BIOFILM FORMATION AND PHENOTYPE MICROARRAY ANALYSIS OF *Listeria monocytogenes* STRAINS FROM READY-TO-EAT FOOD

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FACULTY OF SCIENCE
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KUALA LUMPUR

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BIOFILM FORMATION AND PHENOTYPE MICROARRAY ANALYSIS OF *Listeria monocytogenes* STRAINS FROM READY-TO-EAT FOOD

ABSTRACT

Listeria monocytogenes is an important foodborne pathogen and the causative agent of human listeriosis. Infection is typically acquired through the ingestion of contaminated foods. Ready-to-eat (RTE) foods are currently very popular due to its instant availability and convenience. The occurrence of *L. monocytogenes* has been reported in RTE smoked fish, seafood, raw meat, sausages and dairy products. In addition, biofilm produced by *L. monocytogenes* is a major nuisance in food manufacturing industries. Foodborne *L. monocytogenes* has been reported to form persistent biofilm structures and withstand the routine disinfection procedure in food processing facilities and eventually cross-contaminate the finished products. *L. monocytogenes* primarily causes infection in the gastrointestinal tract that results in gastritis, meningitis and meningoencephalitis. Therefore, listeriosis is one of the major foodborne illnesses at present. While previous studies emphasized on the intracellular life cycle of *L. monocytogenes,* knowledge regarding the substrate utilization and metabolic adaptations of *L. monocytogenes* in the environment are still scarce. Therefore, the objectives of this study were to investigate the carbon and nitrogen substrate utilization of three Malaysian foodborne *L. monocytogenes* strains, to determine their biofilm forming ability and lastly to identify and correlate the genes involved in catabolism and biofilm formation. Biolog Inc. Phenotype Microarray (PM) technique was used to analyse the catabolic activity of the foodborne strains in 190 carbon and 380 nitrogen sources. PM analysis showed that the carbon and nitrogen catabolic activity of the studied strains were considerably limited, although they all utilized detergents, such as Tween 40 and Tween 80, which are frequently used as sanitizing agents on various surfaces in meat processing industries.

The ability to utilize and grow in different substrates provides fitness advantage in stress conditions. Biofilm forming ability was determined using crystal violet assay in nutrient-rich (LB broth) and nutrient-limited (M9 minimal) media and 15 carbon and 8 nitrogen sources were supplemented with minimal medium. All three strains were strong biofilm producers in LB and minimal media. However, thymidine inhibited the biofilm formation in all strains, thereby suggesting a possible role in biofilm control. The whole genome sequencing data of *L. monocytogenes* strains was analysed and compared with the reference strain *L. monocytogenes* EGD-e to identify the related genes using the KEGG pathway mapping tools and confirmed by NCBI Nucleotide BLAST analysis. The genomic analysis identified 136 genes associated with biosynthesis, metabolism and biofilm formation, including the genes responsible for surface attachment (*flaA, bapL, motA, degU* and *inlA*) and biofilm initiation (*secA, recA* and *relA*). Identification of the genes and regulatory pathways involved in different stages of biofilm formation can be beneficial in minimizing biofilm development in food processing industries.

Keywords: Biofilm formation, foodborne pathogen, *Listeria monocytogenes*, Phenotype Microarray, ready-to-eat food.

PEMBENTUKAN BIOFILEM DAN ANALISIS MIKROATUR FENOTIP BAGI STRAIN *Listeria monocytogenes* DARIPADA MAKANAN SEDIA DIMAKAN

ABSTRAK

Listeria monocytogenes adalah sejenis patogen bawaan makanan yang penting dan penyebab punca utama bagi penyakit listeriosis di manusia. Jangkitan ini kebiasaanya merebak melalui pemakanan makanan yang tercemar. Makanan sedia dimakan (ready-to-eat, RTE) adalah makanan yang disukai ramai orang kerana ia senang, cepat dan mudah diperolehi. *L. monocytogenes* telah dilaporkan ditemui di dalam RTE seperti ikan salai, makanan laut, daging mentah, sosej dan produk tenusu. Tambahan pula, perkembangan biofilem menjadi masalah besar bagi persekitaran pembuatan makanan. *L. monocytogenes* telah dilaporkan membentuk struktur biofilem yang utuh dan masih mampu hidup setelah prosedur disinfeksi rutin untuk kemudahan pemprosesan makanan dan akhirnya menyebabkan cemar silang di dalam produk akhir. *L. monocytogenes* menjadi punca utama jangkitan pada saluran perut usus dan mengakibatkan penyakit gastritis, meningitis dan meningoensefalitis. Pada masa terkini, listeriosis merupakan salah satu penyakit bawaan makanan yang utama. Kajian sebelum ini telah mekankan kitar hidup intrasel bagi *L. monocytogenes*. Namun begitu, pengetahuan berkenaan pengunaan substrat dan penyesuaian metabolik bakteria ini dalam persekitaran adalah sangat terhad. Objektif bagi kajian ini adalah untuk menyiasat penggunaan substrat karbon dan nitrogen dalam tiga jenis strain *L. monocytogenes* bawaan makanan Malaysia, mengenal pasti potensi pembentukan biofilem dan mengenalpasti serta menghubungkan gen-gen yang terlibat dalam pembentukan katabolisma dan biofilem. Teknik *Phenotype Microarray* (PM) telah digunakan untuk menganalisis aktiviti katabolik bagi strain bawaan makanan menggunakan 190 karbon dan 380 nitrogen. Analisis PM menunjukkan katabolik aktiviti bagi karbon dan nitrogen agak terhad walaupun kesemua detergen yang

