55)	3,665 (48)	4,457 (71)	7,806 (92)	8,291 (78)	5,285 (52)	31,772 (396)

()-number of fatal cases Ministry of Health Malaysia, 2017

According to Table 2.2, Selangor reported has highest occurrence of leptospirosis cases in Malaysia with 1,832 cases and followed by Kelantan with 1,030 leptospirosis cases in 2014. It also shows the diversity of serovar distribution in different localities in East Malaysia. In Sarawak out of 723 isolates *Leptospira* from rats, soil and water samples, the

study found that 6.4% pathogenic *Leptospira*, 1.9% intermediate *Leptospira* and 2.2% of saprophytic *Leptospira* (Pui et al., 2017).

Ministry of Health Malaysia (Health Indicator 2011-2016) reported the annual incidence rate of leptospirosis as 100, 000 populations every year. A total of 31,772 leptospirosis cases and 396 fatal cases were reported from 2011 to 2016 in Malaysia. Among the 15 states in Malaysia, Selangor has the highest cases of 6,559 cases with 62 fatal cases, followed by Kelantan which recorded 4,557 cases with 51 fatal cases. One of the main factors for the high prevalence of leptospirosis in Kelantan, Perak and Selangor may be due to heavy rainfall and flooding. In rural areas, leptospirosis cases are often related to poor hygiene, overcrowding and poverty (Lau et al., 2010). The *Leptospira* sample was mostly collected during the flooding season (Samsudin et al., 2015).

Besides rainy season, occupation such as town cleaners may exposes to leptospirosis disease (Levett, 2001). In Selangor, a study conducted among the healthy municipal service workers proved a total of 34.8% leptospirosis cases (Samsudin et al., 2015). Besides, many studies only focus on animal leptospirosis in Malaysia and therefore researcher shifted the domestic animal studies to *in vivo* studies extensively. However, serovars are not traditionally isolated from domestic animals and *Leptospira* spp. has been isolated more on environmental samples such as soil and water (Garba et al., 2017). Based on the statistical data of leptospirosis in Malaysia, Sarawak also poses high prevalence of leptospirosis cases. The leptospirosis cases were reported 434 cases in 2013 and doubled to 800 and 844 leptospirosis cases in 2015 and 2016 respectively.

2.5 Microbial Biofilm

One of the factors that contribute to pathogenesis is the presence of biofilm in microbes. The multiple species of microbial communities are often associated with each other and their environment. Hence, the arrangement in biofilm architecture of microbes in microcolonies state has important function in microbial communities. According to Davey and O'toole (2000), the microbial biofilm able to position themselves in a niche where they can propagate. One of the most common positioning mechanisms is attachment. During attachment process, the microbes can adapt and position themselves on a surface and form a microbial communities by obtain additional benefit of their phenotypic versatility beside their neighbours. Microbial communities are known to have good interaction to a surface and they encounter with attraction and repelling forces which vary according to the presence of interface properties, nutrient availability, the composition of microbial community and hydrodynamics. These numerous conditions can affect the structure of biofilm communities. One of the common bacteria *Escherichia coli*, the presence of biofilm matrix contribute to development of phenotypic resistance of pathogenic *E. coli* biofilms and hence lead to persistent infections (Justice et al., 2004; Beloin et al., 2008). On the other hand, biofilm prefers to form on smoother surfaces which are stronger and more resistant to rupture. On the rough surface, biofilm tends to possess a low tensile strength and break easily. According to Characklis (1981), the extent of microbial colonization was heightened with increase of the surface roughness because to shear forces are reduced and the surface area of rough surfaces is larger.

In medical context, bacterial biofilm have proven to affect human health. The attachment of bacteria on medical devices can cause indirect transfer infectious disease to

the human body. The biomedical devices such as intravascular catheters, urinary catheters and orthopaedic implants are commonly found to be able to cause bacterial adhesion (biofilm) (Francolini & Donelli, 2010). *Acinetobacter baumannii*, a nosocomial pathogen also causes nosocomial infections by forming biofilm on hospital equipment and indwelling medical devices (Djeribi et al., 2012). It can cause a wide range of urinary tract infections, pneumonia, soft tissue infections and sometimes mortality.

Biofilm is a complex structure that helps bacteria cells to protect themselves from antimicrobial action and also for survival. Almost all bacteria cells are able to form biofilm including lestospiral. The first bacterial biofilm was reported in 1978 with the role of polysaccharide glycocalyx formation by *Streptococcus mutants* on teeth (Hamada & Slade, 1980; Matsumoto-Nakano, 2018). Biofilm consists of layers of adherent microbial cells attach on the lower layer, amorphous layer and the fluid layer. In microbial community, the microbes attached with polysaccharide matrix consisting organic and inorganic materials on the lower layer of biofilm. The upper layer is known as amorphous layer and further extends to outer surrounding (Chandki et al., 2011). The inner layer of biofilm consists of fluid layer and was covered by stationary and dynamic sub layers. There are two classification of biofilm based on their location and pathogenicity which was also known as cariogenic and periopathogenic. In gram positive and acidogenic bacteria, the biofilm acquire pathogenicity as cariogenic while in gram negative and basophilic bacteria they retain their pathogenicity is known as periopathogenic (Ximénez-Fyvie et al., 2000).

There are a few factors that affect the biofilm formation of bacteria due to their genetic adaptation which enables the bacteria to easily adapt to the sudden shifts in nutrient availability and their response towards environmental conditions. The mode of biofilm

formation has been studied widely among the researchers due to their role of adaptation that can contribute to the bacteria pathogenicity. The most common mode of bacterial adaptation is that biofilm can highly tolerate antimicrobial agents at high concentration of 10-100 times that can kill genetically planktonic bacteria (Jefferson, 2004). Besides, biofilm is also identified to extraordinary resistance to phagocytosis, so biofilm can hardly be removed from the living hosts. Therefore, it increases the concentration of antibiotics that is needed to treat biofilm related infections.

In previous study, *Leptospira* planktonic cell was studied on the antimicrobial susceptibility minimum bactericidal concentration (MBC) using penicillin G (25-100 U/ml), ampicillin (12.5–50 mg/ml), tetracycline (50-100 ug/ml) and doxycycline (50-100 ug/ml). It was noticed that antimicrobial concentration on biofilm formation carried higher resistance compared to the antimicrobial concentration in planktonic cells. Bacterial cell that produces biofilm favours high resistance which can reach up to 1000 folds of normal antibiotic for inhibition. It was suggested that high concentration of antibiotic to be uses to inactivate the organisms growing in a biofilm (Mah & O'Toole, 2001). The first study on high tolerance of antibiotics that are related to biofilm were reported by van Leeuwenhock in 1684 (Shi & Zhu, 2009). It was observed that animals (bacterial infection) within the scurf (the plaque) on teeth were found outside the plaques which were killed. *Leptospira* are also known to have a good tolerance with stressful environment such as low nutrition condition that can cause the bacteria to grow extremely elongated which then result in decreased motility and poor cell health.

Another factor that play important role in microbial biofilm growth is time course study in microbes. Time course study was observed through the contact time between

biofilm and the surfaces under different time intervals. However, there is lack of knowledge of time course study in *Leptospira* spp. A study conducted by Tang et al. (2012) documented the time course study of *Salmonella enterica* serovar Typhimurium in forming biofilm on the fresh produce during processing, harvest or any point of production. In this study, mango and guava fruits were used as test surfaces and the OD value for mango was larger than OD value for guava. OD value represents the quantity of biofilm and it differs between guava and mango. Other than *S. Typhimurium*, *Listeria monocytogenes* that can be found in foods also has the ability to form biofilm at different conditions (Lee et al., 2014). Biofilm formation increases with time of incubation and was found to be higher at temperature of 45 ^{o}C. This contributes to the critical points in food processing line in which the persistent and contamination of *L. monocytogenes* need to be controlled.

2.6 Biofilm formation development

The biofilm formation comprises four main stages of biofilm forming ability which known as attachment, microcolonies, mature biofilm and detachment (Crouzet et al., 2014). Briefly, four stages involved in biofilm formation are includes attachment on the surfaces, then accumulation and multiplication to create micro-colonies of bacteria layers monitored by accumulation-associated protein. Then, it becomes matured biofilm, and finally detaches back to planktonic cells. It is very crucial to study the genes encoded for the mechanism that is responsible for development of biofilm layers which facilitate the bacterial adhesion and colonization of bacteria. Figure 2.1 shows the schematic diagram of biofilm formation in bacteria.

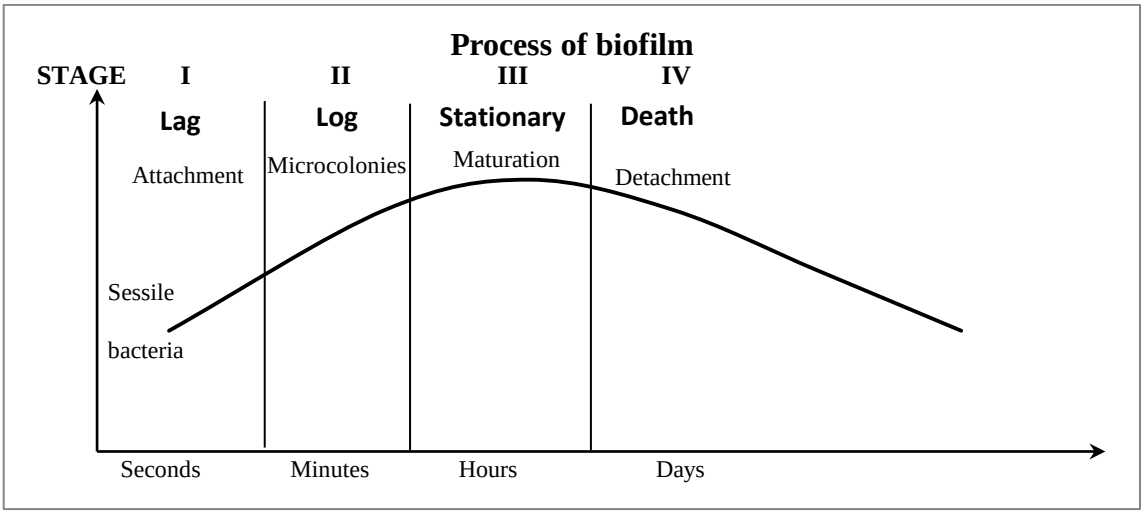


Figure 2.1: The schematic drawing represents the cycle process of biofilm formation (Chandki, 2011).

Interestingly, other study on life cycle of biofilm includes stages of conditioning, first contact, adsorption, growth, production of extracellular producers, attachment and re-entrainment (Gerbersdorf & Wieprecht, 2015). To further investigate how biofilm develop itself over the time, we must understand their first step which is known as conditioning. Conditioning is a process of adapting to the new habitat of the contact surfaces in a certain time (Loeb & Neihof, 1975; Suwarno et al., 2016). The transportation of molecules and small particles is fastidious and can cause the adsorption of conditioning films simultaneously. Therefore, the film will help to change the characteristics of the substratum and alter the critical surface tensions. Secondly, the main step of biofilm formation is the first contact of microbial communities on abiotic and biotic surfaces. Generally, the microbial communities in fluid form tend to transport via mass transport mechanisms such as sedimentation, motility, molecular diffusion, convection and diffusive transport (Gerbersdorf & Wieprecht, 2015).

Next, absorption process occurs using weak interactions with low specificity such as electrostatic or van der Waals forces where the cells are ready to absorb reversibly or irreversibly and cause the early colonies to form on the surface (Flemming et al., 2016). It is mediated through a specific and non-specific physiochemical association with the biofilm. This is followed by the growth of irreversible microbial cells by repetitive absorption and the rate of nutrient needed in supplying for their growth are extremely important. Subsequently, extracellular products undergone transition from planktonic to attached form and this step is regulated by encoding gene that responsible for the products (Iraola et al., 2016). Hence, the attachment process is now completed.

The colonized cells are formed form from bulk fluid that adheres to the existing biofilm. After attachment is done, the co-aggregation process takes place and bacterial cells attach through specific molecules in which the single cells in bulk fluid recognize the surface to adhere and develop biofilm. Lastly, the detachment of biofilm takes place where the bacterial cells are detached from the adhered surfaces and returned to it bulk fluid and planktonic form for their growth (Sanchez et al., 2016; Armbruster et al., 2018). A few factors that cause the detachment are erosion, starvation of nutrients, abrasion and either passive or active process which can lead the bacterial cell to survive or colonize.

As an evolution, the biofilm have caused the microbial infections which mainly caused in the dental cares and environment. Surprisingly, 80% of microbial infections include dental cares, periodontal disease, otitis media, musculoskeletal infections, necrotizinng fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native valve endocarditis, meloidosis, cystic fibrosis peneumonia and peri-implantitis (Socransky

& Haffajee, 2002; Nobile & Johnson, 2015). However, biofilm is normally associated with chronic persistent infections.

Leptospira can produce biofilm which is important for survival outside the host. Approximately 90% of the biofilm biomass consists of a self-produced extracellular matrix. The components of matrix that comprise extracellular polymeric substances (EPS) and carbohydrate binding proteins therefore assist the microbial biofilm to favour at specific environment(Cegelski et al., 2009; Flemming & Wingender, 2010).

2.7 Genes Associated with Biofilm

Biofilm is an essential process for a planktonic cell to adhere to the surfaces. Previous study showed the expression of gene during the first attachment and final detachment of biofilm in *Leptospira* (Ristow et al., 2008; Iraola et al., 2016). According to Iraola et al (2016), the leptospires cell can produce biofilm in order to survive when they are exposed to the environment. Besides using extracellular matrix (ECM) for biofilm formation, *Leptospira* also utilise cell migration, adhesion and aggregation with the support of gene regulation along the process. The importance of gene regulation can be a useful tool in genetic manipulation, drug design and vaccine development. Like any bacteria was found often persists on the surface and how biofilms play a role in pathogenesis of *Leptospira* is extremely useful for *in vivo* study. However, environmental conditions have an impact on the transition of biofilm that can trigger biofilm development.

Extensive study on saprophytic strain, *Leptospira biflexa* strain Patoc strain Patoc 1 (paris) found that profiling of gene expression of biofilm formation on abiotic surface with planktonic cells was carried out using RNA sequencing (Iraola et al., 2016). Their study

highlighted a few genes which play an important role of metabolic pathways that are related to biofilm formation. The *icaADBC* genes encode for proteins used in the synthesis of polysaccharide intercellular adhesion (PIA) or polymeric N-acetyl-glucosamine (PNAG) by ica operon encoded enzymes that have important roles during attachment, accumulation and production of an extracellular polysaccharide adhesin (Maira- Litran et al., 2002). One of the most upregulated genes of outer membrane protein (*OmpA*) homologs is associated with cell aggregation that is commonly found in *E. coli* and *Acinetobacter baumannii.* This gene can be related to biofilm aggregation in *L. biflexa* (LEPBI_I1873) which encodes for OmpA-like protein (Iraola et al., 2016). Another mechanism that may overexpress during the biofilm formation which can help in bacterial motility of *L. biflexa* is using flagellar gens, *FlaA* and *FlaB* that have unknown pleiotropic function in biofilm homeostasis and hence their motility mechanism may be insightful in future work. Besides, biofilm is also associated with overexpression of outer membrane protein which has been identified to be upregulated during formation of biofilm in *Leptospira*. The study demonstrated the important role of overexpression of *L. biflexa* genes that play an important role in maintenance of biofilm structure and give support during the attachment.

Apart from that, biofilm also need the main carbon sources for energy production and storage to maintain the structure of biofilm. Sugar and lipids (galactose) are the main nutrients for biofilm production especially in exopolysaccharides (EPS) and fatty acids (Rajput & Kumar, 2018). In *L. biflexa*, GalK (LEPBI_I0073) and one GalE-like gene coding for a UDP-glucose 4-epimerase (LEPBI_I0113) were downregulated in mature biofilm (Iraola et al., 2016). The study suggested the galactose metabolism play central cole in EPS production in *L. biflexa.*

2.8 Bacterial Virulence Study

The bacterial virulence study used to increase the fundamental understanding on mechanism of virulence factors and the potential novel target for antibacterial therapy in microorganisms. To study virulence of bacteria, several model organisms has been used such as *Caenorhabditis elegans*, hamster, zebrafish and *Galleria mellonella* (Davis et al., 2009). In most bacterial studies, bacterial virulence depends on infectious doses introduced into the host. According to Gomes-Solecki et al. (2017), the lethal infection doses needed for *Leptospira* sp. was between 10^6 to 10^8 cfu/ml. For 5 weeks old of hamster, continuous lethal doses in *Leptospira* spp. was needed with 50% of survival rate of *Leptospira kirschneri*, pathogenic strains needed between 10^2 to 10^6. In a six weeks old mice, a 50% of mortality rate was observed and resulted in chronic renal infections (Spradbrow, 1963). Following that, a study on the long term colonization of kidney required 10^7 bioluminescent version of *L. interrogans* serovar Manilae strain L495 to infect the mice (Retet et al., 2014). However, other early study reported that Syrian mice were susceptible to *Leptospira icterohemorrhagiae* (Morton, 1942).

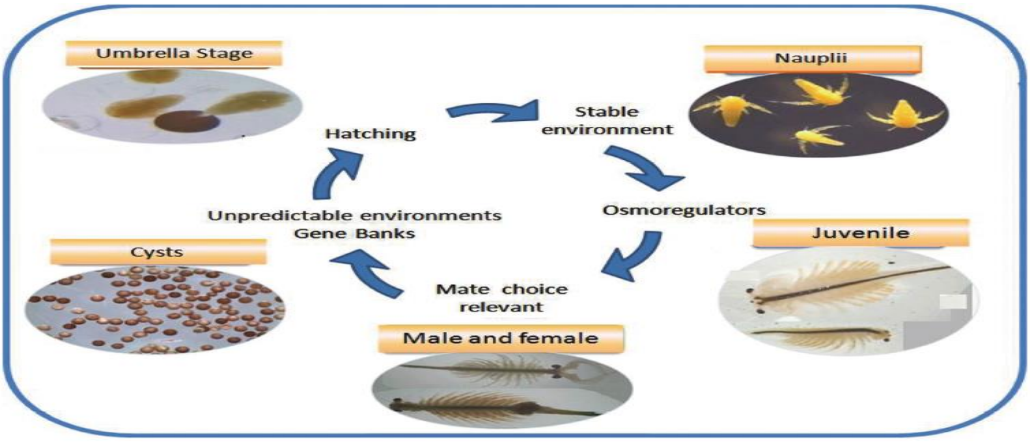


Figure 2.2: The life cycle of *Artemia salina* (Gajardo & Beardmore, 2012).

Artemia salina Leach or more commonly known as sea monkey is a type of marine invertebrates with 1 mm in size and produces dormant eggs (cysts) as shown in, Figure 2.2. The dormant cysts can be stored for long term and hatched without any equipment (Lieberman, 1999). Figure 2.3 shows the cross section of *Artemia salina* leach.

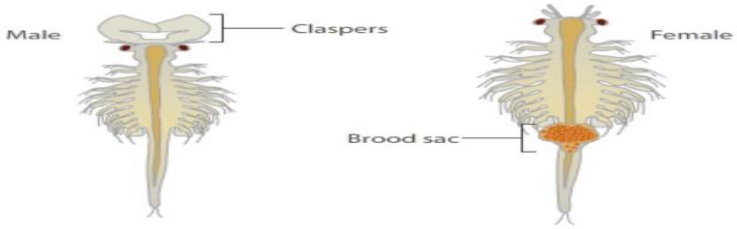


Figure 2.3: The different cross section of male and female *Artemia salina* leach. Male *Artemia salina* composed of claspers while female *Artemia salina* has brood sac to store their cysts.

Besides high salinity concentration, its short life span and good resilience favour it to become one of the widely used model host organism (Gajardo & Beardmore, 2012). This test is also widely used to examine the virulence and toxicity tests in *Stapylococcus aureus*, *P. aeruginosa*, *Burkholderia vietnamiensis*, *E. coli* and *V. vulnificus* (Lee et al., 2014). Brine shrimp were chosen due to several reasons such as they do not require maintenance of stock culture which can be obtained directly from the dormant cysts, short life span, easy hatching in massive number, and gnotobiotically grown in the laboratory. In summary, the bacterial culture used to feed the nauplii brine shrimp used the similar initial concentration of *Leptospira* spp. on the bacterial virulence assay.

CHAPTER 3

MATERIALS AND METHOD

3.1 *Leptospira* Growth Condition and Maintenance

A total of 30 isolates from intermediate (n=15) and saprophytic (n=15) *Leptospira* strains were obtained from the culture collection of Molecular Microbiology Laboratory in Faculty of Resource Science and Technology, UNIMAS. These cultures were previously isolated from rats (103 samples liver and kidney) and environmental samples (50 soil and 60 water samples) (Pui et al., 2015). The positive references used for pathogenic, intermediate and saprophytic strains were *L. interrogans* serovar Copenhageni (Institute for Medical Research Malaysia), *L. wolfii* serovar Khorat strain Khorat-H2 (locally isolated) and *Leptospira biflexa* serovar Patoc (Institute for Medical Research Malaysia). For positive control in brine shrimp assay, pathogenic *Leptospira interrogans* (n=1) was used in this study. The list of intermediate and saprophytic *Leptospira* strains used was in Table 3.1 and Table 3.2.