biasa digunakan, termasuk Tween 40 dan Tween 80, adalah agen sanitasi bagi pelbagai permukaan dalam industri pemprosesan daging. Kemampuan strain-strain ini untuk menggunakan substrat berbeza dan tumbuh didalamnya memberikan kelebihan daya dalam keadaan stres. Kemampuan membentuk biofilem ditentukan oleh media asai ungu hablur yang kaya nutrient (kaldu LB) dan kurang nutrien (minimal M9) dimana 15 karbon dan 8 sumber nitrogen telah dibekalkan dalam medium minimal. Kesemua tiga strain ini merupakan pengeluar biofilem utama di dalam LB dan media minimal. Namun begitu, pengurangan pembentukan biofilem oleh timidina dalam kesemua strain memungkinkan peranannya sebagai perencat biofilem. Data penjujukan penuh genom bagi strain *L. monocytogenes* telah dianalisis dan dibandingkan dengan strain rujukan *L. monocytogenes* EGD-e untuk mengenal pasti gen yang berkaitan, menggunakan pengakalan data KEGG dan disahkan menggunakan analisis NCBI Nucleotide BLAST. Kajian genomik telah mengenal pasti 136 gen yang terlibat dengan proses biosintesis, metabolisma dan pembentukan biofilem termasuk gen-gen yang bertanggungjawab untuk perlekatan permukaan (*flaA, bapL, motA, degU* dan *inlA*) dan perkembangan biofilem (*secA, recA* dan *relA*). Identifikasi gen dan tapak jalan kawal atur yang terlibat dalam pelbagai peringkat pembentukan biofilem akan memberi manfaat bagi mengurangkan perkembangan biofilem dalam industri makanan.

Kata kunci: Pembentukan biofilem, patogen bawaan makanan, *Listeria monocytogenes,* fenotip mikroatur, makanan sedia dimakan.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
%	:	Percentage
<	:	Less than
=	:	Equal
>	:	Greater than
≤	:	Less or equal to
≥	:	Greater or equal to
°C	:	Degree Celsius
ABC	:	ATP-binding Cassette
ADI	:	Arginine deiminase
AGR	:	Accessory Gene Regulator
ATP	:	Adenosine triphosphate
AUC	:	Area Under the Growth Curve
BLAST	:	Basic Local Alignment Search Tool
CA	:	California
CDC	:	Centers for Disease Control and Prevention
CFU	:	Colony Forming Unit
CFU mL^{-1}	:	Colony Forming Unit per Millilitre
CO2	:	Carbon dioxide
dH2O	:	Distilled water
DNA	:	Deoxyribonucleic Acid
EPS	:	Extracellular Polymeric Substance
FDA	:	Food and Drug Administration

G + C	:	Guanine + Cytidine
GAD	:	Glutamate decarboxylase
KAAS	:	KEGG Automatic Annotation Server
KEGG	:	Kyoto Encyclopaedia of Genes and Genomes
LB	:	Luria Bertani
LTA	:	Lipoteichoic Acid
mM	:	Millimolar
MR-VP	:	Methyl Red-Voges Proskauer
MW	:	Molecular Weight
NaCl	:	Sodium chloride
NADH	:	Nicotinamide Adenine Dinucleotide Hydrogen
NCBI	:	National Centre for Biotechnology Information
O.D.c	:	Cut-off Optical Density
OD	:	Optical Density
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
PFGE	:	Pulsed Field Gel Electrophoresis
pH	:	Potential Hydrogen
PM	:	Phenotype Microarray
PPP	:	Pentose Phosphate Pathway
PTS	:	Phosphotransferase System
QAC	:	Quaternary Ammonium Compound
RNA	:	Ribonucleic Acid
Rpm	:	Revolutions per minute
RTE	:	Ready-To-Eat
SIM	:	Sulfide, Indole, Motility

TCA	:	Tricarboxylic acid
TM	:	Trademark
TSI	:	Triple Sugar Iron
US	:	United States
UV	:	Ultraviolet
v/v	:	Volume per volume
w/v	:	Weight per volume
WHO	:	World Health Organization
µL	:	Microliter