Leptopira strains were cultured and maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) broth and 1.5% (w/v) EMJH agar at 30 °C. The 1 L of EMJH broth consists of 2.3 g of EMJH base (Difco, USA), 900 ml of distilled water, 0.1 g of 5-fluorouracil and 100 ml of EMJH enrichment (Difco, USA) added after autoclaved with temperature 121 ^{o}C (pressure at 15 psi) for 15-20 mins. Fluorouracil was used to inhibit the growth of bacteria and did not alter the growth or the morphology of leptospirae (Johnson & Rogers, 1964; Ristow et al., 2008; Pui et al., 2015). On the other hand, 1.5% (w/v) EMJH agar comprises of 2.3 g of EMJH base, 1.5 g of bacteriological agar, 0.1 g of 5-fluorouracil and 100 ml of EMJH enrichment agar were used for purification and

selection of colonies. For culture storage, 20% (w/v) of glycerol stock in EMJH broth was used and the vials were stored at -20 ^{o}C until further use. *Leptospira* has an optimum growth temperature ranging from 28 ^{o}C to 30 ^{o}C without shaking in obligate aerobic environment of OD_{420}= 0.3 (± 0.05) for 30 days.

For the isolated intermediate and saprophytic strains, they achieved bacterial concentration of 10^6-10^8 cfu/ml (OD_{420} ~ 0.3-0.4) started from day 5 to day 7. A total of 15 strains of intermediate and 15 strains of saprophytic *Leptospira* were studied. The positive controls of *Leptospira* were also included. Table 3.1 and Table 3.2 show the list of intermediate and saprophytic *Leptospira* strains used in this study.

Table 3.1: List of intermediate *Leptospira* microbial culture collection

No.	ID	Sources	Intermediate *Leptospira*	Strain	Locality
1.	G4	Soil	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Bako National Park
2.	G14	Soil	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Kubah National Park
3.	G16	Soil	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Kampung Barieng
4.	G12	Soil	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Miri National Training Centre
5.	G3	Water	*Leptospira inadai* serovar Aguaruna strain MW4	Khorat-H2	Tanjung Datu National Park
6.	G5	Water	*Leptospira wolffii* strain LS0914U	LS0914U	Bako National Park
7.	G7	Water	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Kota Samarahan
8.	G9	Water	*Leptospira sp*. MS341	MS341	Miri National Training Centre
9.	G11	Water	*Leptospira wolffii* serovar Khorat strain Khorat-H2	MS341	Miri National Service Training Centre
10.	G13	Water	*Leptospira idonii*	-	Kubah National Park
11.	G15	Water	*Leptospira inadai* serovar Aguaruna strain MW4	MW4	Kubah National Park
12.	G17	Water	*Leptospira inadai* serovar Aguaruna strain MW4	MW4	Kampung Barieng
13.	G19	Water	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Kampung Barieng
14.	G21	Water	*Leptospira sp*. Neco007	Neco007	Juara National Service Training Centre
15.	G29	Rat liver	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Khorat-H2	Kampung Sungai Mata
16.	G2	Soil	*Leptospira wolffii* serovar Khorat strain Khorat-H2 (Positive Control) (NR_044042)	Khorat-H2	Tanjung Datu National Park

Table 3.2: List of saprophytic *Leptospira* microbial culture collection

No.	ID	Sources	Saprophytic *Leptospira*	Strain	Locality
1.	S4	Soil	*Leptospira meyeri* strain Semaranga_DB49	Semaranga_DB49	Kota Samarahan
2.	S11	Soil	*Leptospira meyeri* strain Semaranga_DB49	Semaranga_DB49	Miri National Service Training Centre
3.	S21	Soil	*Leptospira meyeri* strain 19CAP	19CAP	Medan Niaga Satok
4.	S3	Water	*Leptospira meyeri* strain Patoc strain "Patoc	Patoc	Juara National Service Training Centre
5.	S5	Water	*Leptospira meyeri* strain Semaranga_DB49	Semaranga_DB49	Miri National Service Training Centre
6.	S12	Water	*Leptospira meyeri* strain Semaranga_DB49	Semaranga_DB49	Kubah National Park
7.	S20	Water	*Leptospira meyeri* strain 19CAP	19CAP	Medan Niaga Satok
8.	S14	Rat liver	*Leptospira meyeri* strain 19CAP	19CAP	Gunung Gading
9.	S34	Rat kidney	*Leptospira meyeri* strain 19CAP	19CAP	Gunung Gading
10.	S15	Rat kidney	*Leptospira meyeri* strain 19CAP	19CAP	Kampung Sebayor
11.	S36	Rat liver	*Leptospira meyeri* strain 19CAP	19CAP	Kampung Sebayor
12.	S16	Rat kidney	*Leptospira meyeri* strain 19CAP	19CAP	Taman Sukma
13.	S17	Rat liver	*Leptospira meyeri* strain 19CAP	19CAP	Medan Niaga Satok
14.	S18	Rat kidney	*Leptospira meyeri* strain 19CAP	19CAP	Kampung Tupong
15.	S19	Rat kidney	*Leptospira meyeri* strain 19CAP	19CAP	Kampung Matang
16.	Patoc	-	*Leptospira biflexa* strain Patoc strain Patoc 1	patoc	Institute Medical Research Malaysia

3.2 Dark Field Microscopic Analysis

Dark field microscopic views were conducted to ensure the cells were viable and motile during bacteria culture and that no contamination occurred. The cultures were observed under 400x magnification of dark field microscope (Olympus Corporation, Japan) at the Faculty of Medicine and Health Sciences, UNIMAS. A total of 100 µl of each leptospiral strains was added on glass slides and viewed under the microscope after subcultured. It was observed as a hook-like end, thin and motile and all images were captured. All strains were positive for *Leptospira* were subcultured into EMJH medium (Becton, Dickinsom & company, USA) for further analysis (Benacer et al., 2013).

3.3 Phase Contrast Microscopy

The structure of biofilm in *Leptospira* spp. can be observed under phase contrast microscope. Phase contrast microscope was used to elucidate the structure of biofilm on the glass surfaces at different time following the method of Ristow et al. (2008). Leptospires cells were studied over time and the cells adhered on glass slides. Glass slides were incubated half into 15 ml centrifuges tube with bacteria suspension at 10^6 cfu/ml. After that, the glass slides were rinsed three times in distilled water and air dried by heat fixation. Then, they were observed under phase contrast microscopy using Olympus Inverted IX51 microscope at 600 magnification (Olympus Corporation, Japan).

3.4 Confirmation of *Leptospira* strains

For confirmation of *Leptospira* species, polymerase chain reaction was used to target the specific genes for saprophytic and intermediate strains. Saprophytic strains target *rrs* genes while intermediate strains target 16S *rRNA* genes following the method of Pui et

al. (2015). For DNA isolation, the DNA of *Leptospira* spp. was isolated and extracted after the cell density achieved 10^6 cfu/ml by using spectrophotometer. DNA extraction was performed using Wizard® Genomic DNA purification kit. This involved nucleic lysis solution, RNase solution, protein purification solution, isopropanol, 70% ethanol and DNA rehydration solution as suggested by the manufacturer. The primer sequence for 16S *rRNA* genes 5' GGCGGCGCGTCTTAAACATG 3' (F) and 5' TTCCCCCCATTGAGCAAGATT 3' (R) with 331 bp and for primer sequence in *rrs* gene was 5' AGAAATTTGTGCTAATACCGAATGT 3' (F) and 5' GGCGTCGCTGCTTCAGGCTTTCG 3' (R) for 240 bp. For the PCR conditions used were initial denaturation at 95 °C in 2 minutes, denaturation at 94 °C in 1 minute, annealing at 55 °C in 30s, extension at 72 °C for 35 cycles and final extension at 72 °C in 5 minutes. For indefinite holding period, 4 ^{o}C was maintained. A 2% of agarose gel (Thermo Fisher Scientific, USA) was prepared on gel casting tray. A 5 µl of amplication products and 100bp DNA ladder (TransGen, China) were loaded before electrophoresed using 1X TBE buffer for 60 minutes and 90V. The gel was stained with 0.1% ethidium bromide before viewed under UV transilluminator (Alpha DigiDoc RT UV).

3.5 Assessment of Biofilm Formation on Abiotic surface

Fifteen intermediate and 15 saprophytic *Leptospira* spp. strains were incubated without shaking at 30°C. Serial dilutions were carried out to ensure the final concentration of *Leptospira* cells normalised to 10^6 CFU/ml OD_{420}=0.3(±0.05) (Ristow et al., 2008). In this study, the microtiter (polystyrene) plate was used to represent abiotic surface. A static microtiter plate assay was used (Ristow et al., 2008; Pui et al., 2017). The 24 well polystyrene microtiter plates (flat bottom wells, TPP, Germany) were filled with 1 ml

bacterial culture. All the experiments were done in triplicate. Fresh EMJH without bacterial culture served as negative control in the assay. The polystyrene plates were sealed with parafilm to avoid desiccation and contamination. All the intermediate and saprophytic *Leptospira* were removed 1 ml every 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h, 240 h and 264 h, respectively.

After that, the wells were gently rinsed 1x with phosphate buffered saline (PBS) to remove planktonic cells and air dry for 15 mins. PBS was used as it is isotonic and it maintains constant physiological pH which does not disrupt the structure of cells. Besides, it provides low difference of salinity level by reducing the chances of cell bursting followed by cell death (Das & Dash, 2015). Then, 2% of sodium acetate was fixed for 5 min and removed before air dried for 15 mins. Finally, the attached cells were stained with 0.1% of crystal violet solution for 20 mins and the solution was removed again. After rinsed 3x with PBS, the remaining attached cells were dissolved in (80/20) ethanol/acetone solution. The adhered biofilm cells were assessed and measured at OD_{600} using spectrophotometer (Metertech Inc, Taiwan).

The OD readings were recorded and calculated using optical density cut off value (OD_c). OD_c is defined as three standard deviations above the mean of optical density of the negative control (Stepanovic et al., 2008). Subsequently, the classes of biofilm were determined accordingly into non-adherent, weakly adherent, moderately adherent and strongly adherent biofilm based on Optical Density Cut-Off value (OD_c).

3.6 Assessment of Biofilm Formation on Biotic Surface

Dyera costula or commonly known as "Jelutung paya" hardwood was used to represent the biotic surface. The hardwood chips were obtained from Faculty Resource Science and Technology, UNIMAS. The chips were cut into 1cm x 1cm x 0.5cm to fit in the 24 wells microtiter plates. Firstly, the wood chips were washed with detergent (Unilever, Breeze, Malaysia) and dried at 30 mins at 75 ^{o}C. Then, the cultures were inoculated into 24 wells microtiter plates. The dried wood chips were then placed in the microtiter plate. The negative control was sterile woodchips without bacterial culture.

The time course study for biofilm assay was set for 11 days, similar as the biofilm assay on abiotic surface. The wood chips were removed aseptically at 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h, 240 h and 264 h for the crystal violet assay. Next, each of the wood chips was washed with 3x PBS to remove the planktonic cells. PBS is very reliable and accurate as it shows consistent removal of unstained crystal violet from surface during washing. Subsequently, the fixation was carried out on the remaining adherent cells using 2.5 ml of methanol solution per chip. The methanol was used to retain the crystal violet steadily and provide more reliable and effective staining data (Minnerath et al., 2009). Thereafter, 0.1% of crystal violet solution was used to stain the wood chips for 15 mins and removed again by washing 3x using PBS. After the chips were air-dried, the dye bound to adherent cells were re-solubilised with 2.5 ml of 33% (v/v) glacial acetic acid. Finally, the optical density at 600 nm for re-solubilised from wood chips was measured using spectrophotometer (Metertech Inc, Taiwan). The measurement was recorded and a graph was plotted. Data analysis for each strain was calculated using optical density cut off value for classification into non-adherent, weak, moderate and strong

biofilm producers. Repetitive measure of one-way ANOVA was used to compare the significance means among each strain for each assay.

3.7 Classification of Biofilm Ability

The biofilm formation classification was classified using optical density cut-off value (OD_c). OD_c was defined as three standard deviation above the mean optical density of the negative control (Stepanovic et al., 2000). The classification were known as $OD \leq OD_c$ (non-biofilm producer), $OD_c < OD \leq 2x\ OD_c$ (weak biofilm producer), $2xOD_c < OD \leq 4x\ OD_c$ (moderate biofilm producer) and $4x\ OD_c < OD$ (strong biofilm producer). Each biofilm producers calculated in percentage to represent the amount of biofilm formation.

3.8 Determination of biofilm genes by PCR

Polymerase Chain Reaction (PCR) was used to determine the presence of biofilm genes during biofilm formation stages. In this study, the strongest and weakest biofilm producers of saprophytic and intermediate *Leptospira* were selected for biofilm genes detection. In *Staphylococcus* spp., the commonly known biofilm genes were found in *icaABCD* operon which use for synthesis of intercellular polysaccharide adhesion (PIA) for cell-to-cell adhesion and mainly encoded for *icaADBC* locus (Cramton et al., 1999).

The first transcriptome sequencing of *Leptospira biflexa* was performed based on RNA- sequence and the transcriptional changes linked to the biofilm growth specifically at 48 hour and 120 hour were analysed (Iraola et al., 2016). The genes are flaB that encodes for the flagellar apparatus in *L. biflexa* as well as *galK* gene that encodes for UDP-glucose-4-epimerase in *L. biflexa*. The *ompL1* gene encodes for outer membrane porin OmpL1

which is a novel leptospiral extracellular matrix (ECM)- binding protein plasminogen receptor. The primers used were listed in Table 3.3.

Table 3.3: The primers list in biofilm detection

Genes	Primer Sequences	Amplicon size (bp)	Reference
icaA	Forward 5'-ACACTTGCTGGCGCAGTCAA -3' Reverse 5'- TCTGGAACCAACATCCAACA-3'	188	Mirzaee et al. (2014)
icaB	Forward 5'- TCCTTATGGCTTGATGAATGACG-3' Reverse 5'- CTAATCTTTTTCATGGAATCCGTCC -3'	190	Mirzaee et al. (2014)
icaC	Forward 5'- ATGGGTTATAACTACGAACGTG -3' Reverse 5'- CGTGCAAATACCCAAGATAAC -3'	192	Mirzaee et al. (2014)
icaD	Forward 5'- ATGGTCAAGCCCAGACAGAG -3' Reverse 5'- AGTATTTTCAATGTTTAAAGCAA -3'	198	Mirzaee et al. (2014)
bap	Forward 5'- AAAGAGCCACATAAACAACAAGAA -3' Reverse 5'- GTAGCCATAGCACGGAACATAG -3'	368	Goyal et al. (2014)
ompL1	Forward 5'- ACTGGCATTCGTTTGTTCGC-3' Reverse 5'-CCAGAAACTCCCCACCATCC -3'	639	Iraola et al. (2016)
flab	Forward 5'-GGTTTTGGCGAAAGCCAGAG -3' Reverse 5'- AGCAGTTGGGTTGAGTCGAG-3'	493	Iraola et al. (2016)
galK	Forward 5'- GACAAGGATTCATGTGCGCC-3' Reverse 5'-AATTGTCCTACCAGCCGCAA -3'	792	Iraola et al. (2016)

3.9 Extraction of DNA and Detection of Biofilm Gene

DNA extraction of *Leptospira* spp. was performed using Wizard™ Genomic DNA Purification Kit (Promega Corporation, USA) following standard protocol from the manufacturer's procedures. Polymerase Chain Reaction was carried out using specific PCR amplication and operated using Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA). The PCR reaction consisted of 5 µl of 5x PCR buffer (Promega Corporation, USA), 0.2 mM dNTPs, 10uM of each primer pair, 2.0 mM $MgCl_2$, 1.25 U *Taq* polymerase and 5 µl of DNA template.

The PCR cycling condition differed from genes to gene, therefore optimization was performed to achieve suitable annealing temperature. For *icaADCB* genes, the PCR cycling condition used for amplication were: Initial denaturation at 94 ^{o}C for 5 min, then 40 cycles denaturation at 94 ^{o}C for 30 s, annealing at 55.5 ^{o}C for 30 s, extension at 72 ^{o}C for 30 s and finally extension at 72 ^{o}C for 10 min (Mirzaee et al., 2014). For *bap, galK* and *flaB* genes, the PCR cycling condition used for amplication were: Initial denaturation at 95 ^{o}C for 5 min, then 40 cycles of denaturation at 94 ^{o}C for 2 min, annealing at 60 ^{o}C for 30 s, extension at 72 ^{o}C for 30 s and finally extension at 72 ^{o}C for 2 min (Iraola et al., 2016). For indefinite holding period, 4 ^{o}C was employed.

3.10 Agarose Gel Electrophoresis

A 2% of agarose gel (Thermo Fisher Scientific, USA) was prepared on gel casting tray. A 5 µl of amplication products and 100 bp DNA ladder (TransGen, China) were loaded before electrophoresed using 1X TBE buffer for 60 mins and 90V. After that, the gel was stained with 0.1% ethidium bromide before viewed under UV transilluminator.

3.11 Bacterial virulence analysis by using Brine Shrimp Test

Virulence study for *Leptospira* spp. was conducted by using brine shrimp test as a host model and was adapted from the method of Lee et al. (2014). The brine shrimp, *Artemia salina* was provided by Chemistry laboratory from Faculty Resource Science and Technology, UNIMAS. The *Leptospira* cultures were normalised to an initial bacterial concentration at 10^6 cfu/ml. The pathogenic P18 (*Leptospira noguchii* strain LT796) was previously evaluated as the strongest biofilm forming ability with OD_{600} at 21.760 ± 0.332 and obtained from FRST, Unimas from Microbiology laboratory. For negative control was sterile artificial seawater. The viable cells of leptospires were examined again under dark field microscope before experiment started to avoid cross contamination. A 3.2 g of cysts were suspended in 166 ml of sterile artificial seawater which was prepared by sea salt.

The cysts were incubated with air pump to oxygenated the water at 28-30°C for 36 hours to grow up to nauplii state for bacterial virulence test. Then, a total of 20 nauplii brine shrimp were transferred into a petri dish (35 x 10 mm) using sterile syringe and washed with phosphate buffered solution (PBS) to remove contaminants. The brine shrimp was infected in various CFUs with added of artificial seawater and the survival rate of the shrimp was counted daily. The initial CFUs was obtained from 10^{-3}, 10^{-4} to 10^{-5} by using serial dilutions method described by. After that, each strain was transferred into different petri dish with 20 selected nauplii brine shrimp and incubated at room temperature. Percentage of survival analysis of brine shrimp was calculated survival number of brine shrimp after treated over total number of brine shrimp used. The negative control was autoclaved leptospiral cultures. This analysis was repeated three times and data were statistically analysed using t-test assuming p-value< 0.05 considered as significant.

3.12 Statistical Data Analysis

All result was represented as mean ± standard deviation mean. All the data on biofilm formation was analysed using repetitive measure ANOVA in Microsoft Excel (version 14.0, release 1.5, California, USA. A significance level of 5% ($p<0.05$) was set for statistical analysis. Mean differences among triplicate measurement for the isolates were evaluated using repeated measures one way ANOVA, followed by Post-Hoc Bonferoni test to differentiate the biofilm forming ability of *Leptospira.* For brine shrimp assay, the survival of shrimp was measured triplicate with t-test with significance level $p<0.05$ when compared to negative control.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth and Morphology of *Leptospira*

All *Leptospira* spp. cultures were incubated at room temperature (30 ^{o}C) in EMJH broth for 30 days. Among the 15 intermediate and 15 saprophytic *Leptospira*, the initial bacterial concentration (OD_{420}) recorded for representative intermediate (G7, *Leptospira wolffii* serovar Khorat strain Khorat-H7) was 0.374 ± 0.029 which also corresponded bacterial concentration of 2.5 x 10^8 cfu/ml and representative saprophytic (S19, *Leptospira biflexa* strain Patoc strain Patoc 1) based on bacterial counts was 0.336 ± 0.068 which corresponded to 2.2 x 10^8 cfu/ml. The acceptable range of OD values for *Leptospira* is between 10^6-10^8 cfu/ml to perform a biofilm assay (Sutton, 2006). According to Bourhy et al (2011), the biofilm assay was used from initial bacterial concentration to a mid-exponential phase culture (10^6-10^8 cfu/ml). The *Leptospira* strains in this study were grown to 30 days. Unlike other microorganism that commonly required 18 to 24 hours to grow, the growth of *Leptospira* required 30 days for the bacterial growth to achieve maximal growth (Ristow et al., 2008).

The morphology of *Leptospira* cannot be observed under normal compound microscope due to their thin, coiled and rapidly moving cells. Therefore, in this study dark field microscopy was used to observe the morphology after the 30 days growth period. During the observation, the movement of bacterial cells in the viable samples could be seen from the monitor screen. The cell was thin, long and spiral shaped. Motile *Leptospira* isolates were observed in all samples, which indicated that the samples were viable and ready to use for biofilm formation study. This finding is in agreement to the findings of

Krishna & Sharma (2008) who observed similar appearance of leptospires cells that were hooked, spiral and thin under dark field microscope. The leptospires cells motility were also observed by the movement of the cell as shown on the computer monitor screen. Figure 4.1 shows the morphology of the motile intermediate and saprophytic *Leptospira* under dark field microscope.

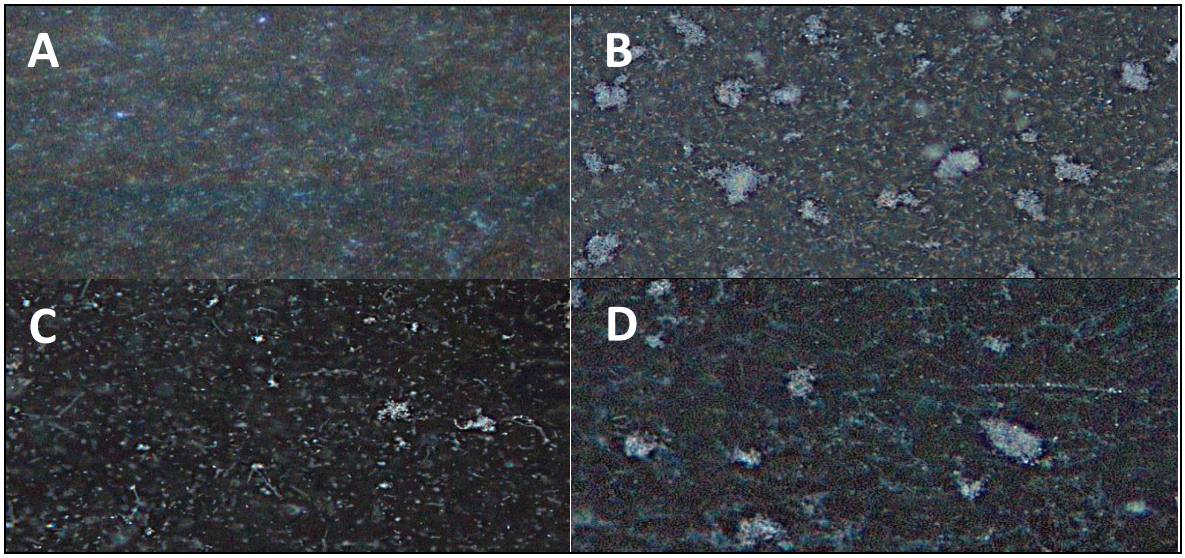


Figure 4.1: A (*Leptospira wolffii* serovar Khorat strain Khorat-H2 (G7-intermediate)); B (*Leptospira inadai* serovar Aguaruna strain MW4 (G15-intermediate)); C (*Leptospira meyeri* strain 19CAP (S19-saprophytic)) and D (*Leptospira biflexa* strain Patoc strain Patoc 1 (S1-Saprophytic)) were showed hooked end and spiral leptospiral cells under dark field microscope under 400X magnification. All leptospiral cells were confirmed active viable at concentration of 10^6- 10^8 CFU/ml or OD_{420}= 0.3-0.4 after 30 days at 30 ^oC incubation at room temperature.

From Figure 4.1, the *Leptospira* cells were seen to be clear under dark field microscope and no clump formation in both strain G7 (section A) and S19 (section C). Both strains were isolated from water and rat kidney respectively (Pui et al., 2017). While for G15 (section B) and S1 (section D) showed clump formation and overlapping colonies. It is important to ensure the growth of leptospires was given an adequate time (above 10^4 cfu/ml) to view live *Leptospira* with characteristic morphology and their mortility (Budihal, & Perwez, 2014; Jaiswal et al., 2015). Although this technique is lack of

sensitivity and specificity, but this technique can still be used for early diagnose and visualize leptospirosis for more cost effective and time saving option.

Like other spirochaetes, *Leptospira* cell also has the ability to alter their morphology depending on the environmental conditions. The changes include the cells aggregation and their colonization of planktonic cells into biofilm mode has become our interest of study. Since, biofilm exist in all natural (biotic) and man-made environments (non-biotic), this study examined two types of surfaces to imitate the environmental conditions. Microtiter plates were used to represent the abiotic surface while wood chips were used to represent the biotic surface. The potential of *Leptospira* in forming biofilm on these surfaces play an important role, not only for survival strategy but also to ensure it is successful in disease transmission and pathogenesis of these species (Iraola et al., 2016). The time course study for intermediate and saprophytic *Leptospira* was performed for 11 days in accordance to their general biofilm formation process. Crystal violet was used based on the ability of this dye to colour the polysaccharide matrix of *Leptospira* and their optical density of light at 600 nm to measure the presence and cells attachment after repeated washing steps (biofilm mass quantification).

In general, the intermediate *Leptospira* (G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2) was formed stronger biofilm on biotic surface 93.99% than abiotic surface with 53.33% at day 5, as shown by the percentage of biofilm forming ability (Table 4.1). While in saprophytic (S19, *Leptospira meyeri* strain 19CAP), it formed stronger biofilm on biotic surface on different day with 86.67% (day 5) and abiotic surface with 40% (day 6) (Table 4.1). This showed the biofilm *Leptospira* formed at different time according to their conditions. Among all the strains studied for biofilm mass quantification,

the strongest biofilm producer reported was intermediate strain *Leptospira wolffii* serovar Khorat strain Khorat-H2 (G7) with OD_{600} (2.561 ± 0.579). The optical density of all *Leptospira* at 600 nm was recorded more than 0.2 value and these results are in good agreement with Ristow et al. (2008). Based on the results of the biofilm formation as shown by OD values, the intermediate strains are noted to form stronger biofilm compared to the saprophytic strains. Although there is lack of information of other studies to compare the OD values between the intermediate and saprophytic *Leptospira*, the biofilm formation can be compared with a pathogenic *Leptospira* P18, *Leptospira noguchii* strain LT796 formed biofilm with OD_{600} at 21.760 ± 0.332, which is 9 folds higher than intermediate *Leptospira* that produces the highest biofilm in this study (2.561 ± 0.579). Comparison of these values showed that the pathogenic *Leptospira* formed higher biofilm formation compared to intermediate and saprophytic *Leptospira*

In the next step, time course study was included to understand the biofilm formation stages and the growth pattern according to their surface of attachment. The biofilm formation was conducted for 11 days with the method by Ristow et al. (2008). From day 1 to day 11, the data of OD_{600} of each isolate was recorded (Appendix B to E) and the mean values were plotted in bar graphs. All assays were done in triplicate to ensure reliability and reproducibility data of biofilm assays.

4.2 Biofilm Formation of Abiotic Surface

Microtiter plate assay (abiotic surface) is well known for biofilm formation with high throughput techniques that can give applications in phenotypic screening of mutant libraries, attachment and early biofilm development studies and biomass quantification with staining (O'toole & Kolter 1998; Franklin et al., 2015). A total of 30 intermediate

(n=15) and saprophytic (n=15) isolates were tested using the microtiter plate assay as described by Ristow et al. (2008). This assay was adapted to evaluate biofilm formation of *Leptospira* after 11 days of incubation at 30 °C in room temperature. Each isolates were inoculated 1 ml of cultures with triplicate microtiter plates at 30 °C. The growth was monitored over time by optical density (OD_{600}) and the colour intensity of stained microtiter plates. The percentage of biofilm was calculated based on the classification of optical density of bacteria (Stepanović et al., 2000). The percentage of classification of optical density cut off value is as shown in Table 4.1 and Table 4.2. From the statistical analysis, there is a significant different, $p < 0.05$ in the effect of time in biofilm formation by leptospires between day 4 to day 7. From the Table 4.1, intermediate (G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2) was identified as strong biofilm forming ability on 3rd and 4th day respectively. As for saprophytic strains, only 40% of isolates was reported as strong biofilm producers on day 6. In comparison of time course study, intermediate *Leptospira* formed faster than saprophytic *Leptospira.*

On the other hand, the weakest biofilm forming producers were G12 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) with 82% for intermediate and S5 (*Leptospira meyeri* strain Semaranga_DB49) with 45% for saprophytic strains. For weak biofilm forming ability, the biofilm formation process only showed two different phases, reversible attachment and detachment process. In this study, the weakest strains recorded were G12 for intermediate strain while S16 for saprophytic *Leptospira*. Biofilm have a structurally complex and dynamic architecture and can develop on many surfaces (Ristow et al., 2008).

Bacterial adhesion on abiotic surfaces and subsequent biofilm formation constitute a serious issue in several sectors such as food industries, water canalizations and medical facilities. Indeed, bacteria find favourable conditions to colonize surfaces and establish

biofilm (Khelissa et al., 2017). In this study, the microtiter plate bottom has less surface tension of the solid surface with less porous materials to evaluate the most favourable bacterial attachment surface. In other bacterial attachment, such as *Staphylococcus aureus* was found adhere preferentially to metals while *Salmonella epidermis* more to polymers surface (Scharff, 2012). Therefore, *S. epidermidis* causes more polymer implant infection while *S. aureus* often cause major pathogen in metal implant infection. Besides, biofilm bacteria can attach to medical devices and surgical sites after long term. Most medical implants such as urinary catheters and implanted prosthetic devices mostly caused serious nosocomial infection (Khelissa et al., 2017). The issue starts when the bacteria unable to irreversible attach from the surface and further developed into biofilm bacteria. It was widely known as chronic polymer associated infection. *Pseudomomas aeruginosa* biofilms can cause nosocomial infection when it colonizes water system in healthcare facilities.

Crystal violet assay is commonly used to determine biofilm quantification based on biofilm mass and colour intensity. From Figure 4.2 and Figure 4.3, the biofilm formation shows the purple ring at the biomass of air-liquid interface. Figure 4.2 shows crystal violet stained cell for intermediate *Leptospira* (G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2) in 11 days of biofilm assay and Figure 4.3 shows crystal violet stained cell for saprophytic (S19, *Leptospira Leptospira meyeri* strain Semaranga_DB49) for 11 days of biofilm assay.

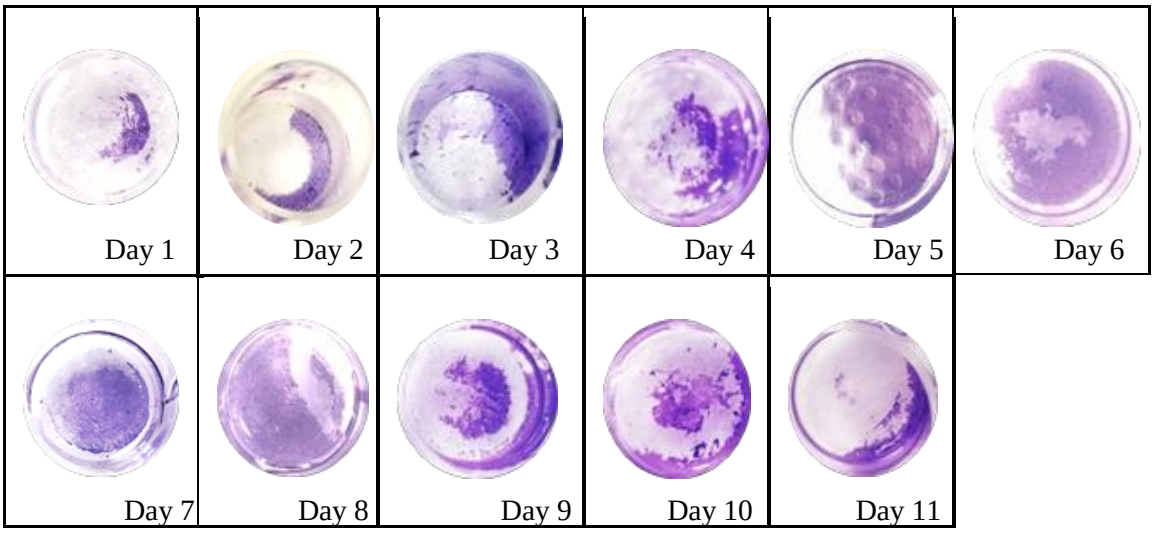


Figure 4.2: Crystal violet stained cell for G7, intermediate *Leptospira* in 11 days. The highest biofilm production was observed from day 5 to day 8. From day 1 to day 4, the biofilm cells attached and formed microcolonies. From day 5 to day 8, the biofilm strongly formed and attached well at the bottom of well. The cells clumped and resist from washing step. At day 9, the cells started to disperse and detachment from bottom plates until day 11.

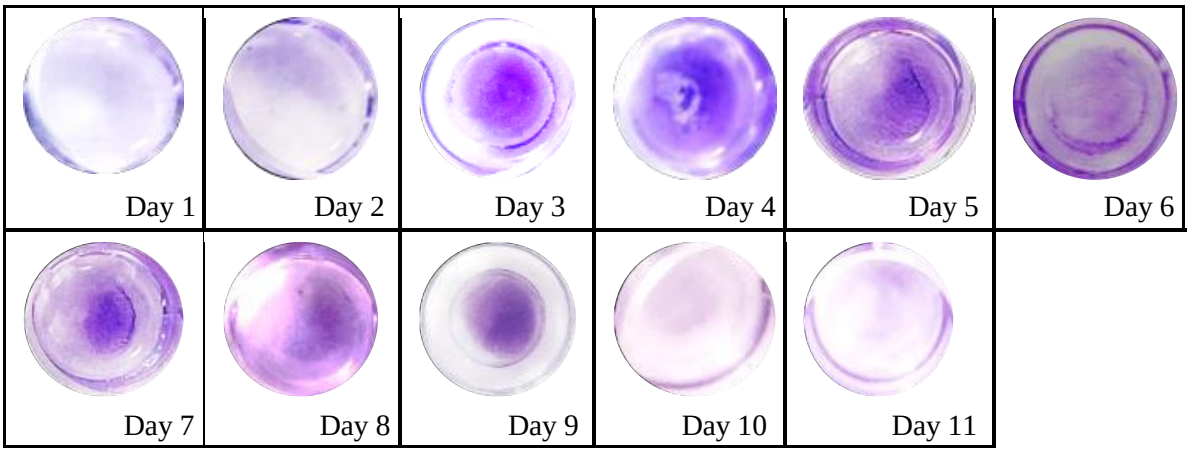


Figure 4.3: The crystal violet stained cell for S19, saprophytic *Leptospira* in 11 days. From day 1 to day 2, no biofilm cell was attached on the bottom wells. At day 3 onwards, the biofilm formed bottom wells intense with multiple colonies resisted until 5 from washing step. At day 6, the biofilm cells attached on bottom well plates started to detach from the well. From day 10 onwards, no biofilm cells were observedhighest biofilm production was observed as the strongest from day 3 to day 5.

Based on colour intensity as shown in Figure 4.2 and Figure 4.3, the amount of biofilm developed on microtiter polystyrene bottom wells could be seen clearly between day 1 and day 11. At day 1, the surface of biofilm of G7 and S19 were observed with only small amount of purple ring on the flat bottom wells which indicated the beginning step of biofilm formation. It was also known as surface attachment. The colour intensity was observed partially filled up the wells from day 2 onwards for intermediate isolate (G7) which corresponds to the microcolonies and biofilm matures as the colour intensity increased on day 5. For saprophytic *Leptospira* (S19), the most intense colour of crystal violet was observed on day 4 until day 6, which corresponding to the occurrence of biofilm maturation. On day 9, the colour intensity gradually faded and the colour of crystal violet can be easily rinsed for both G7 and S19 isolates.

A similar trend in biofilm formation was observed from time course study graph (mean value biofilm formation) and the colour intensity stained over time. Long term incubation of bacteria can stress survival adapt at extreme condition. It is important to note that the assay presented here measured biofilm production under a minimal nutrient environment and sustain during *Leptospira* transmission (Djordjevic et al., 2002). During leptospirosis transmission, it depend on several factors including the reservoir species and its Leptospira carriage prevalence, the dilution into watered environment and the survival time of leptospires can sustain during nutrient depletion and extreme environmental conditions (Thibeaux et al., 2017).

Interestingly, result from this study showed that the intermediate strain can strongly resisted the rinsing process from day 9 until day 11 compared to saprophytic strain which can be rinsed from day 10 onward. This may indicated the intermediate *Leptospira* shas a

good mechanism of biofilm attachment on abiotic surface. Although there is no study on biofilm intermediate *Leptospira*, previous study on the pathogenic *Leptospira* revealed strongly attachment capabilities until day 10 of biofilm assay (Pui et al., 2017). According to Pui et al. (2017), biofilm attachment slowly developed surface sensing responses and attached to the conditioning film that trapped the surrounding nutrients and cell debris.

Quantification of Intermediate *Leptospira* Biofilm on Microtiter Plate Assay

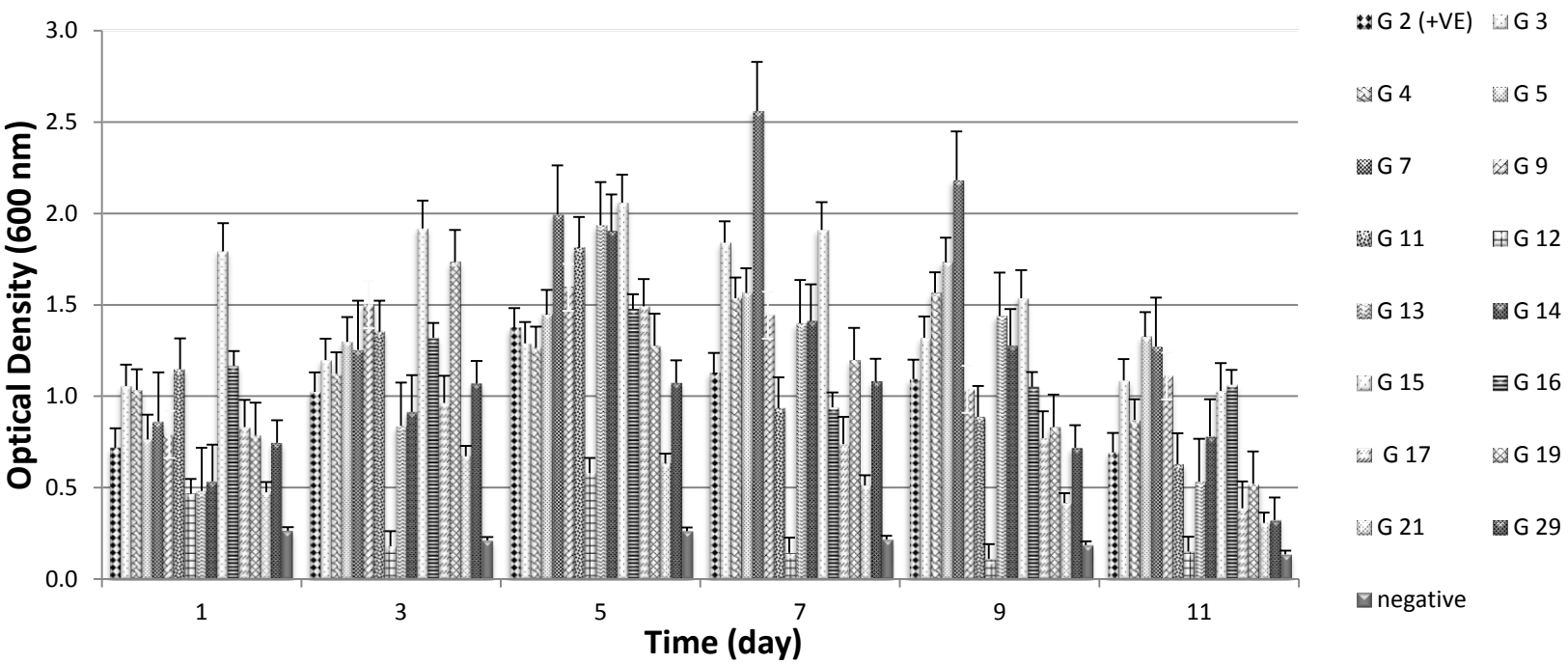


Figure 4.4: Quantification of biofilm formation (OD_{600}) for 15 intermediate *Leptospira* comprises of 4 soil samples, 10 water samples, 1 rat liver sample tested on abiotic surface (24 well microtiter plates) compared with positive control from FRST, Unimas. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments.

Quantification of Saprophytic *Leptospira* Biofilm On Microtiter Plate Assay

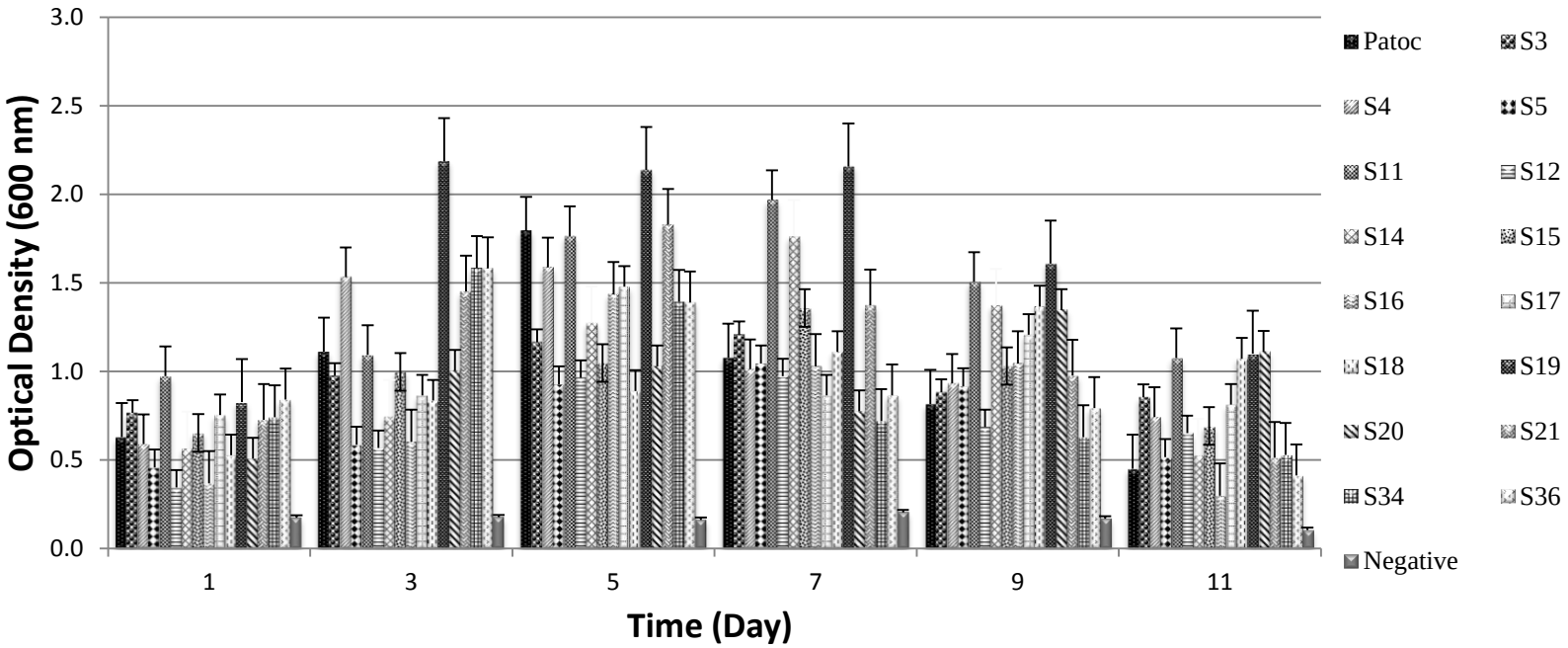


Figure 4.5: Quantification of biofilm formation (OD_{600}) for 15 saprophytic *Leptospira* comprises of 3 soil samples, 4 water samples, 8 rat liver samples tested on abiotic surface (24 well microtiter plates) compared with positive control from IMR, Malaysia. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments.