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of Research

Listeria monocytogenes is a Gram-positive, facultative anaerobe, non-sporulating, rod shaped bacterium with a low G + C content and ubiquitously spread in soil, sewage, water, decaying plant material, raw and processed food products (NicAogáin & O'Byrne, 2016; Doijad et al., 2015; Karlin et al., 2004). It can survive and grow over a wide range of environmental conditions, such as refrigeration temperatures (0 - 4°C), low pH (2 - 4) and high salt concentration (up to 10% NaCl), and therefore making it very difficult to eliminate (Gardan et al., 2003; Liu et al., 2005). It is an emerging foodborne pathogen that causes human listeriosis worldwide. Infection is typically acquired through ingestion of contaminated food products and the most common site of infection is intestinal epithelium. *L. monocytogenes* infection has several clinical conditions, including meningitis, encephalitis, gastroenteritis, septicaemia, abortions, convulsions with high mortality (20 - 30%) rates and extremely susceptible to pregnant women, neonates, elderly and immunocompromised patients (Buchanan et al., 2017; Lomonaco et al., 2015). It is accountable for majority of deaths caused by foodborne epidemics in Europe and the United States (US). According to Centers for Disease Control and Prevention (CDC), the annual incidence of laboratory confirmed listeriosis outbreaks in the US is about 0.24 cases per 100,000 populations and nearly 1,600 cases remain undetected each year (CDC, 2016). Listeriosis is considered as the third foremost cause of death due to foodborne illness with around 260 deaths each year (CDC, 2016). In Malaysia, foodborne *L. monocytogenes* had been detected in raw and ready-to-eat (RTE) foods and the majority of incidences resulted from the contamination of *L. monocytogenes* in various street-side foods, salad, vegetables, raw and processed deli meat and fish products (Jamali et al., 2013; Jeyaletchumi et al., 2012; Marian et al., 2012; Ponniah et al., 2010; Wong et

al., 2011). Since listeriosis is not a notifiable disease in Malaysia, official reports on foodborne listeriosis are currently unavailable.

Listeria monocytogenes is one of the leading foodborne pathogens in both developed and developing countries and forms resistant biofilm structures in food processing environment (Kadam et al., 2013; Lomonaco et al., 2015). Biofilms are bacterial communities which are surrounded by extracellular polymeric substances attached to biotic or abiotic surfaces (Zhou et al., 2012). *L. monocytogenes* biofilms have been reported to show increased resistance to cleaning, disinfectants, desiccation and UV exposure leading to long term persistence in processing plants (Gandhi & Chikindas, 2007). Biofilm structures can disperse and contaminate the food products during processing and packaging, thus making them a food safety concern (Donlan, 2002; O'Toole et al., 2000). Industrially processed foods, such as cheese and meats have been reported to be contaminated with *L. monocytogenes* (Alonso et al., 2014; Doijad et al., 2015). Moreover, previous studies showed that serotype 1/2a, 1/2b and 4b are responsible for 95% of the clinical cases of listeriosis worldwide (Kadam et al., 2013; Lomonaco et al., 2015). In the current study, three Malaysian foodborne *L. monocytogenes* strains belonging to the pathogenic serogroups were studied for their biofilm forming ability.

Phenotype Microarray is a well-established platform to determine the cellular phenotypes of various microorganisms under different growth conditions (Bochner, 2001). Studies with other bacteria have successfully determined their metabolic activity in a variety of substrates (Chelvam et al., 2015; Chong et al., 2017; Farrugia et al., 2013; Tang et al., 2010). Although there is a relatively high incidence of foodborne *L. monocytogenes* in raw and RTE foods in Malaysia (Jamali et al., 2013; Kuan et al., 2013; Marian et al., 2012; Ponniah et al., 2010; Wong et al., 2011), studies regarding the catabolic activity of *L. monocytogenes* are insufficient. Microbial biofilms have been

reported to show increased resistance against industrial chemicals and cleaning process (Møretrø et al., 2017). While previous studies focused on the biofilm formation by different serotypes of this pathogen (Borucki et al., 2003; Doijad et al., 2015; Stepanović et al., 2004), there are no reports about the importance of substrate utilization during biofilm formation. A comparative genomic and phenotypic analysis of the foodborne strains were performed to determine the genes involved in biofilm formation and catabolism of different substrates.

1.2 Objectives

Therefore, the objectives of this study were:

i. To characterize the carbon and nitrogen catabolic profiles of *L. monocytogenes* strains based on the phenotype microarray data
ii. To determine the biofilm forming capability of the selected *L. monocytogenes* strains
iii. To identify the relevant genes involved in substrate utilization and biofilm formation

CHAPTER 2: LITERATURE REVIEW

2.1 The *Listeria* genus

The Gram-positive bacterium *Listeria monocytogenes* was first discovered by Murray in 1924 when an epidemic of septicemic disease affected guinea pigs and rabbits in England. Initially, Murray and his colleagues named it '*Bacterium monocytogenes*' due to its production of large mononuclear leukocytosis and described it as the most remarkable feature of the microorganism (Murray et al., 1926). The first case of human listeriosis was reported in 1929 in Denmark (Vázquez-Boland et al., 2001). A few decades later, another human epidemic case was reported by Schlech et al. (1983) when it caused a fatal invasive disease in Canada with a high-mortality rate due to the consumption of contaminated food product. Since this outbreak, researchers are occupied into elucidating the virulence nature and epidemiology of this bacterium and hence *L. monocytogenes* became one of the well-studied bacterial pathogens.

The *Listeria* genus consists of six species, including *L. innocua, L. ivanovii, L. grayi, L. monocytogenes, L. seeligeri* and *L. welshimeri.* Among them *L. monocytogenes* is a human pathogen and *L. ivanovii* is an animal pathogen (Gouin et al., 1994; Liu, 2006; Nightingale, 2010). Later, two more species have been identified (*L. marthii* and *L. rocourtiae*) and added to the genus *Listeria* (Graves et al., 2010; Leclercq et al., 2010).

2.1.1 *Listeria monocytogenes*

Listeria monocytogenes has four evolutionary lineages containing 13 serotypes based on the serological reactions of somatic (O) and flagellar (H) antigens. The serogroups consist of 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Zhang et al., 2018). Lineage I primarily includes the serotypes 1/2b, 3b, 4b, 4d, 4e and 7; Lineage II contains serotypes 1/2a, 1/2c, 3a and 3c; while Lineage III contains serotypes 4a and 4c (Liu, 2008; Roberts et al., 2006). Based on the sequence data analysis, strains from serotypes 4a, 4c

and some atypical 4b strains were assigned to lineage IV (Orsi et al., 2011). Lineage I strains are mainly associated with human listeriosis cases, lineage II strains are typically present in food and the environment; while lineage III and lineage IV strains rarely detected and usually cause diseases in animals (Liu, 2006, 2008).

2.1.2 Listeriosis

Listeria monocytogenes is an opportunistic pathogen that causes listeriosis among immunocompromised adults, pregnant women and neonates. The infection is characterized by gastroenteritis, meningitis, encephalitis and maternofetal infection in humans (Swaminathan & Gerner-Smidt, 2007). The primary site of infection is the intestinal epithelium and after crossing the intestinal barrier it spreads through blood and lymph to the liver and spleen, where it actively proliferates and causes infection. Due to hematogenous dissemination the pathogen reaches to brain and placenta. Therefore, *L. monocytogenes* can overcome three host barriers: the intestinal barrier, the blood barrier, and the maternofetal barrier. *L. monocytogenes* possess several cell surface proteins and virulence factors that prevent the intracellular killing by macrophages after phagocytosis and promotes invasion in non-phagocytic cells (Camejo et al., 2009).

2.1.3 Incidences of Listeriosis Worldwide

The U.S. Food and Drug Administration (FDA) listed *L. monocytogenes* as a 'pathogen of concern' due to its frequent occurrence in raw and processed RTE foods and high mortality rates (20 - 30%) in pregnant women and newborns, hence it has been listed among the top five domestically acquired foodborne pathogen leading to fatal illnesses (Scallan et al., 2011).

In 2018, an outbreak of listeriosis in South Africa affected 700 patients and killed 200 (28.6%) of which 42% cases were newborns who were infected during pregnancy. This outbreak was accounted for the widespread consumption of ready-to-eat processed meat,

known as ‘polony’. The outbreak strain has also been detected in the manufacturing environment of the associated product (WHO, 2018). It is considered as the largest listeriosis outbreak so far. Another notable listeriosis outbreak in Singapore which caused illness among five people due to the consumption of contaminated rock melons imported from Australia. The authorities described that the genome sequence of the Singapore strain showed similarity to the strain associated with the deadly listeriosis outbreak in Australia earlier this year (Xinhua, 2018).

The major listeriosis epidemic in U.S. history occurred in 2011, causing 147 illnesses, 33 deaths and 1 miscarriage among the residents of 28 states, on account of the consumption of contaminated cantaloupe from a single farm (CDC, 2011). The 2014-2015 multistate outbreaks resulted in 35 illnesses in 12 states including 7 deaths associated with *L. monocytogenes* contamination in caramel apples (CDC, 2015). In Europe, the topmost incidences of *L. monocytogenes* contamination are related to foods sold at retail level, including hard cheeses, fermented sausages, RTE meat and fish products and semi-soft cheeses (Lomonaco et al., 2015).

In Malaysia, there are no official records of Listeriosis outbreaks. However, Listeria spp. and *L. monocytogenes* have been detected in contaminated raw and processed RTE foods in the last few decades. Previous reports of incidences of *L. monocytogenes* contamination in various foods in Malaysia are summarized and cited in Table 2.1 below.

Table 2.1: Summary of published studies on the prevalence of foodborne *L. monocytogenes* in Malaysia (1994 – 2017).

Reference	Year	Source	Prevalence
Tang et al.	1994	Raw vegetables; n = 280	5/280 (1.8%)
Arumugaswamy et al.	1994	Raw vegetables, RTE food; n = 234	44.7%
Hassan et al.	2001	Fermented fish and meat	65/142 (45.8%)
Tan et al.	2007	Poultry and seafood	46.9%, 32.5%
Ponniah et al.	2010	Raw salad vegetables	22.5%
Jeyaletchumi et al.	2012	Salad vegetables	5.4 - 75.3%
Goh et al.	2012	Raw chicken meat	42/210 (20%)
Wong et al.	2012	Vegetarian burger patties; n = 108	9.3%
Marian et al.	2012	Raw and RTE foods	12/140 (8.6%)
Adzitey et al.	2013	Ducks and their environment	15/531 (2.8%)
Kuan et al.	2013, 2014	Beef offal	21/63 (33.3%)
Jamali et al.	2013	RTE foods	45/396 (11.4%)
Tang et al.	2017	Raw vegetables; n = 327	3.8%, 7.3%

2.1.4 Pathogenesis and Virulence Mechanism

There are several virulence factors contributing to listerial infection, such as cell surface proteins, internalins, hemolysin protein listeriolysin O and phospholipases. These factors mediate adhesion to host cell surface, uptake into macrophages or non-phagocytic cells, evasion from the vacuole, polymerization of actins to promote cell-to-cell spread and thus carry out the intracellular infection process. *Listeria monocytogenes* also secret molecules that can alter host cell transcription, post-translational modifications, innate immune signalling and cytoskeletal changes (Radoshevich & Cossart, 2018).