According to Figure 4.4 and Figure 4.5 show bar graphs of quantification of biofilm formation of 15 intermediate and 15 saprophytic *Leptospira* isolates. The trend of bar graphs show the biofilm formation stages from initial attachment, forming of microcolonies, biofilm maturation and biofilm disperse back to planktonic cells. From day 1 to day 2, the initiation of cell attachment on the surface with nutrient rich environment assist leptospire cells to increases the layer of cells. The free floating bacteria can be observed on the surface of the wells before the cell aggregated on the surface (day 3 to day 5). According to Renner and Weibel (2011), the physicochemical properties of both cell and material surfaces are play important role affecting the attachment of bacteria and formation of biofilm. These micro colonies started expanded and multiplied into mature state as macro colonies with three dimensional structures (Pui et al., 2017).

At rinsing process, the biofilm cells had matured and became irreversible. Moreover, the bacterial attachment is influenced by many factors such as their environmental conditions, pH, bacterial concentrations and nutrient requirements in order to against formation of biofilm (Di Bonaventura et al., 2008). From the graph, the biofilm intermediate, G15 and saprophytic, S19 are relatively stagnant (stationary phase) on day 3 to day 7 which indicated the biofilm maturation occurred for these days. During stationary phase, the leptospire cells switch to survival mode of metabolism and cells are less susceptible to antibiotics (Martinez & Baquero, 2002). The cells indirectly build up barrier and protection against host defense mechanisms and antibiotics. Consequently, many biofilm bacteria developed and resistance against antimicrobial agents such as Methicillin resistance *Stapylococcus aureus* (MRSA), *Listeria monocytogenes*, *E.*coli and etc.

Among 30 isolates of intermediate and saprophytic *Leptospira*, the biofilm formation was further classified to no biofilm, weak biofilm, moderate and strong biofilm. The classification was based on the calculation of optical density cut off value (OD_c) which method was adapted from Stepanović et al., 2000.

Biofilm cells mass can be used to determine the level of biofilm forming ability in each strain. Generally, the classification of biofilm mass can be calculated by using the optical density cut-off value. The definition of optical cut off value, OD_c is the three times standard deviations above the mean OD of the negative control. The optical density of each strain was calculated from the arithmetic mean value of the three wells. Based on the formula, the OD of the bacterial film was compared to the OD_c of the day and further classified into four categories.

The four classes of biofilm known as: $OD \leq OD_c$ = no biofilm producer; $OD_c < OD \leq 2X\ OD_c$ = weak biofilm producer; $2X\ OD_c < OD \leq 4X\ OD_c$ = moderate biofilm producer and $4X\ OD_c < OD$ = strong biofilm producer. Table 4.1 show the number of tested intermediate *Leptospira* with different biofilm forming abilities on different day and Table 4.2 show the number of tested saprophytic *Leptospira* with different biofilm forming abilities on different day.

Table 4.1: Number of tested intermediate *Leptospira* with different biofilm forming abilities on abiotic surface at different day

Day	Biofilm Forming Ability (n=15)			
	Non	**Weak**	**Moderate**	**Strong**
1	0(0.00%)	4(26.67%)	9(60.00%)	1(6.67%)
2	1(6.67%)	3(20.00%)	8(53.33%)	2(13.33%)
3	1(6.67%)	1(6.67%)	11(73.33%)	2(13.33%)
4	1(6.67%)	0(0.00%)	7(46.67%)	8(53.33%)
5	0(0.00%)	1(6.67%)	5(33.33%)	7(46.67%)
6	1(6.67%)	0(0.00%)	7(46.67%)	7(46.67%)
7	1(6.67%)	4(26.67%)	8(53.33%)	2(13.33%)
8	1(6.67%)	3(20.00%)	8(53.33%)	3(20.00%)
9	2(13.33%)	9(60.00%)	3(20.00%)	1(6.67%)
10	3(20.00%)	4(26.67%)	7(46.67%)	1(6.67%)
11	4(26.67%)	3(20.00%)	8(53.33%)	0(0.00%)

Table 4.2: Number of tested saprophytic *Leptospira* with different biofilm forming abilities on abiotic surface at different day

Day	Biofilm Forming Ability (n=15)			
	Non	**Weak**	**Moderate**	**Strong**
1	2(13.33%)	8(53.30%)	5(33.33%)	0(0.00%)
2	0(0.00%)	5(33.33%)	10(66.67%)	0(0.00%)
3	0(0.00%)	0(0.00%)	11(73.33%)	4(26.67%)
4	0(0.00%)	4(26.67%)	10(66.67%)	1(6.67%)
5	0(0.00%)	0(0.00%)	14(93.33%)	1(6.67%)
6	0(0.00%)	0(0.00%)	9(60.00%)	6(40.00%)
7	0(0.00%)	0(0.00%)	10(66.67%)	5(33.33%)
8	0(0.00%)	0(0.00%)	13(86.67%)	2(13.33%)
9	0(0.00%)	2(13.33%)	11(73.33%)	2(13.33%)
10	1(6.67%)	4(26.67%)	10 (66.67%)	0(0.00%)
11	2(13.33%)	13(86.67%)	0(0.00%)	0(0.00%)

The number of tested intermediate and saprophytic *Leptopsira* with different biofilm forming ability on different are represented on Table 4.1 and Table 4.2. Among 30 isolates and 2 positive controls examined, intermediate *Leptospira* recorded the strongest biofilm forming ability on day 4 and day 5 with 53.33% of biofilm forming ability while saprophytic *Leptospira* reported the strongest on day 6 with 40% of biofilm forming ability. Majority of isolates produced biofilm from day 2 to day 6 during microcolonies forming and biofilm maturation phases. This was an agreement to previous report that in pathogenic *Leptospira* produced moderately and strongly attachment of biofilm from day 2

until day 5 but remained strongly forming biofilm until day 10 (Pui et al., 2017). In other studied, previous researchers explained that during this phase, the surface contact triggers responses that lead to gene expression on the extracellular matrix of the bacteria. For example in *P. aeruginosa*, the extracelluar matrix composition responses vary depending on the environmental conditions (Harmsen et al., 2010). In another study, *P. aeruginosa* EPS component (alginate) developed resistance to antibiotic treatments and host immune defences during chronic infection (Leid et al., 2005). At last phase during biofilm dispersal, biofilm forming ability of both strains was unable to resist the washing step of biofilm assays and bacteria evolved ways to perceive environmental changes and resume a planktonic lifestyle (Kostakioti et al., 2013).

4.3 Biofilm formation on biotic surface

As a comparison for biofilm assays, the biotic surface was also analysed. In order to differentiate biofilm formation between abiotic and biotic surfaces, wood chips from *Dyera postula* was used to represent biotic surface in this study. Similarly, 11 days for time course study biofilm formation was conducted to compare the biofilm forming ability between intermediate and saprophytic *Leptospira*. From the result, biotic surface retained stronger biofilm forming ability compared to abiotic surface. At day 5, intermediate *Leptospira* recorded 93.99% strong biofilm formation while saprophytic *Leptospira* reported 86.67% strong biofilm forming ability. Figure 4.6 and Figure 4.7 show the biofilm formation of intermediate and saprophytic *Leptospira* using mean value of optical density (OD_{600}).

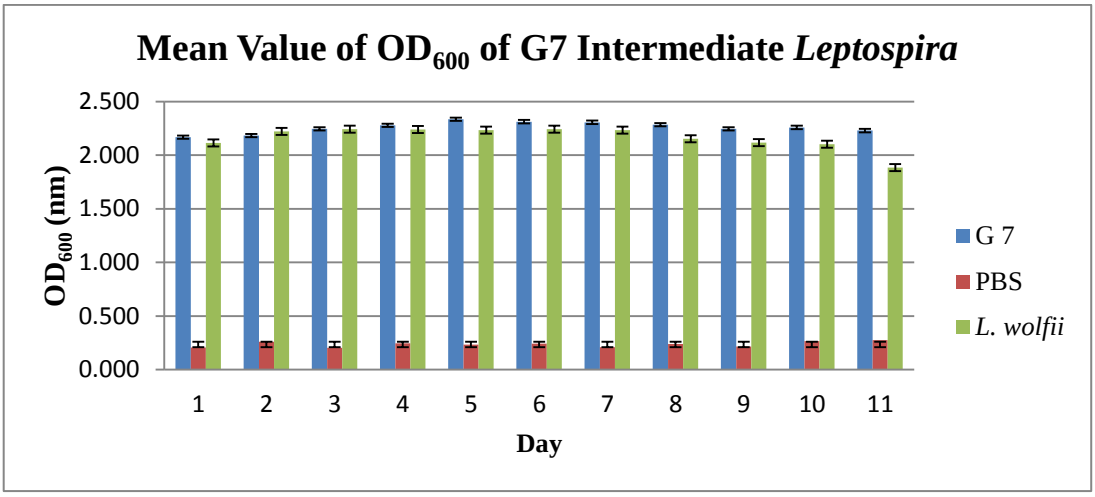


Figure 4.6: OD_{600} of biofilm formation on wood surface in G7 (*L. wolffii* serovar Khorat strain Khorat-H2). The OD for strongest biofilm producer of intermediate strain was reported as 2.335 ± 0.052 on day 5.

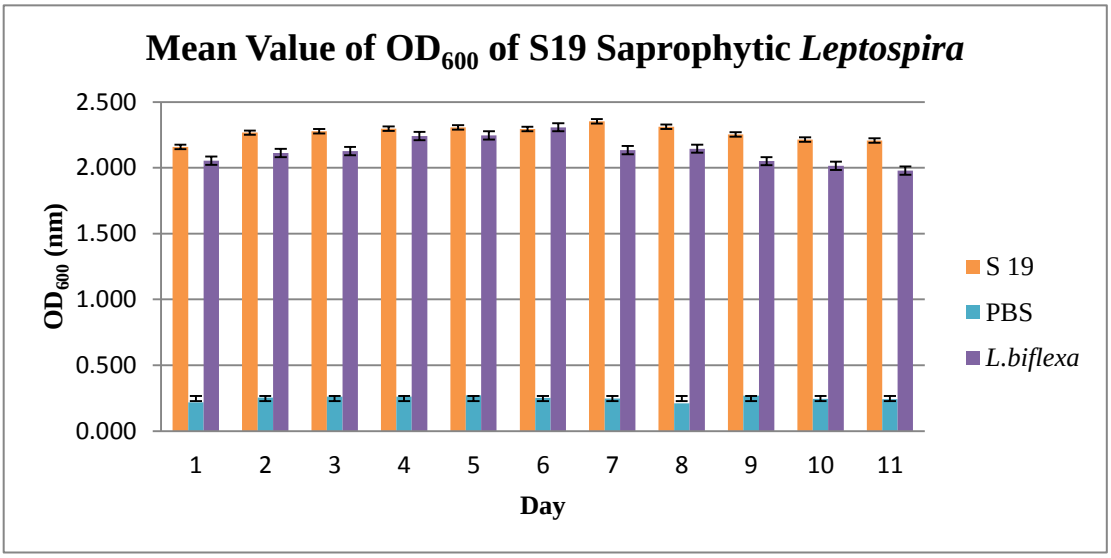


Figure 4.7: OD_{600} of biofilm formation on wood surface in S19 (*L. meyeri* strain 19CAP). The OD for saprophytic strain was recorded as 2.308 ± 0.043 on day 5 of biofilm assay.

From Figure 4.6 and Figure 4.7 show the comparison using bar graphs of biofilm formation by using mean value of OD_{600}. The graphs show similar trend and only slight different value of optical density from each day. From day 1 cell attachment, the mean value of OD_{600} recorded for G7 intermediate was 2.168 ± 0.085 and the value S19, saprophytic reported was 2.078 ± 0.052 with 0.090 different. Day 2 to day 5, the OD_{600}

retained 2.0 value until day 5 where the microcolonies and biofilm matured phases done. After day 6, the mean value of optical density for both strains started to consistent reduce until day 11 but still retained OD_{600} value more than 2.0.

Biofilm bacteria can form and withstand biofilm at different conditions. The present study found that *Leptospira* spp. developed biofilm on the two different surfaces, biotic and abiotic with incubation time. Interestingly, in this study wood chips surface produced stronger biofilm formation compared to the microtiter plate surface. Plant surfaces especially wood surface has a rough texture, appearance and irregular surface that favoured for bacterial cell attachment which eventually will develop a robust biofilm (Apun et al., 2019). Often leptospirosis outbreak poses during flooding and seasonal rainfall which increases the risk of exposure to contaminated water and other natural resources. Leptospires is transmitted by exposure to contaminated water or urine from infected animals and may survive for days to months in freshwater, soil or mud and wood surface (Rood et al., 2017). However, the environmental survival capacities of *Leptospira* depend on the species and strains. Wood surface biofilm has been intensively study in microbial communities especially in terms of food safety. In terms of food processing, biofilm formation is the most common issue that lead to serious hygienic problems which can lead to potential chronic infections. Hence, it has been one of the most challenging within food and industrial environment problematic. However, there is lack of information and inadequate data to compare the value of biofilm formation in *Leptospira* in this study. The observation of biofilm attachment under stereo microscope has been used to study the structure of biofilm assays for 11 days. Figure 4.8 and Figure 4.9 show the stained cell of intermediate and saprophytic *Leptospira.*

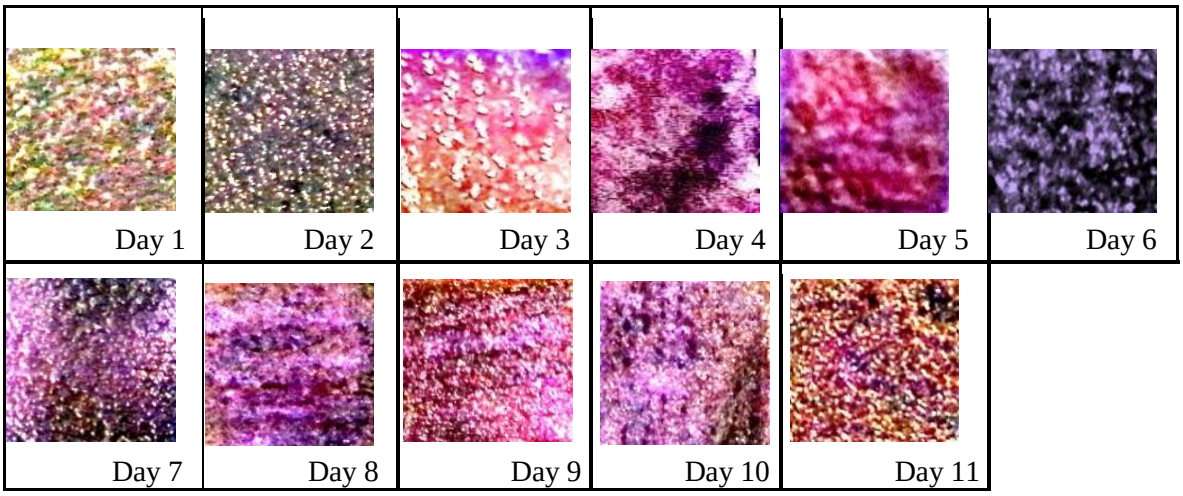


Figure 4.8: The stained cell of intermediate, G7 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) from water sample viewed under phase contrast microscope. On day 1, the leptospires do not stained well on the woodchip. However, on day 2 onwards the crystal violet had retained well on the wood chip surface. At this phase, the dye was resistant to the washing step. The dye started faded on day 11 due to biofilm detachment.

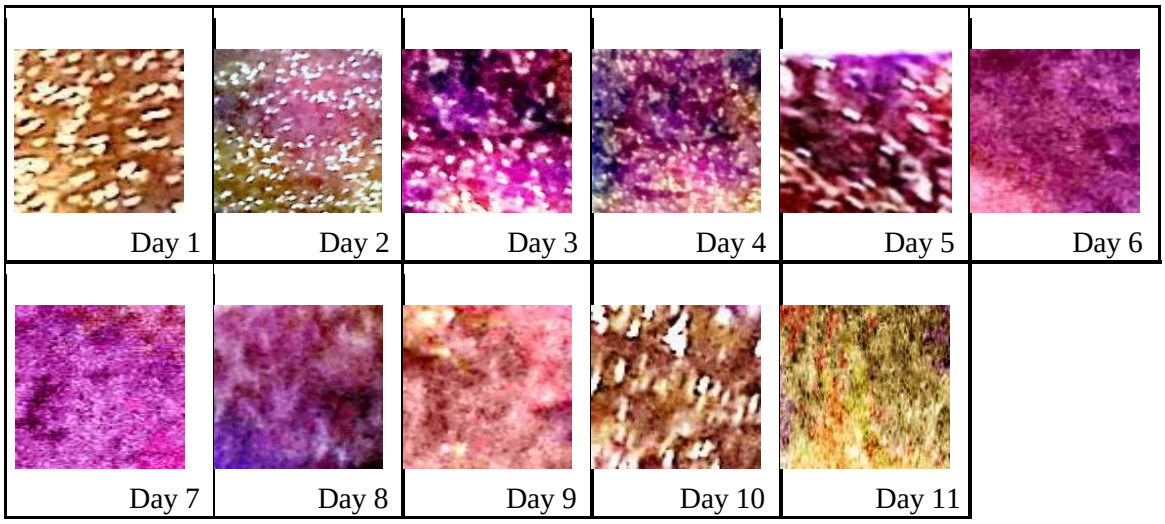


Figure 4.9: The stained cell of saprophytic, S19 (*Leptospira meyeri* strain 19CAP) from water sample viewed under phase contrast microscope. On day 1, the leptospires do not stained well on the woodchip. However, on day 2 onwards the crystal violet had retained well on the wood chip surface. At this phase, the dye was resistant to the washing step. The dye started disappeared on day 10 due to biofilm detachment.

From Figure 4.8 and Figure 4.9, the visualization and colour intensity were observed under stereo microscope (Raxvision) at 40x magnification. The original colour of the wood was yellowish brown. The microscopic viewed the end grain of the wood surface

with large pores when crystal violet stain covered the surface of the wood. The intensity of crystal violet showed that intermediate *Leptospira* spp. stained and retained well on wood surface compared to microtitre plate surface. Although not much different, the colour intensity of crystal violet on intermediate was stronger than saprophytic *Leptospira*. The biofilm formation on biotic surface in intermediate and saprophytic *Leptospira* were relatively stronger (OD_{600}> 2.0) compared to abiotic surface. Other studies on *L. monocytogenes* showed that wood surface retained stronger biofilm than steel and glass surfaces (Adetunji & Isola, 2011).

Woods are hydrophobic materials and it provides better bacterial adherence. This finding obtained is in agreement by Fletcher and Loeb (1979) documented large numbers of bacteria attached on the hydrophobic surfaces with neutral charge substrate compared to hydrophilic surface. Previous study on *E.*coli O157:H7 found that biofilm attached stronger on wood surface (0.59 ± 0.02).than steel (0.18 ± 0.01) and glass (0.10 ± 0.01) (Adetunji & Isola, 2011). Their report revealed that the biofilm ability on wood surface of *E.*coli O157:H7 is stronger than *E.*coli with increases of biofilm formation with an extension of incubation time. Hence, it is important to develop a new biofilm modelling system that more closely resembles the clinical and terrestrial relevance of biofilm in *Leptospira*. The bar graphs of quantification of intermediate and saprophytic *Leptospira* were plotted on Figure 4.10 and Figure 4.11.