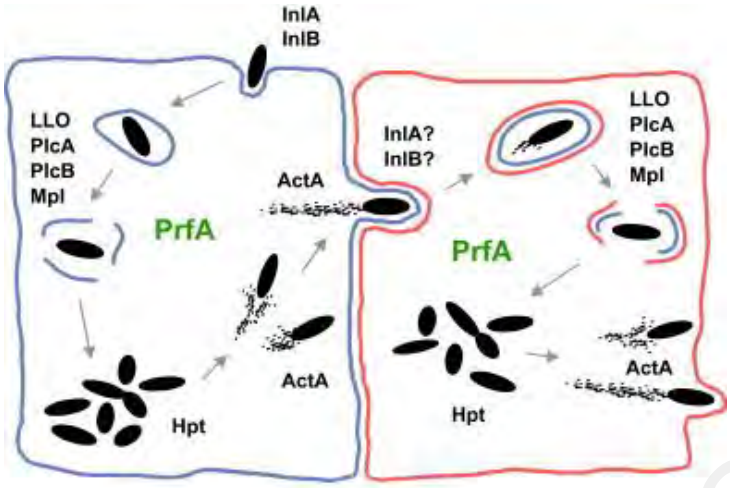

Figure 2.1: A depiction of the intracellular life cycle of pathogenic *Listeria monocytogenes*. The virulence proteins in PrfA regulon are indicated near each phases of infection cycle in which they are involved. Diagram adapted from Scortti et al. (2007).

2.2 Metabolism of *L. monocytogenes*

Like all living beings, microorganisms also require the fundamental constituents for their structure and morphology. These vital elements consist of carbon, nitrogen, phosphate and sulphur. The sources for these building blocks exist differently in bacterial host cell and the environment, signifying that the pathogen must acquire these nutrients for adaptation process (Haber et al., 2017). *Listeria monocytogenes* is a heterotrophic organism and its metabolic pathways are vastly similar to other comprehensively studied low G + C bacteria, such as *Bacillus subtilis* (Karlin et al., 2004). However, there are fundamental differences between the nutrient requirements and enzymatic pathways of these organisms (Joseph & Goebel, 2007). According to published literature, *L. monocytogenes* requires cysteine, methionine, leucine, isoleucine, valine, arginine, riboflavin, thiamine, biotin, lipoic acid and phenylalanine as important growth factors**.** Furthermore, glucose and glutamine has been described as primary sources of carbon and nitrogen for optimal growth in previous studies (Rocourt & Buchrieser, 2007; Premaratne et al., 1991).

Listeria monocytogenes has glucose oxidase and NADH oxidase activities. Respiration occurs aerobically and the respiratory chain contain menaquinone, instead of ubiquinone (Joseph & Goebel, 2007). It can utilize glucose, fructose, maltose, lactose, mannose, cellobiose, dextrin and salicin under aerobic and anaerobic conditions. Acetate and acetoin is produced as end products when glucose is utilized aerobically, whereas lactate is the main fermentation product in anaerobic conditions in which only pentose and hexose sugars support the anaerobic growth (Rocourt & Buchrieser, 2007).

Listeria monocytogenes metabolizes glucose and synthesizes glucose-6-phosphate using the glycolysis and pentose phosphate pathways. The glycolysis genes that are involved in carbon metabolic pathway are *gap, pgk, tpi, pgm* and *eno* and expressed highly in low G + C bacteria including *L. monocytogenes* (Karlin et al., 2004). In addition, the presence of glucose inhibits the positive regulatory factor (PrfA) activity which is associated with the virulence gene expression in *L. monocytogenes*. In the absence of glucose, glucose-6-phosphate and glycerol regulate the PrfA activity by inducing strong expression of virulence genes in the host cell cytoplasm (Deutscher et al., 2014; Haber et al., 2017). Furthermore, *L. monocytogenes* has an incomplete citric acid cycle due to the absence of an enzyme α-ketoglutarate dehydrogenase and thus it is incapable of generating oxaloacetate from citrate (Eylert et al., 2008). Therefore, oxaloacetate is produced by the carboxylation of pyruvate by the enzyme pyruvate carboxylase (*pycA*). Other key intermediates of the citric acid cycle, namely malate and succinate are produced using a reducing branch of citrate cycle from oxaloacetate where CO_2 acts as an important substrate during the synthesis of oxaloacetate by pyruvate carboxylase (Eisenreich et al., 2006; Joseph & Goebel, 2007). Previous studies showed that an increased expression of the glycolysis genes when *L. monocytogenes* is grown in a complex medium where glucose was the sole carbon source (Goldfine & Shen, 2007). On the contrary, there was an increased expression of the pentose phosphate pathway (PPP) genes, whereas a

reduced expression of the glycolysis genes when grown in minimal medium (Joseph et al., 2006). Similar phenomenon is observed during the intracellular growth, therefore suggesting that the PPP is the major catabolic pathway when other carbon sources are present in the medium instead of glucose. This is attributable to the lack of PTS sugars in the cytosol of the host cell (Shahraz, 2013).