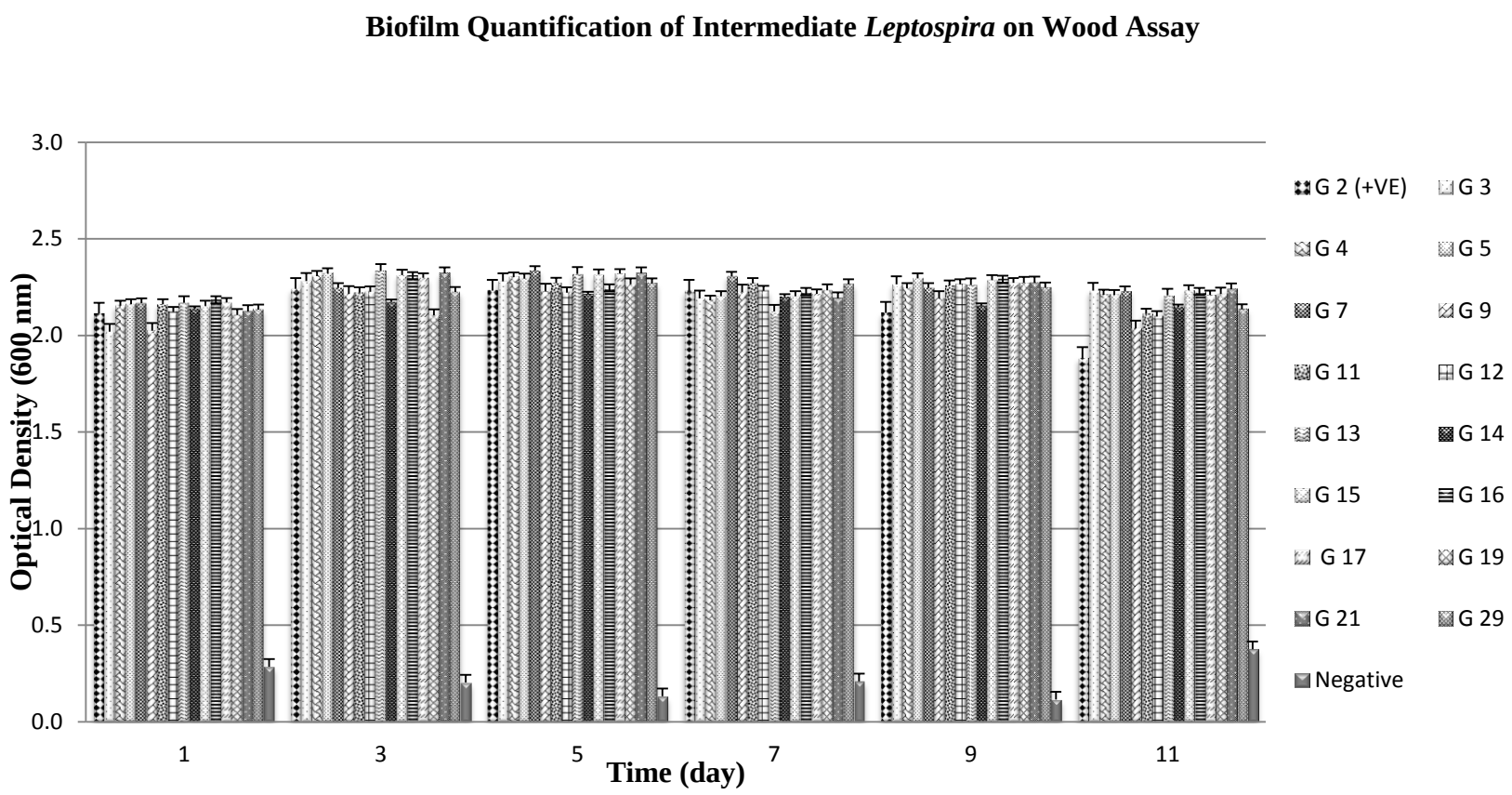


Figure 4.10: Quantification of biofilm formation (OD_{600}) for 15 intermediate *Leptospira* comprises of 3 soil samples, 4 water samples, 8 rat liver samples tested on biotic surface (*Dyera Costulata*) compared with positive control from FRST, Unimas. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments.

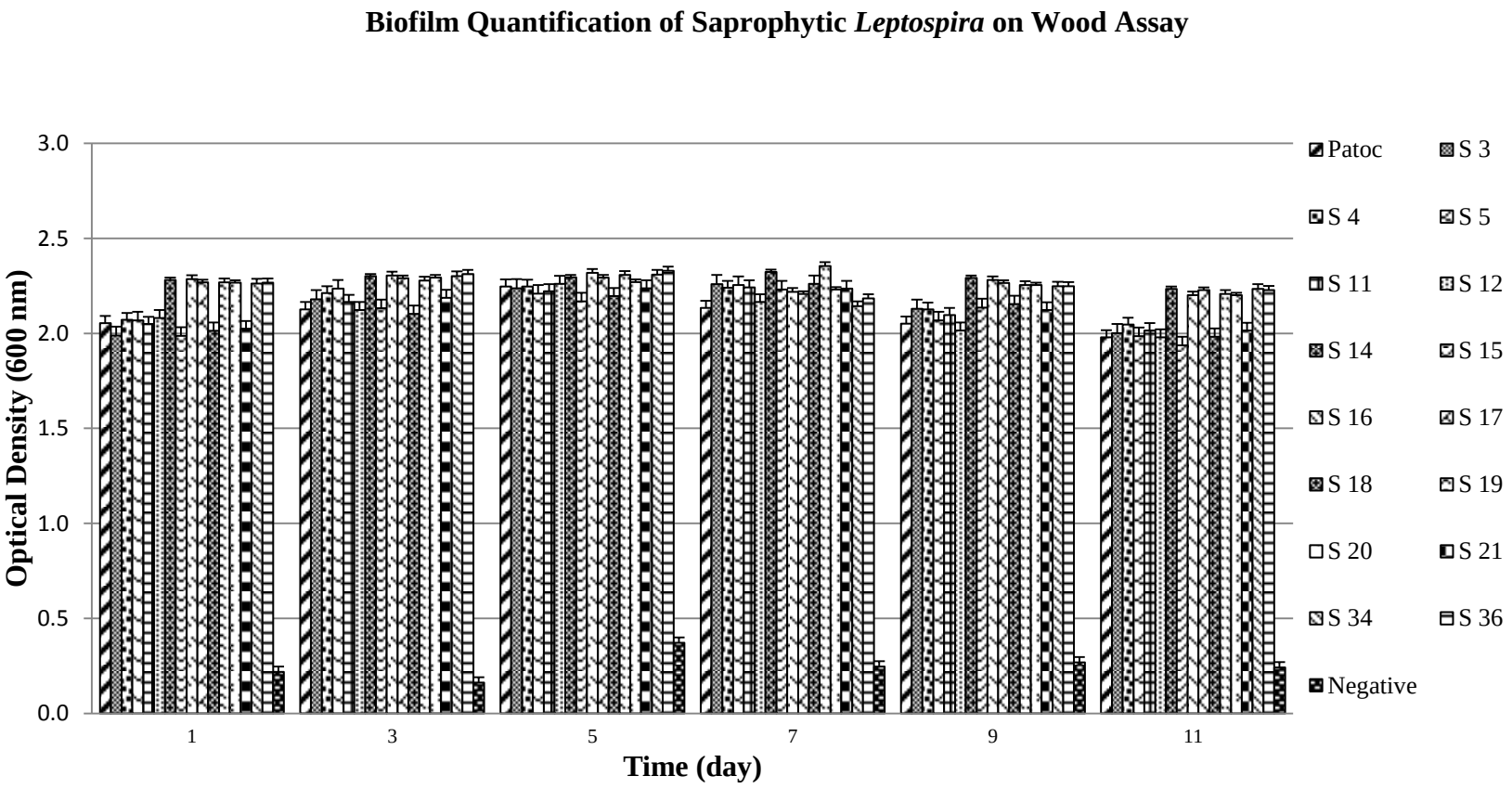


Figure 4.11: Quantification of biofilm formation (OD_{600}) for 15 saprophytic *Leptospira* comprises of 3 soil samples, 4 water samples, 8 rat liver samples tested on biotic surface (*Dyera Costulata*) compared with positive control from IMR, Malaysia. The amount of biofilm was quantitatively measured at OD_{600}. Error bars are standard errors derived from triplicate experiments.

In Figure 4.10 and Figure 4.11 show the trend of biofilm formation by intermediate and saprophytic *Leptospira.* Out of the 15 intermediate strains, strain G7 (2.335 ± 0.052) reported the highest optical density while saprophytic strain S19 recorded the highest biofilm production with 2.308 ± 0.043. However the ability of attachment of intermediate and saprophytic *Leptospira* were not similar. From the graph, the highest strongest isolate The OD_{600} of all isolate was included in Appendix D and E. The trend varied depending on the strain's ability to form biofilm. From the graph, there is not much difference on the optical density value as seen in Figures 4.10. The average OD readings of the strains had reached a certain maximum limit depending on their strength to form biofilm. From the statistical analysis, the biofilm formation produced by these bacteria were significant different in the effect of time, $p<0.05$. This variation was significant different among 15 intermediate and 15 saprophytic *Leptospira* on biotic surfaces (two sample *t*-tests by day, $p<0.05$).

Biofilm formation on biotic surface in *Leptospira* has been less intensively studied, although a variety of microbial communities especially *E.coli, L. monocytogenes* have been reported to colonize on biotic surface. The current study is the first of its kind to determine the biofilm forming abilities among *Leptospira* isolates from environmental samples, which possibly can contribute toward the understanding of leptospirosis infection process. Previous study on *Vibrio parahaemolyticus* and *Vibrio alginolyticus* on biotic surface by using Hep2 cells and showed the results of high level of adhesion (4%-18%) during the initial attachment to epithelial cells. From the results, the statistical analysis also revealed a statistically significant positive correlation between the hydrophobicity and biofilm production in *Vibrio* spp. (Lamari et al., 2018). Thus, hydrophobic interaction favour the adhesion and colonization ability on biotic surface compared to abiotic surface

in this study. Table 4.3 and Table 4.4 show the number of tested intermediate and saprophytic *Leptospira* with different biofilm forming abilities on different day. Biofilm forming ability were calculated using optical density cut off (OD_c) value and further classified into different biofilm forming ability classes (Stepanovic et al., 2008).

Table 4.3: Number of tested intermediate *Leptospira* with different biofilm forming abilities on biotic surface (wood chips) at different day

Day	Biofilm Forming Ability (n=15)			
	No	**Weak**	**Moderate**	**Strong**
1	0(0.00%)	1(6.67%)	5(33.33%)	9(60.00%)
2	0(0.00%)	0(0.00%)	6(40.00%)	9(60.00%)
3	0(0.00%)	0(0.00%)	4(26.67%)	11(73.33%)
4	0(0.00%)	0(0.00%)	2(13.33%)	13(86.67%)
5	0(0.00%)	0(0.00%)	1(6.67%)	14(93.33%)
6	0(0.00%)	0(0.00%)	3(20.00%)	12(80.00%)
7	0(0.00%)	0(0.00%)	4(26.67%)	11(73.33%)
8	0(0.00%)	0(0.00%)	6(40.00%)	9(60.00%)
9	0(0.00%)	0(0.00%)	7(46.67%)	8(53.33%)
10	0(0.00%)	1(6.67%)	8(53.33%)	6(40.00%)
11	0(0.00%)	2(13.33%)	8(53.33%)	5(33.33%)

Table 4.4: Number of tested saprophytic *Leptospira* with different biofilm forming abilities on biotic surface (wood chips) at different day

Day	Biofilm Forming Ability (n=15)			
	No	**Weak**	**Moderate**	**Strong**
1	0(0.00%)	3(20.00%)	4(26.67%)	8(53.33%)
2	0(0.00%)	0(0.00%)	6(40.00%)	9(60.00%)
3	0(0.00%)	0(0.00%)	5(33.33%)	10(66.67%)
4	0(0.00%)	0(0.00%)	4(26.67%)	11(73.33%)
5	0(0.00%)	0(0.00%)	2(13.33%)	13(86.67%)
6	0(0.00%)	0(0.00%)	2(13.33%)	13(86.67%)
7	0(0.00%)	0(0.00%)	5(33.33%)	10(66.67%)
8	0(0.00%)	0(0.00%)	6(40.00%)	9(60.00%)
9	0(0.00%)	0(0.00%)	7(46.67%)	8(53.33%)
10	0(0.00%)	4(26.67%)	4(26.66%)	7(46.67%)
11	0(0.00%)	2(13.33%)	6(40.00%)	7(46.67%)

From Table 4.3 and Table 4.4 show the number of tested intermediate and saprophytic *Leptospira* with different biofilm forming ability on different day.

Intermediate *Leptospira* was recorded 60% (day 1) of strong biofilm producer while for saprophytic was reported 53.33% of strong biofilm producer on day 1 on biotic surfaces. The biofilm production was strongly formed and consistently increases from day 1 to day 7. However, the strong biofilm producer started to decline to 60% and this occurred due to nutrients depletion and caused the detachment of biofilm.

During formation of biofilm, it was observed that the amount of biofilm was highly influenced by the availability of water and nutrients on the biotic surface which bacteria multiplication was faster under nutrient rich condition. As it continued to multiply, the biofilm reached nutrient depletion and started to detach and allowed them to return back as planktonic cells. Recently, co-aggregation and biofilm forming ability of leptospires with other environmental bacteria was demonstrated, which in turn helped leptospires to cope with environmental stress (Vinod Kumar et al., 2015). Other factor such as hydrophobicity of bacteria and surface roughness also play an important role in biofilm formation. Wood surface is the main component of our study. Other *Leptospira* biofilm studied on the paddy leaf, while our study tried to demonstrate the attachment of biofilm on the wood surface (Vinod Kumar et al., 2016). The surface chosen have the highest roughness retention and biofilm can attach stronger with greater amount of biofilm formation. Many researchers have widely studied on the bacterial-surface interaction and discovered the physical forces which interact and influenced their motility. The physical forces such as Brownian diffusion, gravitational settling and hydrodynamic forces, electrostatic interactions, Acid Base Lewis interaction and van der Waals interaction normally used for normal bacterial adhesion (Ammar et al., 2015). A study on a fresh produce found that the surface roughness was influenced by the retention of *S. Typhimurium* during the rinsing process

from cucumber surface (Tang et al., 2012). They also reported that the optical density of biofilm obtained from the surface of cucumber was higher compared to mango and guava surfaces. The study also found that cucumber surface was stronger attachment surface than mango and guava. This explained why the biofilm on biotic surface was rough compared to microtiter plate surface. Hence, the amounts of bacterial cells attached on biotic surface were higher compared to abiotic surface.

4.4 Phase contrast Light Microscopic

Phase contrast light microscope was used to view the leptospires cell aggregates over time. Phase contrast microscopic views were conducted for 11 days to compare the process of biofilm formation on abiotic and biotic surfaces. During the first hour of incubation in Figure 4.12, a single layer of bacteria attached on glass at the air liquid interface (A). From single layer of bacteria cell, the cell started to accumulate clumps of cells (B and C-Figure 4.12). The cells aggregated and shaped a network like structure to link between cells (D- Figure 4.14). In intermediate strain, the network like structure was no longer be seen on 168 hours (Figure 4.12 & Figure 4.13). However, saprophytic strain retained their structure as seen in Figure 4.14 and Figure 4.15 (E). Finally, the cells dispersed from the network and became one-single cell (F) back (Figure 4.12).

Intermediate *Leptospira*

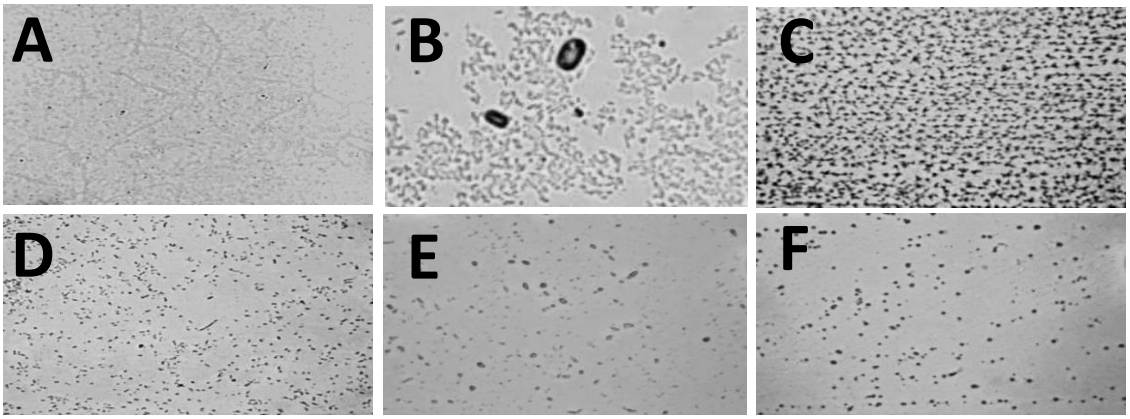


Figure 4.12: Light micrographs of intermediate, G7 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) formed biofilm on glass slide (A to F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. The cells on (A) formed a matrix from cell to cell. The highest number of cell was observed on 24 h (C). The number of cells started to decrease in (E) and (F).

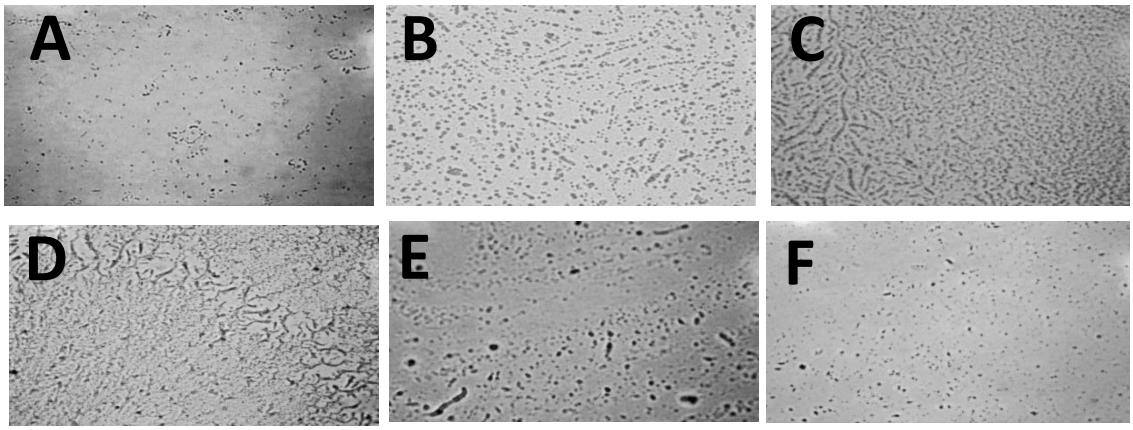


Figure 4.13: Light micrographs of intermediate, G15 (*Leptospira inadai* serovar Aguaruna strain MW4) formed biofilm on glass slides (A) to (F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. The cells appeared to be in a single cell, (B) to (D) appeared to clump and become a matrix. The highest number of cell was observed on 24 h (C). The number of cells became lesser as in (E) and (F).

Saprophytic *Leptospira*

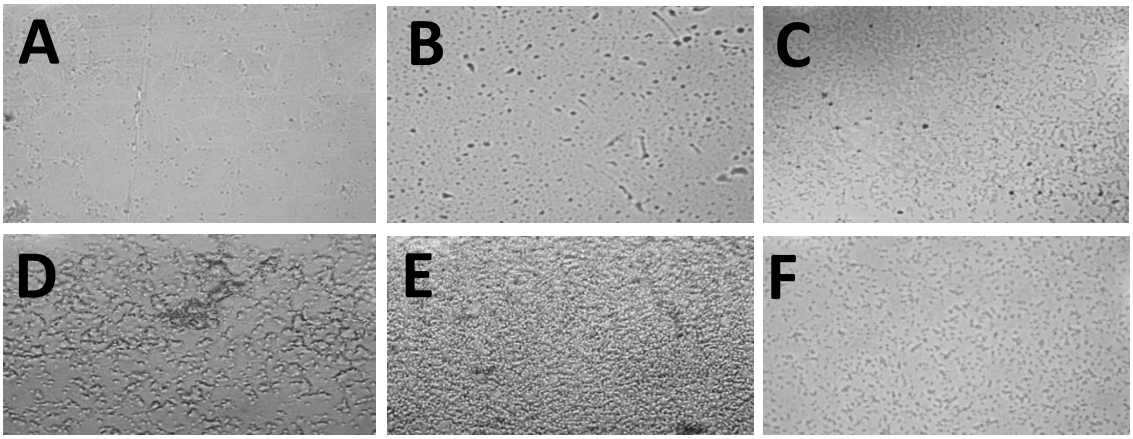
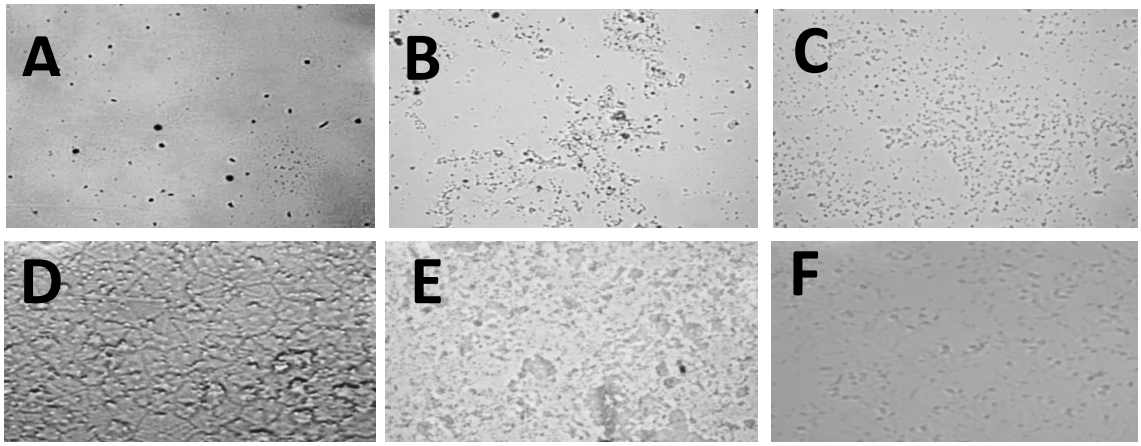


Figure 4.14: Light micrographs of saprophytic, S11 (*Leptospira meyeri* strain Semaranga_DB49) formed biofilm on glass slides (A) to (F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. A does not show a single cell. The cells formed matrix from (C) onwards and started to decrease at (F). The highest number of cell was observed on 24 h (C).