2.2.1 Carbon Metabolism

The facultative bacterium *L. monocytogenes* contains four major transport mechanisms for carbohydrate transport and utilization: (1) phosphotransferase (PTS) system, (2) ATP binding cassette (ABC) transporters, (3) facilitated diffusion, and (4) ion-driven transporters (Deutscher et al., 2014). Based on the genome sequence and functional data, the heterotrophic *L. monocytogenes* contain more than 40 different PTS-related genes for carbohydrate metabolism and the majority of these genes are associated with the uptake of single sugar molecules, such as glucose, fructose, mannose, cellobiose, gentobiose, trehalose and β-glycosides, including salicin, arbutin, esculin and amygdalin (Deutscher et al., 2014; Shahraz, 2013; Glaser et al., 2001).

Listeria monocytogenes contains eight carbohydrate-specific ABC transporters which consist of a sugar-binding protein and two membrane spanning permeases. The ATP-hydrolysing protein gene is absent in the ABC transporters of *L. monocytogenes*; therefore, it utilizes Lmo0278 protein, which has similar functions as MsmX protein in *Bacillus subtilis* (Deutscher et al., 2014). Maltose and maltodextrose are taken up by an ABC transporter containing Lmo2123, Lmo2124 and Lmo2125 protein genes, whereas Lmo0278 function as ATPase during the transport. The maltose operon includes three additional genes for the enzymes, e.g. maltogenic amylase (*lmo2126*), maltodextrose utilization protein, MalA (*lmo2122*) and maltose phosphorylase (*lmo2121*). Gopal et al. (2010) reported that ABC transporters might be the only uptake mechanism for maltose

and maltodextrose present in *L. monocytogenes.* The seven other ABC transport systems in *L. monocytogenes* is apparently associated with the uptake of disaccharides and plant oligosaccharides by substrate-specific catabolic enzymes (Deutscher et al., 2014).

The uptake of triol glycerol takes place through an energy-independent mechanism called facilitated diffusion and catalysed by a pore-forming membrane protein. The three sugar transport systems, e.g. PTS, ABC and ion-driven transporters require energy for carbohydrate metabolism. The gene *lmo1539* encodes the protein GlpF, which has similar functions as the protein in *B. subtilis*. Upon entering the cell, glycerol is either phosphorylated to glycerol-3-phosphate by glycerol kinase (*glpK*) or oxidized to dihydroxyacetone (Mertins et al., 2007).

When *L. monocytogenes* is grown in an L-rhamnose containing medium, the genes encoding *lmo2850-2846* operon is expressed. L-rhamnose is transported via facilitator superfamily protein, Lmo2850 by a proton transport mechanism. Lmo2850 shows a high sequence identity to other L-rhamnose transporter operons found in several Gram-positive and Gram-negative bacteria. The *lmo2850-2846* operon encodes many enzymes that catalyse the degradation of L-rhamnose into dihydroxyacetone phosphate and L-lactaldehyde. First, L-rhamnose-1-epimerase (*lmo2846*) and L-rhamnose isomerase (*lmo2848*) converts L-rhamnose into L-rhamnulose. Then L-rhamnulose kinase (*lmo2849*) phosphorylates L-rhamnulose and forms L-rhamnulose-1-phosphate and then L-rhamnulose-1-phosphate aldolase (*lmo2847*) catalyses L-rhamnulose-1-phosphate into dihydroxyacetone phosphate and L-lactaldehyde. L-lactaldehyde is reduced to 1,2-propanediol which is further degraded into propanol or propionic acid (Deutscher et al., 2014).

2.2.2 Nitrogen Metabolism

Listeria monocytogenes cannot metabolize atmospheric nitrogen since it does not contain the enzyme nitrogenase, hence it acquires the organic form: ammonia. L-glutamine is the primary nitrogen for this intracellular pathogen due to its abundance in the host cell cytosol, therefore making it easier to metabolize (Haber et al., 2017). A recent study by Haber et al. (2017) showed that L-glutamine significantly enhances the expression of virulence genes in *L. monocytogenes* during intracellular infection. Glutamine acquisition is regulated by a high-affinity L-glutamine ABC transporter, GlnPQ. Although, soil and water are highly enriched with the nitrogen source ammonia, it is incapable of activating the virulence gene transcription in *L. monocytogenes* (Haber et al., 2017). Previous study showed that glutamine is replaced by ammonium when grown in a defined minimal medium (Tsai & Hodgson, 2003). The concentration of ammonia is relatively low in the serum of mammalian cells, whereas the concentration of L-glutamine is quite high (500 μM to several mM), thus aiding the localization process after invading the host cell (Haber et al., 2017; Shimmura et al., 2011). *L. monocytogenes* also utilizes arginine, ethanolamine and glucosamine as alternative nitrogen sources when L-glutamine is not present in the medium (Joseph & Goebel, 2007; Kutzner et al., 2016).

Kaspar et al. (2014) showed that the transcriptions of genes (*citC*, *citB* and the gene for pyruvate oxidase) involved in citric acid cycle were induced when ammonium was present in the medium. Therefore, their study concluded that *L. monocytogenes* growth rate was considerably higher in ammonium as nitrogen source than glutamine under different temperatures. The author also mentioned that the central nitrogen metabolism genes *glnR, glnA, amtB, glnK* and *gdh* were upregulated during a temperature-dependent transcription when glutamine and ammonium were utilized as sole nitrogen sources. Ammonium is converted to glutamine and glutamate by the enzyme glutamate synthase

in the presence of 2-oxogluterate due to the incomplete citric acid cycle, there is an accumulation of 2-oxoglutarate in *L. monocytogenes* (Shahraz, 2013).