Figures 4.15: Light micrographs of saprophytic, S19 (*Leptospira meyeri* strain 19CAP) strain biofilm on glass slides (A) to (F) with intervals (1, 6, 24, 96, 168, 264 h) at 2000X magnification. (A) only show a few cells, (B) to (E) shows clump and (F) shows less number of cell. The highest number of cell was observed on 24h (C).

The structure of biofilm formed by *Leptospira* spp. was studied at different time using a phase contrast light microscopy. To elucidate the structure of biofilm developed and give an insight on their biofilm behaviour, six strains were chosen for microscopic view including the positive control and both intermediate and saprophytic *Leptospira*. From biofilm assay, S19 (*Leptospira meyeri* strain 19CAP) and S11 (*Leptospira meyeri* strain Samaranga_DB49) saprophytic were the strongest biofilm producers in non-pathogenic *Leptospira* while G7 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) and G15 (*Leptospira inadai* serovar Aguaruna strain MW4) were the strongest biofilm producers in intermediate *Leptospira*.

A single cell of bacteria could be observed without any complex structure in the first hour of incubation. As it continued to form biofilm, the presence of polysaccharide could be observed under microscope after 6 hours of incubation and it increased over time. This was in agreement with Pui et al. (2011), who stated that the bacterial cells need time to adapt their new environment before finally attach on the surface and begin to cover themselves with exopolysaccharides. Ristow et al. (2008) also demonstrated that Leptospires were attached to one another in the biofilm and they were enclosed by an exopolysaccharide matrix that hampered the translational motility once the biofilm matured. Other studies found that sugars, galactose and lipids are essential element in building biofilm matrix component in *Leptospira* (Iraola et al., 2016).

4.5 Detection of biofilm genes

Twenty isolates that comprises of 10 intermediate and 10 saprophytic *Leptospira* were selected to determine the presence of biofilm genes based on their strongest biofilm forming ability. The *ica ADCB* genes and *bap* genes were used in this study. The PCR result shows that *ica C* gene (192 bp) was present in all tested strongest biofilm strains for three times repetition. No band was detected for *ica ADCB* genes in the tested intermediate and saprophytic *Leptospira.* Figure 4.16 and Figure 4.17 show the result of PCR detection on *ica C* gene for identification of biofilm gene in 10 intermediate and 10 saprophytic *Leptospira* isolates with strong biofilm forming ability.

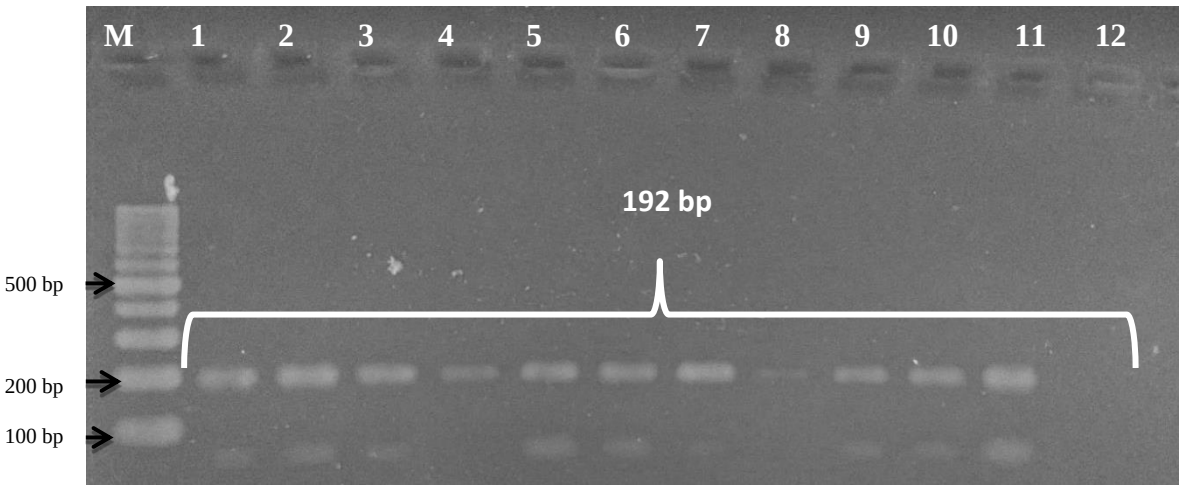


Figure 4.16: The representative amplication of random sequence of *ica C* gene for identification of biofilm gene in 10 intermediate *Leptospira* isolates with strong biofilm forming ability. Lane M showed the 100bp DNA gene ruler (Transgen), lane 1 represented positive control (*Leptospira wolffii* serovar Khorat strain Khorat –H2). Lane 2 to 11 represented the representative biofilm isolates, lane 2-G7, lane 3-G15, lane 4- G5, lane 5-G3, lane 6-G13, lane7-G16, lane 8-G17, lane 9-G29, lane 10-G21, lane 11-G12. Lane 12 represented the negative control.

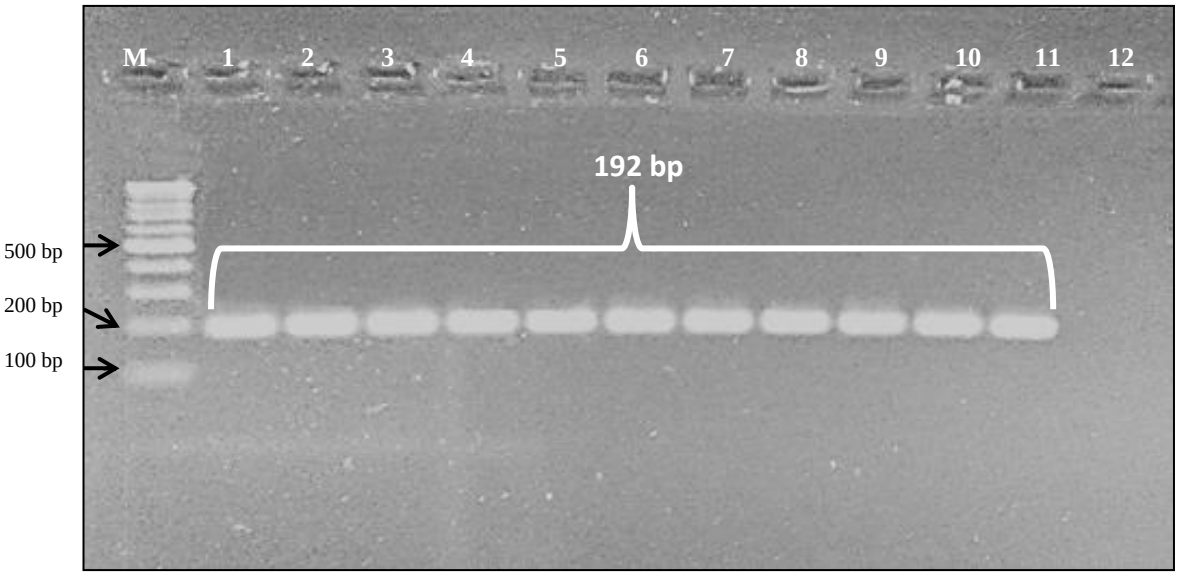


Figure 4.17: The representative amplication of of *ica C* gene for identification of biofilm gene in 10 saprophytic *Leptospira* isolates with strong biofilm forming ability. Lane M showed the 1kb DNA gene ruler (Transgen), lane 1 represented positive control (*Leptospira biflexa* serovar Patoc strain patoc 1). Lane 2 to 11 represented the representative biofilm isolates, lane 2-S19, lane 3-S11, lane 4- S21, lane 5-S14, lane 6-S12, lane7-S20, lane 8-S34, lane 9-S16, lane 10-S5, lane 11-S3. Lane 12 represented the negative control.

In this study, *icaADCB* genes and bap genes were used to study the presence of biofilm in *Leptospira*. The *icaADBC* genes encode for proteins used in the synthesis of polysaccharide intercellular adhesion (PIA) or polymeric N-acetyl-glucosamine (PNAG) by ica operon encoded enzymes that have important roles during attachment, accumulation and production of an extracellular polysaccharide adhesin (Maira- Litran et al., 2002). It was known to assist in bacteria persistence.

Biofilm formation is always related to the role of surface proteins in intercellular adhesion and accumulation of multi-layered cells clusters in most microorganism which still remains unknown. In the past decades, biofilm development association was associated with a group of surface proteins with sequence similar to the biofilm associated protein (Bap) in *S. aureus, Burkholderia cepacia* and *Staphylococcus epidermis* (Huber et

al., 2002 & Tormo et al., 2005). Bap-related proteins are large size and need metabolic effort to produce protein in certain bacteria which result in the presence of high number of repeats. This suggests that proteins can mediate homophilic or heterophilic intercellular interactions. The *bap* gene in *S. aureus* has been known as an important virulence factor that contributes to initial attachment, intercellular adhesion and biofilm maturation (Goh et al., 2013). BAP family of proteins revealed the presence of more than 100 proteins which were similar to Bap homologs database in majority of bacteria.

In this present study, *ica C* gene were detected in all (100%, 20/20) tested *Leptospira* strains with 192 bp fragment size. However, none of *icaA, icaB and icaD* was found in the examined *Leptospira* and there was no presence of bap gene in this study. It was reported that surface protein *bap* gene and its homologues could be found in *Acinetobacter baumanii, Bordetella pertussis, Enterococcus faecalis, E. coli, Lactobacillus reuteri, P. aeruginosa, Salmonella enterica* serovar Enteritidis, *S. Typhimurium* and *S. aureus* (Valle et al., 2012; Colagiorgi et al., 2016). The result obtained in this study also implied that other biofilm-associated genes may have present in *Leptospira* strains to ensure the process of biofilm formation was successfully completed.

4.6 Bacterial virulence study using brine shrimp test

For bacterial virulence assay, the strongest biofilm producers of pathogenic P18 (*Leptospira noguchii* strain LT796), intermediate G7 (*Leptospira wolffii* serovar Khorat strain Khorat-H2) and saprophytic S19 (*Leptospira meyeri* strain 19CAP) *Leptospira* were used as representative of *Leptospira* in this study. The survival analysis of brine shrimp nauplii were counted from 24 hours to 72 hour after infected by the *Leptospira* at different leptospires concentration of 10^3, 10^4 and 10^5 cfu/ml. Percentage of survival analysis of

brine shrimp was calculated survival number of brine shrimp after treated over total number of brine shrimp used (n=20). From the Figure 4.18, the percentage of survival of brine shrimp on day 3 is the lowest (0%) treated with pathogenic compared to intermediate (5%) and saprophytic (25%) *Leptospira.* The result showed that the survival of brine shrimp in *Leptospira* spp. after treated using bacterial cell with 20% lower than to negative control. Pathogenic was included in this study to act as positive control. The pathogenic P18 (*Leptospira noguchii* strain LT796) was previously evaluated as the strongest biofilm forming ability with OD_{600} at 21.760 ± 0.332. The result showed there is significant different ($P<0.05$) between the *Leptospira* strains and doses used (CFUs.) Various CFUs (10^3, 10^4, 10^5) of *Leptospira* strains were infected to compare the bacterial virulence and their infectious doses.

The percentage of survival rate is shown in Figure 4.18 to Figure 4.20. From Figure 4.18, the pathogenic *Leptospira* have strong virulence to brine shrimp when tested in low dosage compared to intermediate and saprophytic *Leptospira*. The brine shrimps were killed in dose dependent manner with different CFUs. From the evaluation graph (Figure 4.20), the significant killing effect of pathogenic *Leptospira* (*L. interrogans*) occurred at 2.6×10^3 cfu/ml.

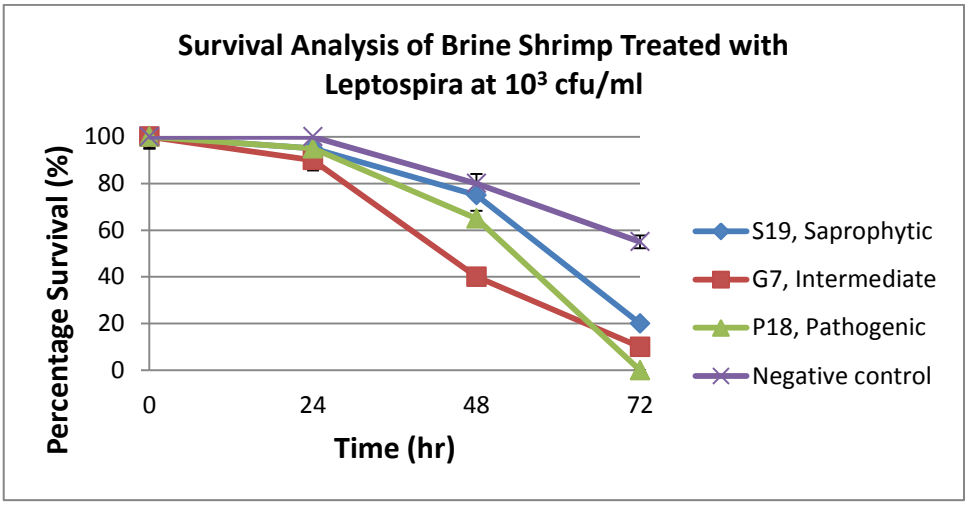


Figure 4.18: Comparison of survival analysis of brine shrimp treated with S19, *Leptospira meyeri* strain 19CAP, G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2, P18, *Leptospira noguchii* strain LT796 (positive control) at 10^3 cfu/ml in 3 days. At day 3, no survival of brine shrimp after treated with pathogenic *Leptospira*, 10% and 20% for intermediate and saprophytic *Leptospira* respectively.

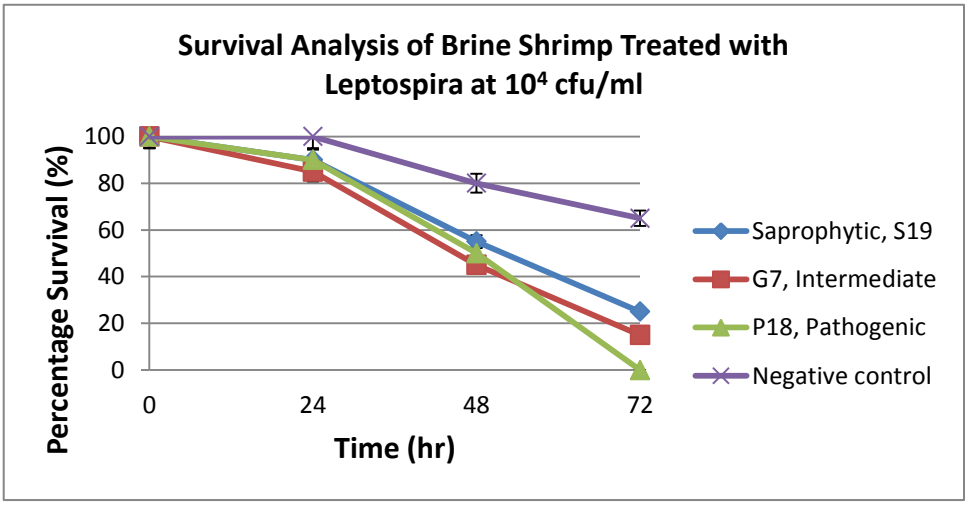


Figure 4.19: Comparison of survival analysis of brine shrimp after treated with S19, *Leptospira meyeri* strain 19CAP, G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2, P18, *Leptospira noguchii* strain LT796 (positive control) at 10^4 cfu/ml in 3 days. At day 3, no survival of brine shrimp after treated with pathogenic *Leptospira*, 15% and 25% for intermediate and saprophytic *Leptospira* respectively.

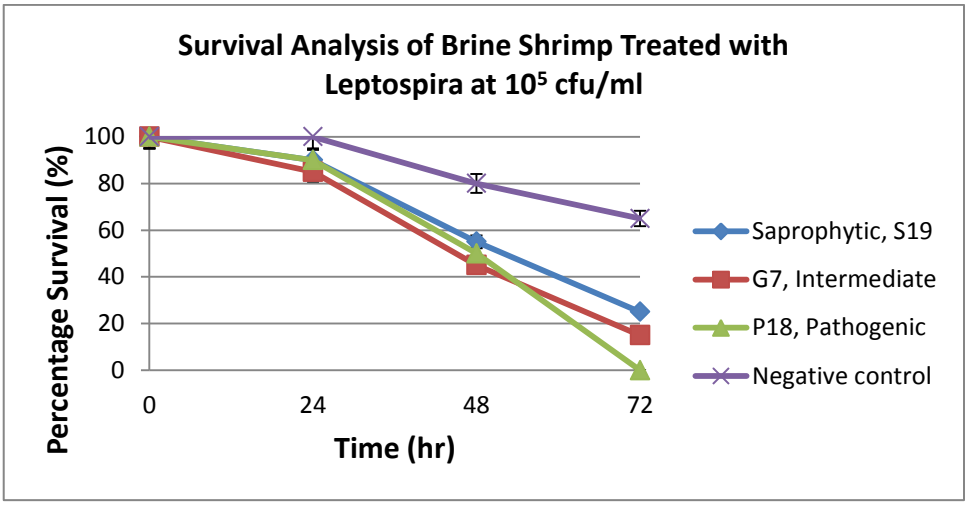


Figure 4.20: Comparison of survival analysis of brine shrimp after treated with S19, *Leptospira meyeri* strain 19CAP, G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2, P18, *Leptospira noguchii* strain LT796 (positive control) at 10^5 cfu/ml in 3 days. At day 3, only 5% of survival rate of brine shrimp after treated with pathogenic *Leptospira*, 20% and 30% for intermediate and saprophytic *Leptospira* respectively.

The first 24 hour of percentage survival of brine shrimp after treated with pathogenic *Leptsopira* (10^3 cfu/ml) was declined to 75% and intermediate and saprophytic were declined to 85% and 95% respectively. Even the smallest dosage of saprophytic *Leptospira* introduced, they can significantly induced the shrimp death. This indicated the brine shrimp can be sensitively assayed with *Leptospira* strains.

After day 2 of incubation, the percentage survival of brine shrimp treated with pathogenic *Leptospira* at concentration of 10^3 cfu/ml declined drastically to 0% compared to intermediate (10%) and saprophytic (20%) as shown in Figure 4.18. The lowest survival rate recorded may be due to the gut infection of the brine shrimp in a reported study (Tkavc et al., 2011). In other host organism, leptospirosis often triggered urinary shedding, body weight loss, hypothermia, and colonization of the kidney in mice after two weeks of infections (Ratet et al., 2014).

Followed by the third day of incubation indicated the percentage survival of brine shrimp treated with pathogenic strains declined up to 0%. Interestingly, intermediate and saprophytic strains could sustain their mortality rates above 10% but no survival on day four. However, the persistence of bacteria was varied between bacteria strains used in this study. This result found that the brine shrimp is susceptible to *Leptospira* strains. Generally, it was a fast and easy method to calculate their survival analysis.

In the brine shrimp assay, brine shrimps were selected as model organism for bacterial virulence study in *Leptospira*. To the best of our knowledge, there has been no study on the *Leptospira* mortality assay using *Artemia salina* (brine shrimp). The bacterial virulence assay was adapted and modified from Lee et al. (2001). After several trials, the suitable bacterial concentration used was at 2.6 x 10^6 cfu/ml or OD at 600nm of 0.32 after bacterial incubation.

It is a well-known model organism for which does not require maintenance of stock culture. This method was commonly known as simple, fast and reliable method for evaluation of among pathogenic, intermediate and saprophytic *Leptospira.* It is usually used to distinguish between pathogenic and non-pathogenic bacteria and further confirmed by statistical analysis (Lal & Zulkarnaen, 2018). In an agreement of Lee et al. (2014), this model organism has advantage over other model organism due to this bacterial virulence analysis does not need maintenance of stock culture and can be cultivated in large amount which help in facilitating replication for statistical analysis.

Other than that, this model organism is also known to have short life span of which only support short analysis virulence assay and it is transparent under microscopic examination. In other microorganisms, brine shrimp was widely used in virulence analysis

of other pathogenic bacteria such as *Bacillus vietnamiensis, P. aeruginosa, S. aureus, V. vulnificus* and *E. coli* (Rumbaugh et al., 2000 & Nair et al., 2011).

In the present study, the infectious doses of pathogenic (*L. interrogans)* at 2.6 x 10^3 cfu/ml. This indicated the small CFU (colony forming unit) of pathogenic *Leptospira* was more virulence than higher CFU of intermediate and saprophytic *Leptospira.* The significant killing effect in brine shrimp occurred at dose of 2.6 x 10^3 cfu/ml for pathogenic *Leptospira.* The highest biofilm producers of intermediate and saprophytic *Leptospira* were used as the feeding food to infect the brine shrimp. Brine shrimp virulence test used *Artemia salina* to test the virulence assay in this study. When different CFUs (colony forming units) of leptospires cells were infected, the brine shrimps were killed in dose-dependent manner. Other studies reported infectious doses of 3.0 x 10^4 on brine shrimp using *P. aeruginosa* (Lee et al., 2014). A study on hamster in *Leptospira* suggested the lethal doses at 10^2-10^3 cfu/ml and it was found to be similar to our present study (Barnett et al., 1999). Another study on the mice was reported using high dose of inoculum of *Leptospira* from 10^6 cfu/ml to 10^8 cfu/ml. The level of pathogenicity was hence determined according to their killing effect on model organism.