Furthermore, glutamine can be synthesized by the enzyme glutamine synthetase and glutamate can be synthesized in three possible ways: (1) enzymatic reaction of ammonia or 2-oxoglutarate by glutamate dehydrogenase, (2) conversion of glutamine to glutamate via glutamate synthase, and (3) direct transamination of alpha-ketoglutarate (Shahraz, 2013). Moreover, *L. monocytogenes* contains specific gene for arginine transporter, which is upregulated during the intracellular replication inside the host cell. Arginine is catabolized by the enzyme arginine deaminase and converted to ammonia and citrulline. Citrulline can be further degraded to ammonia and ornithine. *L. monocytogenes* also utilizes adenine as an alternative nitrogen source. It contains the gene (*lmo1742*) for the enzyme adenine deaminase which catabolizes adenine and produces hypoxanthine and ammonia during intracellular infection (Camejo et al., 2009).

A comparative genomics study by Glaser et al. (2001) reported that the genome sequence of *L. monocytogenes* lacks the catabolic genes encoding nitrate and nitrite reductases, therefore it shows high dependency on reduced nitrogen sources. In addition, arginine, cysteine and methionine are essential amino acids for growth and several branched chain amino acids, namely valine, leucine and isoleucine significantly improves bacterial growth when grown in minimal medium (Tsai & Hodgson, 2003). Since *L. monocytogenes* do not possess the sulfate reductase in their genome, they are solely dependent on the host cell for sulfur containing amino acids, especially cysteine and methionine (in the absence of cysteine) which are produced in excess amount by the host cell (Joseph & Goebel, 2007).

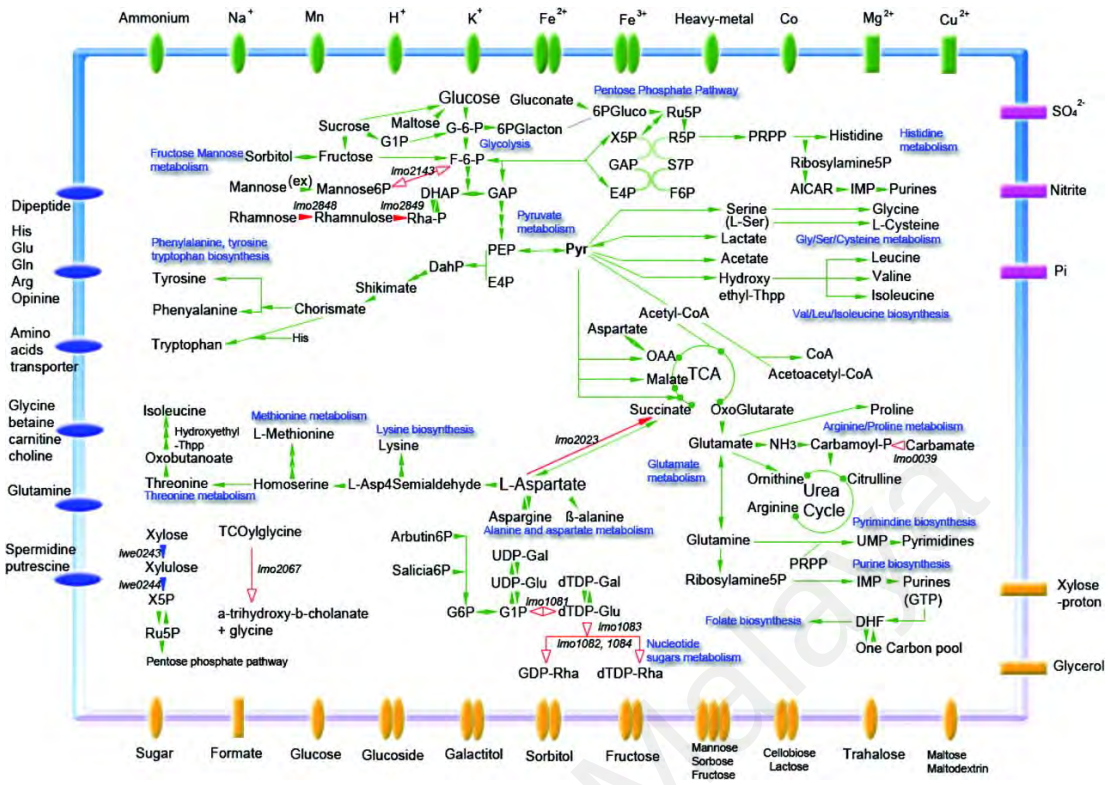

Figure 2.2: An Illustration of the biochemical pathways for energy production in *L. monocytogenes.* Glycolysis, starch metabolism, TCA cycle, PPP, amino acid pathways, nucleic acid metabolism are written in blue. Proteins are assembled by substrate specificity and transporters for cations (green), anions (purple), carbohydrates (orange), amino acids and peptides (blue) are as depicted. Ion-coupled permeases and ABC transporter systems are shown as ovals. Lipid and fatty acid pathways are excluded since they are incompletely present. Diagram adapted from Hain et al. (2006).