In general, the development of biofilm formation process in intermediate and saprophytic *Leptospira* is very similar to pathogenic *Leptospira* which comprises three main stages namely stationary, exponential and death phase. For initial stage of biofilm attachment, s single cell was visualized on different types of surfaces during the first 24 hours of incubation. The self-produced matrix of extracellular polymetric substance (EPS) was then found embedded on the adherent cells. Hydrophobicity of bacteria and surface attachment of surface were important criteria in biofilm formation.

CHAPTER 5

CONCLUSION

This study has successfully demonstrated the importance and significance of biofilm production in saprophytic and intermediate *Leptospira*. This is important due to the facts that biofilm forming ability could be highly associated with the successfulness of bacteria pathogenesis in the host. Our study used time course to illustrate the strength of biofilm production on biotic and abiotic surfaces in certain days. At day 5, intermediate *Leptospira* (G7, *Leptospira wolffii* serovar Khorat strain Khorat-H2) was formed stronger biofilm on biotic surface 93.99% than on abiotic surface with 53.33%. While in saprophytic *Leptospira* (S19, *Leptospira meyeri* strain 19CAP), it formed stronger biofilm on biotic surface with 86.67% and abiotic surface with 40%. Furthermore, the biofilm genes involved could be a crucial factor in gene regulation to regulate the biofilm forming ability at certain stages of biofilm. In brine shrimp assay, all tested *Leptospira* have significant virulence to brine shrimp, especially in pathogenic *Leptospira* showed the strongest virulence but weaker on intermediate and saprophytic *Leptospira*. As the *Leptospira* strains used in this study are from local environment, the results provided revealed the current status of leptospirosis in Sarawak. Any contact of *Leptospira* spp. via abiotic and biotic surfaces must take preventive measure and the current status of biofilm formation in *Leptospira* especially pathogenic is highly virulence. Hence, this information would be useful for the authority in formulating strategy to control and prevent infection of leptospirosis.

Biofilm genes carry important role for successful infection store in host body and also to ensure long term survival. Results of this study revealed that intermediate

Leptospira strains may have the ability to evolve as human pathogen in the near future. This is due to its unique characteristics in forming biofilm in short period of time and the ability to form biofilm on abiotic surfaces. This ability may lead to higher risk of infections by intermediate strain. In addition, the brine shrimps assay was also successfully used to show pathogenic potential of *Leptospira.* This assay is simple, rapid and reliable so it can be applied for further study to test bacterial virulence in *Leptospira*.

In summary, time course study on the ability of biofilm formation in microbial community is extremely important as the strength of biofilm layer can illustrate the pathogenicity of bacteria in a host. Variability among all the isolates of intermediate and saprophytic *Leptospira* yielded different results. The common fact on both intermediate and saprophytic isolates is that they are able to detach from the surface area according to their preference. The cells detach as clusters when the environment is not favourable to form biofilm. In conclusion, infections by the intermediate strains can be prevent if the colonization process stop before it reaches maturation stage. More researchers are needed in order to understand the biofilm forming ability of intermediate *Leptospira* on abiotic surfaces in future.

Limitation of Study

The limitation of this study is the lack of time to determine the effect of antimicrobial agents during biofilm formation in intermediate and saprophytic *Leptospira.*

Recommendations

For future works, it is recommended to study the novel genes used in biofilm formation by *Leptospira* using transposon mutagenesis study. The genes detected may further be used for gene knock out and create mutant library application. Furthermore, the model organism can be improvised into nearest model organism that can resemble to human adaption such as, *Galleria mellonella* and *Caenorhabditis elegans* which can be used to study the virulence or killing effect of doses in human. Due to time constraint of slow growth bacteria, the maintenance and subculturing process can be improvise to twice a month in order to achieve maximal growth for growing bacteria

REFERENCES

Adler, B., & de la Peña Moctezuma, A. (2010). *Leptospira* and leptospirosis. *Veterinary Microbiology, 140*(3), 287-296.

Adetunji, V. O., & Isola, T. O. (2011). Adhesion of E. coli and E. coli O157: H7 isolates from a typical tropical abattoir on wood, steel and glass surfaces. *Research Journal of Microbiology, 6*(1), 669-677.

Alexander, A. D., Evans, L. B., Toussant, A. J., Marchwicki, R. H., & McCrumb, F. R. (1957). Leptospirosis in Malaya. II. Antigenic analysis of 110 leptospiral strains and other serological studies. *American Journal of Tropical Medicine and Hygiene, 6*(1), 871–889.

Alston, J. M., Broom, J. C., & Doughty, C. J. A. (1958). *Leptospirosis in man and animals*. Edinburgh, Livingstone.

Ammar, Y., Swailes, D., Bridgens, B., & Chen, J. (2015). Influence of surface roughness on the initial formation of biofilm. *Surface and Coatings Technology, 284*(1), 410-416.

Armbruster, C. R., & Parsek, M. R. (2018). New insight into the early stages of biofilm formation. In proceedings of the *National Academy of Sciences, 115*(17), 4317-4319.

Apun, K., Jalan, J., Pui, C. F., Bilung, L. M., Hashim, H. F., Ahsan, A. A. N. B. M., & Rupert, R. (2018). Biofilm forming ability of intermediate and saprophytic *Leptospira* on abiotic and biotic surfaces. *Malaysian Journal of Microbiology, 14*(4), 313-319.

Bahaman, A. R., Ibrahim, A. L., & Adam H. (1987). Serological prevalence of leptospiral infection in domestic animals in West Malaysia. *Epidemiology Infection*, *99*(2), 379-392.

Bahaman, A. R., & Ibrahim, A. L. (1988). A review of leptospirosis in Malaysia. *Veterinary Research Communications*, *12*(2-3), 179-189.

Babudieri, B. (1961). Laboratory diagnosis of leptospirosis. *Bulletin of the World Health Organization*, *24*(1), 45-56.

Barnett, J. K., Barnett, D., Bolin, C. A., Summers, T. A., Wagar, E. A., Cheville, N. F., & Haake, D. A. (1999). Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. *Infection and Immunity*, *67*(2), 853-861.

Beloin, C., Roux, A., & Ghigo, J. M. (2008). *Escherichia coli* biofilms. Berlin, Heidelberg.

Benacer, D., Zain, S. N. M., Amran, F., Galloway, R. L., & Thong, K. L. (2013). Isolation and molecular characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* isolates from the urban rat populations of Kuala Lumpur, Malaysia. *The American Journal of Tropical Medicine and Hygiene*, *88*(4), 704-709.

Bennett, J. E., Dolin, R., & Blaser, M. J. (2016). *Mandell, Douglas and Bennett's Infectious Disease Essentials E-Book*. Elsevier Health Sciences.

Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., & Vinetz, J. M. (2003). Leptospirosis: a zoonotic disease of global importance. *The Lancet Infectious Diseases*, *3*(12), 757-771.

Brading, M. G., Boyle, J., & Lappin-Scott, H. M. (1995). Biofilm formation in laminar flow using *Pseudomonas* fluorescens EX101. *Journal of Industrial Microbiology, 15*(4), 297-304.

Cetinkaya, B., Ertas, H. B., Ongor, H., & Muz., A. (2000). Detection of *Leptospira* species by polymerase chain reaction (PCR) in urine of cattle *Turkish Journal of Veterinary and Animal Sciences, 24*(2), 123-130.

Cegelski, L., Pinkner, J. S., Hammer, N. D., Cusumano, C. K., Hung, C. S., Chorell, E., Aberg, V., Walker, J. N., Seed, P. C., & Almqvist F. (2009). Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nature Chemical Biology, 5*(1), 913-919.

Chai, Y., Beauregard, P. B., Vlamakis, H., Losick, R., & Kolter, R. (2012). Galactose metabolism plays a crucial role in biofilm formation by *Bacillus subtilis*. *Microbiology Biology, 3*(4), 174-192.

Characklis, W. G. (1981). Bioengineering report: fouling biofilm development: a process analysis. *Biotechnology and Bioengineering*, *23*(9), 1923-1960.

Cosson, J. F., Picardeau, M., Mielcarek, M., Tatard, C., Chaval, Y., Suputtamongkol, Y., & Morand, S. (2014). Epidemiology of Leptospira transmitted by rodents in Southeast Asia. *PLoS Neglected Tropical Diseases, 8*(6), 902-923.

Chiriboga, J., Barragan, V., Arroyo, G., Sosa, A., Birdsell, D. N., España, K., & Pinargote, C. (2015). High prevalence of intermediate *Leptospira* spp. DNA in febrile humans from urban and rural Ecuador. *Emerging Infectious Diseases*, *21*(12), 2141-2153.

Chadsuthi, S., Bicout, D. J., Wiratsudakul, A., Suwancharoen, D., Petkanchanapong, W., Modchang, C., & Chalvet-Monfray, K. (2017). Investigation on predominant Leptospira serovars and its distribution in humans and livestock in Thailand, 2010-2015. *PLoS Neglected Tropical Diseases, 11*(2), 5208-5228.

Chandki, R., Banthia, P., & Banthia, R. (2011). Biofilms: a microbial home. *Journal of Indian Society of Periodontology, 15*(2), 111-121.

Christensen, G. D., Baldassarri, L., & Simpson, W. A. (1995). Methods for studying microbial colonization of plastics. *Methods in Enzymology, 253*(1), 477-500.

Ciofu, O., Rojo-Molinero, E., Macià, M. D., & Oliver, A. (2017). Antibiotic treatment of biofilm infections. *Journal of Pathology and Immunology, 125*(4), 304-319.

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., & Ko, A. I. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Neglected Tropical Diseases, 9*(9), 1-19.

Colagiorgi, A., Di Ciccio, P., Zanardi, E., Ghidini, S., & Ianieri, A. (2016). A look inside the *Listeria monocytogenes* biofilms extracellular matrix. *Microorganisms, 4*(3), 22-34.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W., & Götz, F. (1999). The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infection and Immunity, 67*(10), 5427-5433.

Crouzet, M., Le Senechal, C., Brözel, V. S., Costaglioli, P., Barthe, C., Bonneu, & Vilain, S. (2014). Exploring early steps in biofilm formation: set-up of an experimental system for molecular studies. *Bio Medical Central Microbiology, 14*(1), 253-287.

Davis, J. M., Haake, D. A., & Ramakrishnan, L. (2009). *Leptospira interrogans* stably infects zebrafish embryos, altering phagocyte behavior and homing to specific tissues. *PLoS Neglected Tropical Diseases, 3*(6), 463-453.

Davey, M. E., & O'toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews, 64*(4), 847-867.

Degeest, B. & de Vuyst L. (2000). Correlation of activities of the enzymes alpha-phosphoglucomutase, UDP-galactose 4 epimerase and UDP- glucose pyrophosphorylase with exopolysaccharide biosynthesis by *Streptococcus thermophilus* LY03. *Applied Environmental Microbiology, 66*(1), 3519-3527.

De Vries, S. G., Visser, B. J., Nagel, I. M., Goris, M. G., Hartskeerl, R. A., & Grobusch, M. P. (2014). Leptospirosis in Sub-Saharan Africa: a systematic review. *International Journal of Infectious Diseases, 28*(1), 47-64.

Djeribi, R., Bouchloukh, W., Jouenne, T., & Menaa, B. (2012). Characterization of bacterial biofilms formed on urinary catheters. *American Journal of Infection Control, 40*(9), 854-859.

Djordjevic, D., Wiedmann, M., & McLandsborough, L. A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology, 68*(6), 2950-2958.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious Diseases, 8*(9), 881-888.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews, 15*(2), 167-193.

Erickson, T. (2017). Epidemiology of Leptospirosis in Mesoamerica: Historical Perspectives on One Health Transmission. *Current Tropical Medicine Reports*, *4*(2), 62-69.

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563-580.

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Review Microbiology Journal*, *8*(1), 623-633.

Fletcher, W. (1928). Recent Work on Leptospirosis, Tsutsugamusfai Disease, and Tropical Typhus in the Federated Malay States. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *21*(4), 12-34.

Francolini, I., & Donelli, G. (2010). Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunology and Medical Microbiology*, *59*(3), 227-238.

Fux, C. A., Costerton, J. W., Stewart, P. S., & Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends in Microbiology*, *13*(1), 34-40.

Garba, B., Bahaman, A. R., Khairani-Bejo, S., Zakaria, Z., & Mutalib, A. R. (2017). Retrospective study of leptospirosis in Malaysia. *EcoHealth*, *14*(2), 389-398.

Ganoza, C. A., Matthias, M. A., Saito, M., Cespedes, M., Gotuzzo, E., & Vinetz, J. M. (2010). Asymptomatic renal colonization of humans in the peruvian Amazon by *Leptospira*. *PLoS Neglected Tropical Diseases*, *4*(2), 600-612.

Garrett, T. R., Bhakoo, M., & Zhang, Z. (2008). Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science, 18*(9), 1049-1056.

Gerbersdorf, S. U., & Wieprecht, S. (2015). Biostabilization of cohesive sediments: revisiting the role of abiotic conditions, physiology and diversity of microbes, polymeric secretion, and biofilm architecture. *Geobiology, 13*(1), 68-97.

Goldstein, S. F., & Charon, N. W. (1990). Multiple-exposure photographic analysis of a motile spirochete. In proceedings of the *National Academy of Sciences, 87*(13), 4895-4899.

Gajardo, G. M., & Beardmore, J. A. (2012). The brine shrimp *Artemia*: adapted to critical life conditions. *Frontiers in Physiology, 3(1)*, 185-198.

Goh, H. S., Beatson, S. A., Totsika, M., Moriel, D. G., Phan, M. D., Szubert, J., & Lipman, J. (2013). Molecular analysis of the *Acinetobacter baumannii* biofilm-associated protein. *Applied and Environmental Microbiology, 79*(21), 6535-6543.

Gomes-Solecki, M., Santecchia, I., & Werts, C. (2017). Animal models of leptospirosis: of mice and hamsters. *Frontiers in Immunology, 8*(1), 58-80.

Gonwong, S., Chuenchitra, T., Khantapura, P., Islam, D., Ruamsap, N., Swierczewski, B. E., & Mason, C. J. (2017). Nationwide Seroprevalence of Leptospirosis among Young Thai Men, 2007–2008. *The American Journal of Tropical Medicine and Hygiene, 97*(6), 1682-1685.

Haake, D. A., & Matsunaga, J. (2010). *Leptospira*: a spirochaete with a hybrid outer membrane. *Molecular Microbiology, 77*(4), 805-814.

Hamada, S., & Slade, H. D. (1980). Biology, immunology, and cariogenicity *of Streptococcus mutans*. *Microbiological Reviews*, *44*(2), 331-345.

Hashem, A. A., El Fadeal, N. M. A., & Shehata, A. S. (2017). In vitro activities of vancomycin and linezolid against biofilm-producing methicillin-resistant staphylococci species isolated from catheter-related bloodstream infections from an Egyptian tertiary hospital. *Journal of Medical Microbiology*, *66*(6), 744-752.

Harmsen, M., Yang, L., Pamp, S. J., & Tolker-Nielsen, T. (2010). An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunology & Medical Microbiology*, *59*(3), 253-268.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A. & Miller, S. I. (2005). Aminoglycoside antibiotics induces bacterial biofilm formation. *Nature, 436* (1), 1171-1175.

Hoyle, B. D., & Costerton, J. W. (1991). Bacterial resistance to antibiotics: the role of biofilms. *Progress in Drug Research*, *37*(1), 91-105.

Huber, W., Von Heydebreck, A., Sültmann, H., Poustka, A., & Vingron, M. (2002). Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*, *18*(1), 96-104.

Hunt, S. M., Werner, E. M., Huang, B., Hamilton, M. A., & Stewart, P. S. (2004). Hypothesis for the role of nutrient starvation in biofilm detachment. *Applied and Environmental Microbiology*, *70*(12), 7418-7425.

Ido, Y., Hoki, R., Ito, H., & Wani, H. (1917). The rat as a carrier of *Spirochaeta icterohaemorrhagiae*, the causative agent of Weil's disease (*spirochaetosis icterohaemorrhagica*). *The Journal of Experimental* Medicine, *26*(3), 341-367.

Iraola, G., Spangenberg, L., Bastos, B. L., Graña, M., Vasconcelos, L., Almeida, Greif, G., Robello, C., Paula Ristow, and Hugo NayaNaya, H. (2016). Transcriptome sequencing reveals wide expression reprogramming of basal and unknown genes in *Leptospira biflexa* biofilms. *MSphere, 1*(2), 142-165.

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology Letters, 236*(2), 163-173.

Johnson, R. C., & Faine, S. (1984). Leptospira. *Keieg NR, Nolt JG. Bergey's manual of systematic bacteriology. Baltimore USA: Williams & Wilkins*, 62-67.

Johnson, R. C., & Harris, V. G. (1967). Differentiation of pathogenic and saprophytic leptospires I. growth at low temperatures. *Journal of Bacteriology, 94*(1), 27-31.

Justice, S. S, Hung, C., Theriot, J. A., Fletcher, D. A., Anderson, G. G., Footer, M. J. & Hultgren, S. J. (2004). Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. In proceedings of the National *Academy of Science of the United State of America, 101*(5), 1333-1358.

Khelissa, S. O., Abdallah, M., Jama, C., Faille, C., & Chihib, N. E. (2017). Bacterial contamination and biofilm formation on abiotic surfaces and strategies to overcome their persistence. *Journal of Material and Environmental Science, 8*(1), 3326-3346.

Kitamura, S., & Hara, S. (1918). Autumnal Fever: Etiology. *Autumnal Fever: Etiology*.

Kostakioti, M., Hadjifrangiskou, M., & Hultgren, S. J. (2013). Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harbor Perspectives in Medicine*, *3*(4), 1-6.

Lal, T. M. & Zulkarnaen, R. (2018). Simple, fast and reliable method to determine pathogenic potential of fish bacterial Pathogen. *International Journal of Science Inventions Today, 7*(2), 198-204.

Lau, C. L., Smythe, L. D., Craig, S. B., & Weinstein, P. (2010). Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *104*(10), 631-638.

Leid, J. G., Willson, C. J., Shirtliff, M. E., Hassett, D. J., Parsek, M. R., & Jeffers, A. K. (2005). The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-γ-mediated macrophage killing. *The Journal of Immunology, 175*(11), 7512-7518.

Levett, P. N., Branch, S. L., Whittington, C. U., Edwards, C. N., & Paxton, H. (2001). Two methods for rapid serological diagnosis of acute leptospirosis. *Clinical Diagnosis Laboratory Immunological*, *8*(2), 349-351.

Levett, P. N., Morey, R. E., Galloway, R. L., & Steigerwalt, A. G. (2006). Leptospira broomii sp. nov., isolated from humans with leptospirosis. *International Journal of Systematic and Evolutionary Microbiology*, *56*(3), 671-673.

Lee, M. N., Kim, S. K., Li, X. H., & Lee, J. H. (2014). Bacterial virulence analysis using brine shrimp as an infection model in relation to the importance of quorum sensing and proteases. *Journal General Applied Microbiology, 60*(1), 169-174.

Lemon, K. P., Higgins, D. E., & Kolter, R. (2007). Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology, 189*(1), 4418-4424.

Levett, P. N. (2015). *Systematics of leptospiraceae*. Berlin, Springer & Heidelberg.

Lieberman, M. (1999). A brine shrimp bioassay for measuring toxicity and remediation of chemicals. *Journal of Chemical Education, 76*(12), 1689-1704.

Loeb, G. I., & Neihof, R. A. (1975). Applied Chemistry at Protein Interfaces, Advances in Chemistry. *American Chemical Society, 145*(1), 319-335.

Mah, T. F. C., & O'toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology, 9(*1), 34-39.

Marshall, R. B. (1992). International Subcommittee on Systematic Bacteriology. Subcommittee on the Taxonomy of *Leptospira*. Minutes of the meetings. *International Journal of Systematic and Evolutionary Microbiology, 42*(1), 330-351.

Martínez, J. L., & Baquero, F. (2002). Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clinical Microbiology Reviews, 15*(4), 647-679.

Matthias, M. A., Ricaldi, J. N., Cespedes, M., Diaz, M. M., Galloway, R. L., Saito, M., & Gilman, R. H. (2008). Human leptospirosis caused by a new, antigenically unique *Leptospira* associated with a *Rattus* species reservoir in the Peruvian Amazon. *PLoS Neglected Tropical Diseases, 2*(4), 200-213.

Matsumoto-Nakano, M. (2018). Role of *Streptococcus mutans* surface proteins for biofilm formation. *Japanese Dental Science Review, 54*(1), 22-29.

Maira-Litrán, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., Goldmann, D. A., & Pier, G. B. (2002). Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity, 70*(8), 4433-4440.

Ministry of Health Malaysia. (2017). Health Indicators 2016: Indicators for Monitoring and Evaluation of Strategy Health for All. Retrieved from http://www.moh.gov.my/index.php/pages/view/239?mid=33.

Minnerath, J. M., Roland, J. M., Rossi, L. C., Weishalla, S. R., & Wolf, M. M. (2009). A Comparison of Heat versus Methanol Fixation for Gram Staining Bacteria. *Bioscene: Journal of College Biology Teaching, 35*(2), 36-41.

Mirzaee, M., Najar Peerayeh, S., & Ghasemian, A. M. (2014). Detection of *icaABCD* genes and biofilm formation in clinical isolates of methicillin resistant *Staphylococcus aureus*. *Iranian Journal of Pathology, 9*(4), 257-262.

Morton, H. E. (1942). Susceptibility of Syrian hamsters to leptospirosis. *Proceedings of the Society for Experimental Biology and Medicine, 49*(4), 566-568.

Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M., Gill, S., & Cheung, A. L. (2011). Whole-genome sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. *Journal of Bacteriology, 193*(9), 2332-2335.

Nobile, C. J., & Johnson, A. D. (2015). *Candida albicans* biofilms and human disease. *Annual Review of Microbiology*, *69*(1), 71-92.

Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., & Akritidis, N. (2008). The globalization of leptospirosis: worldwide incidence trends. *International Journal of Infectious Diseases*, *12*(4), 351-357.

Picardeau, M., Bulach, D. M., Bouchier, C., Zuerner, R. L., Zidane, N., Wilson, P. J., Creno, S., Kuczek, E. S., Bommezzadri, S., Davis, J. C., & McGrath, A. (2008). Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PloS One*, *3*(2), 1607-1623.

Priya, C. G., Hoogendijk, K. T., Berg, M. V. D., Rathinam, S. R., Ahmed, A., Muthukkaruppan, V. R., & Hartskeerl, R. A. (2007). Field rats form a major infection source of leptospirosis in and around Madurai, India. *Journal of Postgraduate Medicine*, *53*(4), 236-251.

Potera, C. (2010). Antibiotic resistance: Biofilm dispersing agent rejuvenates older antibiotics. *Environmental Health Perspectives*, *118*(7), 280-288.

Pui, C. F., Bilung, L. M., Su'ut, L., & Apun, K. (2015). Prevalence of *Leptospira* species in environmental soil and water from national parks in Sarawak, Malaysia. *Borneo Journal of Resource Science and Technology*, *5*(1), 49-57.

Pui, C. F., Bilung, L. M., Apun, K., & Su’ut, L. (2017). Diversity of *Leptospira* spp. in rats and environment from urban areas of Sarawak, Malaysia. *Journal of Tropical Medicine*, *2017*(1), 1-8.

Rajput, A., & Kumar, M. (2018). Anti-biofilm Peptides: A New Class of Quorum Quenchers and Their Prospective Therapeutic Applications. In proceeding of *Biotechnological Applications of Quorum Sensing Inhibito*rs, *2018*(1), 98-110.

Ragunath, C., Komathi, S., & Ragunathan, R. (2018). Molecular Characterisation of *Leptospira interrogans*, Isolated from Animal Samples. *International Journal of Pure & Applied Bioscience*, *6*(2), 995-1004.

Ratnam, S. (1994). Leptospirosis: an Indian perspective. *Indian Journal of Medical Microbiology*, *12*(1), 228-239.

Renner, L. D., & Weibel, D. B. (2011). Physicochemical regulation of biofilm formation. *MRS Bulletin*, *36*(5), 347-355.

Ren, S. X., Fu, G., Jiang, X. G., Zeng, R., Miao, Y. G., Xu, H., & Jiang, H. Q. (2003). Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature*, 422(6934), 880-888.

Richardson, J. L., Burak, M. K., Hernandez, C., Shirvell, J. M., Mariani, C., Carvalho-Pereira, T. S., Pertile, A. C., Panti- May J. A., Pedra, G. G., Serrano, S., & Taylor, J. (2017). Using fine-scale spatial genetics of Norway rats to improve control efforts and reduce leptospirosis risk in urban slum environments. *Evolutionary Applications*, *10*(4), 323-337.

Ristow, P., Bourhy, P., Kerneis, S., Schmitt, C., Prevost, M. C., Lilenbaum, W., & Picardeau, M. (2008). Biofilm formation by saprophytic and pathogenic leptospires. *Microbiology*, *154*(5), 1309-1317.

Rood, E. J., Goris, M. G., Pijnacker, R., Bakker, M. I., & Hartskeerl, R. A. (2017). Environmental risk of leptospirosis infections in the Netherlands: Spatial modelling of environmental risk factors of leptospirosis in the Netherlands. *PloS one, 12*(10), 111-121.

Rumbaugh, K. P., Griswold, J. A., & Hamood, A. N. (2000). The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes and Infection, 2*(14), 1721-1731.

Sahal, G., & Bilkay, I. S. (2014). Multi drug resistance in strong biofilm forming clinical isolates of *Staphylococcus epidermis. Brazilian Journal of Microbiology, 45*(2), 539-544.

Samsudin, S., Masri, S. N., Tengku Jamaluddin, T. Z. M., Saudi, S. N. S., Md Ariffin, U. K., Amran, F., & Osman, M. (2015). Seroprevalence of leptospiral antibodies among healthy municipal service workers in Selangor. *Advances in Public Health*, *2015*(1), 401-412.

Saito, M., Villanueva, S. Y., Chakraborty, A., Miyahara, S., Segawa, T., Asoh, T., & Yoshida, S. I. (2013). Comparative analysis of *Leptospira* strains isolated from environmental soil and water in the Philippines and Japan. *Applied and Environmental Microbiology*, *79*(2), 601-609.

Sanchez, L. M., Cheng, A. T., Warner, C. J., Townsley, L., Peach, K. C., Navarro, G., & Linington, R. G. (2016). Biofilm formation and detachment in gram-negative pathogens is modulated by select bile acids. *PloS One*, *11*(3), 1-3.

Shi, X., & Zhu, X. (2009). Biofilm formation and food safety in food industries. *Trends in Food Science and Technology, 20*(9), 407-413.

Slack, A. T., Kalambaheti, T., Symonds, M. L., Dohnt, M. F., Galloway, R. L., Steigerwalt, A. G., & Smythe, L. D. (2008). *Leptospira wolffii* sp. nov., isolated from a human with suspected leptospirosis in Thailand. *International Journal of Systematic and Evolutionary Microbiology*, *58*(10), 2305-2308.

Slack, A. T., Khairani-Bejo, S., Symonds, M. L., Dohnt, M. F., Galloway, R. L., Steigerwalt, A. G., & Smythe, L. D. (2009). *Leptospira kmetyi* sp. isolated from an environmental source in Malaysia. *International Journal of Systematic and Evolutionary Microbiology*, *59*(4), 705-708.

Socransky, S. S., & Haffajee, A. D. (2002). Dental biofilms: difficult therapeutic targets. *Periodontology 2000*, *28*(1), 12-55.

Spradbrow, P. B., & Seawright, A. A. (1963). *Leptospira pomona* infection in calves. *Australian Veterinary Journal*, *39*(11), 423-433.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, *40*(2), 175-179**.**

Suwarno, S. R., Hanada, S., Chong, T. H., Goto, S., Henmi, M., & Fane, A. G. (2016). The effect of different surface conditioning layers on bacterial adhesion on reverse osmosis membranes. *Desalination*, *387*(1), 1-13.

Tang, P. L., Pui, C. F., Wong, W. C., Noorlis, A., & Son, R. (2012). Biofilm forming ability and time course study of growth of *Salmonella Typhimurium* on fresh produce surfaces. *International Food Research Journal*, *19*(1), 19-34.

Thayaparan, S., Robertson, I. D., Fairuz, A., Suut, L., & Abdullah, M. T. (2013). Leptospirosis, an emerging zoonotic disease in Malaysia. *Malaysian Journal of Pathology*, *35*(2), 123-132.

Trueba, G., Zapata, S., Madrid, K., Cullen, P., & Haake, D. (2004). Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. *International Microbiology*, *7*(1), 35-40.

Tormo, M. A., Knecht, E., Götz, F., Lasa, I., & Penades, J. R. (2005). Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? *Microbiology*, *151*(7), 2465-2475.

Toutain, C. M., Caizza, N, C., Zegans, M. E., & O'Toole, G. A. (2007). Roles for flagellar stators in biofilm formation by *Pseudomonas aeruginosa*. *Research in Microbiology*, *158*(1), 471–477.

Van Leeuwenhoek A. Microscopical observations about animals in the scurf of the teeth. *Philosophical Transactions of the Royal Society of London Biological Science*, *14*(1), 568-574.

Van Thiel, P. H. (1948). *The leptospiroses*. Universitaire Pers, Leiden.

Valle, J., Latasa, C., Gil, C., Toledo-Arana, A., Solano, C., Penadés, J. R., & Lasa, I. (2012). Bap, a biofilm matrix protein of *Staphylococcus aureus* prevents cellular

internalization through binding to GP96 host receptor. *PLoS Pathogens, 8*(8), 2843-2854.

Vein, J., Perrin, A., Berny, P. J., Benoit, E., Leblond, A., & Kodjo, A. (2012). Adaptation of a real-time PCR method for the detection and quantification of pathogenic leptospires in environmental water. *Canadian Journal of Microbiology, 58*(7), 828-835.

Vinod Kumar, K., Lall, C., Vimal Raj, R., Vedhagiri, K., & Vijayachari, P. (2016). Molecular detection of pathogenic leptospiral protein encoding gene (lipL32) in environmental aquatic biofilms. *Letters in Applied Microbiology, 62*(4), 311-315.

Vinod Kumar, K., Lall, C., Raj, R. V., Vedhagiri, K., & Vijayachari, P. (2015). Coexistence and survival of pathogenic leptospires by formation of biofilm with *Azospirillum. FEMS Microbiology Ecology, 91*(6), 51-64.

Wagner, V. E., & Iglewski, B. H. (2008). *P. aeruginosa* biofilms in cystic fibrosis infection. *Clinical Reviews in Allergy & Immunology, 35*(3), 124-134.

Weil, A. (1886). Ueber eine eigenthümliche, mit Mitztumor, Icterus und Nephritis einhergehende, acute Infektionskrankheit. *The journal of Laboratory and Clinical Medicine, 39*(1), 209-215.

Wilson, R., & Fujioka, R. (1995). Development of a method to selectively isolate pathogenic *Leptospira* from environmental samples. *Water Science and Technology, 31*(5-6), 275-282.

Winn, W. C. (2006). *Koneman's color atlas and textbook of diagnostic microbiology*. Lippincott: Williams & Wilkins.

World Health Organization. (1999). Leptospirosis worldwide. *Weekly Epidemological Record*, *74*(1), 237-242.

World Health Organization. (2006). Informal Consultation on Global Burden of Leptospirosis: Methods of Assessment. *Geneva: World Health Organization.*

World Health Organization. (2011). World Health Organization Geneva: Leptospirosis: an emerging public health problem. *Weekly Epidemological Record*, *86*(6), 45-50.

Wolbach, S. B., & Binger, C. A. L. (1914). Notes on a filterable spirochete from fresh water. Spirocheta biflexa (new species). *The Journal of Medical Research*, *30*(1), 17-23.

Wu, S., Li, X., Gunawardana, M., Maguire, K., Guerrero-Given, D., Schaudinn, C., & Webster, P. (2014). Beta-lactam antibiotics stimulate biofilm formation in non-typeable *Haemophilus influenzae* by up-regulating carbohydrate metabolism. *PLoS One*, *9*(7), 204-213.

Ximénez-Fyvie, L. A., Haffajee, A. D., & Socransky, S. S. (2000). Comparison of the microbiota of supra-and subgingival plaque in health and periodontitis. *Journal of Clinical Periodontology*, *27*(9), 648-657.

Zhang, W., Wang, Y., Bougouffa, S., Tian, R., Cao, H., Li, Y., & Zhang, X. (2015). Synchronized dynamics of bacterial niche-specific functions during biofilm development in a cold seep brine pool. *Environmental microbiology*, *17*(10), 4089-4104.

Zhukova, A., Fernandes, L. G., Hugon, P., Pappas, C. J., Sismeiro, O., Coppée, J. Y., & Yang, F. X. (2017). Genome-wide transcriptional start site mapping and sRNA

identification in the pathogen *Leptospira interrogans*. *Frontiers in Cellular and Infection Microbiology*, *7*(1), 10-18.

APPENDICES

Appendix A: Publication

1. Apun, K., Jalan, J., Pui, C. F., Bilung, L. M., Hashim, H. F., Ahsan, A. A. N. B. M., & Rupert, R. (2018). Biofilm forming ability of intermediate and saprophytic *Leptospira* on abiotic and biotic surfaces. *Malaysian Journal of Microbiology*, *14*(4), 313-319.

2. Pui, C. F., Apun, K., Jalan, J., Bilung, L. M., Su'ut, L., & Hashim, H. F. (2017). Microtiter Plate Assay for the Quantification of Biofilm Formation by Pathogenic *Leptospira*. *Research Journal of Microbiology*, *12*(2), 146-153.

3. Jalan, J., Apun, K., Pui, C. F., Bilung, L. M., & Fatma, H. H. Biofilm Genes Involved in Biofilm Formation of Locally Isolated Intermediate and Saprophytic *Leptospira*. *International Congress of Malaysian Society for Microbiology* (ICMSM), 4-7 December, 2017, Putrajaya, Malaysia.

4. Jalan, J., Apun, K., Pui, C. F., Bilung, L. M., Hashim, H. F., & Hassim, A. D. Biofilm Forming Ability and Time Course Study of Intermediate and Saprophytic *Leptospira*. *Malaysian Society for Microbiology Postgraduate Seminar* (MSMPS), 24 August, 2016, Universiti Kebangsaan Malaysia (UKM), Malaysia.

Appendix B: Mean value of OD_{600} on abiotic of 15 isolates Intermediate *Leptospira*

Isolate/Day	1	2	3	4	5	6	7	8	9	10	11
G 2 (+VE)	0.694	0.893	0.957	1.305	1.452	1.562	1.130	1.044	1.094	0.787	0.614
G 3	1.032	1.234	1.199	1.395	1.495	2.011	1.841	1.635	1.320	1.114	1.072
G 4	0.885	1.039	1.127	1.199	1.267	1.731	1.587	1.450	1.566	1.098	0.871
G 5	0.765	0.953	1.235	1.354	1.448	2.144	1.566	1.516	1.287	0.591	0.487
G 7	1.202	1.390	1.255	1.637	1.996	2.099	2.398	2.213	2.182	1.908	1.273
G 9	0.792	1.010	1.501	1.438	1.493	1.394	1.043	1.040	1.039	1.103	1.110
G 11	1.229	1.361	1.354	1.562	1.812	1.462	0.935	0.817	0.888	0.709	0.684
G 12	0.182	0.257	0.181	0.270	0.582	0.197	0.145	0.205	0.110	0.102	0.151
G 13	0.893	0.992	1.031	1.212	1.936	1.953	1.600	1.324	1.440	1.298	1.119
G 14	0.492	0.742	0.915	1.368	1.902	1.844	1.698	1.546	1.108	1.010	0.769
G 15	1.793	1.980	1.918	1.854	2.059	2.171	1.909	1.958	1.537	1.207	1.029
G 16	1.087	1.178	1.321	1.533	1.336	1.256	1.109	1.163	1.052	0.981	1.064
G 17	0.792	0.841	0.964	1.200	1.493	1.051	1.043	0.974	0.771	0.469	0.231
G 19	0.789	0.902	1.734	1.532	1.298	1.139	0.972	1.069	0.831	0.631	0.321
G 21	0.479	0.489	0.580	0.592	0.602	0.864	0.793	0.673	0.416	0.379	0.403
G 29	0.832	0.975	1.071	1.197	1.297	1.490	1.743	1.538	1.378	1.076	0.963
Negative	0.265	0.267	0.211	0.298	0.263	0.290	0.217	0.168	0.186	0.161	0.136

Appendix C: Mean value of OD_{600} on abiotic of 15 isolates saprophytic *Leptospira*

Isolate/Day	1	2	3	4	5	6	7	8	9	10	11
Patoc(+VE)	0.628	0.957	1.111	1.582	1.793	1.424	1.077	0.911	0.816	0.559	0.450
S 3	1.449	1.026	0.974	1.002	0.762	1.305	0.753	1.205	1.287	0.766	0.853
S 4	1.479	1.134	1.532	1.702	1.033	1.404	1.012	1.354	1.465	1.029	1.187
S 5	0.745	0.484	0.585	0.748	0.506	1.489	1.044	1.004	0.915	0.801	0.516
S 11	1.459	1.180	1.618	1.484	1.114	2.117	1.304	1.805	1.505	1.196	1.580
S 12	0.344	0.285	0.187	0.478	0.223	1.880	0.236	0.643	0.685	0.330	0.652
S 14	1.265	0.827	0.747	0.794	1.275	1.899	0.753	1.534	1.657	0.897	1.456
S 15	1.625	1.016	0.997	0.682	1.047	1.499	0.777	1.071	1.030	0.807	0.692
S 16	1.199	0.565	0.602	0.929	0.496	1.648	0.380	1.134	1.044	0.306	0.715
S 17	1.534	1.279	0.864	0.909	0.586	1.490	0.865	1.166	1.208	0.771	0.812
S18	1.876	1.216	0.835	1.283	0.890	1.686	1.110	1.374	1.367	1.009	1.072
S 19	1.559	0.984	0.772	1.275	0.799	1.654	0.819	1.841	1.610	0.838	1.101
S 20	1.508	1.172	1.003	1.175	0.642	1.686	0.775	1.214	1.346	1.163	1.110
S 21	1.520	1.289	1.045	1.972	1.013	1.683	1.373	1.715	1.322	1.082	1.285
S 34	1.478	1.002	0.673	0.729	0.534	0.806	1.065	1.008	1.388	0.969	1.645
S 36	1.535	1.706	1.277	0.893	0.703	1.640	1.063	1.512	1.412	1.269	1.901
Negative	0.174	0.114	0.176	0.159	0.160	0.161	0.133	0.134	0.168	0.126	0.103

Appendix D: Mean value of OD_{600} on biotic of 15 isolates Intermediate *Leptospira*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
G 2 (+VE)	2.113	2.222	2.241	2.238	2.232	2.243	2.232	2.154	2.117	2.101	1.883
G 3	2.019	2.074	2.139	2.302	2.280	2.253	2.192	2.184	2.173	2.136	2.110
G 4	2.154	2.274	2.308	2.284	2.301	2.267	2.181	2.244	2.245	2.281	2.211
G 5	2.162	2.267	2.322	2.303	2.294	2.316	2.203	2.300	2.295	2.211	2.209
G 7	2.168	2.182	2.246	2.278	2.335	2.312	2.306	2.284	2.246	2.258	2.229
G 9	2.025	2.116	2.216	2.219	2.228	2.300	2.224	2.200	2.190	2.164	2.037
G 11	2.161	2.135	2.222	2.262	2.271	2.283	2.270	2.266	2.258	2.146	2.112
G 12	2.121	2.124	2.226	2.210	2.222	2.236	2.230	2.275	2.264	2.121	2.099
G 13	2.169	2.258	2.335	2.288	2.319	2.327	2.124	2.281	2.261	2.246	2.207
G 14	2.138	2.165	2.174	2.284	2.214	2.234	2.201	2.200	2.154	2.132	2.147
G 15	2.153	2.285	2.313	2.301	2.315	2.295	2.203	2.281	2.286	2.243	2.234
G 16	2.184	2.271	2.309	2.263	2.246	2.309	2.227	2.710	2.291	2.207	2.228
G 17	2.170	2.279	2.298	2.307	2.320	2.286	2.215	2.262	2.273	2.269	2.209
G 19	2.106	2.138	2.104	2.167	2.264	2.284	2.234	2.206	2.272	2.261	2.216
G 21	2.125	2.281	2.321	2.322	2.321	2.304	2.192	2.293	2.273	2.235	2.238
G 29	2.134	2.233	2.225	2.239	2.270	2.300	2.265	2.240	2.248	2.180	2.136
Negative	0.185	0.261	0.203	0.245	0.232	0.143	0.334	0.269	0.350	0.265	0.166

Appendix E: Mean value of OD_{600} on biotic of 15 isolates saprophytic *Leptospira*

	1	2	3	4	5	6	7	8	9	10	11
Patoc(+VE)	2.054	2.112	2.127	2.242	2.247	2.308	2.134	2.145	2.051	2.015	1.979
S 3	1.989	2.150	2.180	2.188	2.238	2.258	2.260	2.160	2.130	2.052	2.002
S 4	2.071	2.110	2.213	2.192	2.247	2.323	2.240	2.234	2.127	2.098	2.046
S 5	2.068	2.095	2.236	2.189	2.210	2.282	2.255	2.174	2.069	2.065	1.985
S 11	2.049	2.130	2.166	2.109	2.221	2.272	2.242	2.162	2.097	2.064	2.016
S 12	2.081	2.133	2.124	2.197	2.261	2.310	2.165	2.146	2.016	1.975	1.980
S 14	2.281	2.273	2.301	2.302	2.295	2.334	2.354	2.321	2.292	2.208	2.235
S 15	1.987	2.034	2.132	2.184	2.168	2.266	2.231	2.204	2.136	2.106	1.937
S 16	2.286	2.280	2.305	2.302	2.319	2.311	2.219	2.281	2.281	2.262	2.201
S 17	2.268	2.244	2.290	2.278	2.293	2.320	2.207	2.226	2.266	2.225	2.228
S 18	2.014	2.125	2.103	2.168	2.196	2.221	2.261	2.246	2.154	2.072	1.982
S 19	2.078	2.267	2.278	2.298	2.308	2.295	2.154	2.311	2.254	2.214	2.207
S 20	2.267	2.254	2.294	2.335	2.271	2.350	2.231	2.300	2.254	2.255	2.201
S 21	2.025	2.121	2.188	2.214	2.238	2.280	2.236	2.196	2.123	2.068	2.015
S 34	2.263	2.267	2.302	2.319	2.310	2.366	2.144	2.343	2.248	2.240	2.235
S 36	2.267	2.269	2.313	2.063	2.329	2.315	2.185	2.253	2.249	2.246	2.228
Negative	0.218	0.154	0.162	0.362	0.372	0.351	0.246	0.310	0.268	0.144	0.241