2.3 Genomic Study of *L. monocytogenes*

Listeria monocytogenes can easily acclimatize to the complex environmental condition maintained at the food processing industries, since they are extremely versatile and adaptive to environmental changes (Srey et al., 2013). Although the major virulence factors associated with host-pathogen interactions are extensively studied in the past, the same virulence determinants are also involved in the environmental adaptations of *L. monocytogenes* outside mammalian cells, including attachment to abiotic surfaces, aggregation and subsequent biofilm formation. Microbial whole genome sequencing (WGS) can be used to characterize foodborne pathogens and provide useful information about the metabolism and transmission of the pathogen (Ortiz et al., 2016). WGS is a

powerful and valuable tool for obtaining genomic information of same and different microbial species. This important molecular method can be used to characterize bacterial pathogens and understand the genetic similarity and dissimilarity between bacterial isolates. It facilitates the identification of contamination routes of foodborne pathogens in food production environment (Hyden et al., 2016; Nastasijevic et al., 2017). It is also a beneficial tool for comparing bacterial isolates in outbreak investigation. A recent study showed that WGS data was employed for the potential epidemiological surveillance of *L. monocytogenes* strains along with other food isolates associated with foodborne illnesses (Kwong et al., 2016).

Since 2000, the determination of whole-genome sequences of *L. monocytogenes and L. innocua* genomes (Glaser et al., 2001) have promoted the way for further post-genomic studies including comparative studies between *Listeria* species and among *L. monocytogenes* strains (Buchrieser, 2007). Genomics study of *L. monocytogenes* strains revealed that the pathogen carry a virulence gene cluster (*prfA, plcA, hly, mpl, actA, plcB*), an *inlAB* locus (genes coding surface proteins) and *hpt* gene encoding a hexose phosphate transporter (Buchrieser, 2007; Schmid et al., 2005). The Hpt protein allows the pathogen to utilize phosphorylated sugars, such as glucose-1-phosphate in the host cell cytosol and supports the intracellular replication (Chico-Calero et al., 2002). The gene coding an enzyme bile salt hydrolase (*bsh*) is involved in the intestinal and hepatic phases of listeriosis (Dussurget et al., 2002). The expression of these virulence genes are regulated by the transcriptional activator PrfA in *L. monocytogenes* genome (Scortti et al., 2007).

A comparative genomics analysis by Lim et al. (2016) showed that two Malaysian foodborne strains (LM115 and LM41) shared 90% homologous genes. The core genome consists of carbohydrate transport metabolism genes, amino acid transport metabolism genes, nucleotide transport metabolism genes, coenzyme transport and secondary

metabolite biosynthesis, lipid transport and metabolism genes. Moreover, the virulence gene cluster (*hly, plcA, plcB, mpl, actA, prfA*) and internalin genes (*inlA, inlB, inlC, inlK, inlF, inlJ*) were identified in their draft genomes that are crucial for intracellular growth in the host cell. A few stress tolerance proteins have also been identified in LM115 and LM41 genomes. Both strains carried glutamate decarboxylase (GAD) operon and arginine deiminase (ADI) operon which are associated with acid resistance in the gastric juice and intestine (Cotter et al., 2005; Lim et al., 2016). Consequently, GAD and ADI operons support the survival of *L. monocytogenes* in food with low pH that typically inhibit bacterial growth. Furthermore, regulatory protein gene known as Sigma-B (*sigB*) factor which controls the osmotic pressure and temperature stress was also present in their genomes. In addition to stress tolerance proteins, LM115 and LM41 harbour cold and heat shock proteins related genes (Lim et al., 2016). Earlier studies showed that food stored in freezing temperature and processed in boiling temperature such as, fried chicken and frozen burger patties were contaminated with pathogenic strains of *L. monocytogenes* in Malaysia (Jamali et al., 2013; Wong et al., 2012).

2.4 Biofilm Formation by *L. monocytogenes*

Biofilm formation is a major nuisance in food manufacturing environment. It is regarded as a challenging issue when formed in industrial and clinical settings. Foodborne pathogens including *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, *Bacillus cereus, Campylobacter jejuni*, *Cronobacter*, *Pseudomonas aeruginosa, Staphylococcus aureus* have the capability to adhere and form biofilms on abiotic surfaces of various food processing equipment (Giaouris & Simões, 2018; Kong et al., 2006; Omar et al., 2017; Srey et al., 2013). Ready-to-eat (RTE) foods are extremely popular due to its instant availability and convenience. RTE foods are considered as one of the high-risk food products due to its direct consumption and lack of further cooking or bactericidal treatment. The chances of microbial recontamination during processing

and handling is relatively high even if the packaging conditions are well-monitored (Srey et al., 2013). The contamination of *L. monocytogenes* in RTE smoked fish, seafood, raw meat and sausages was reported due to biofilm formation in food processing environment (Pinto et al., 2010; Wagner et al., 2007). The psychrotrophic nature of foodborne *L. monocytogenes* is another notable factor for the contamination of refrigerated RTE food products. Therefore, *L. monocytogenes* contamination has been reported in soft cheese, raw milk and dairy processing facilities in previous studies (Simões et al., 2010).

Biofilms are surface associated microbial cells surrounded by an extracellular polymeric substance (EPS) matrix, often produced by altered phenotype or gene expression (Donlan, 2002). *Listeria monocytogenes* biofilms are a serious problem in the food industry since it forms biofilms on various contact surfaces even in a regulated environment. The manifestation of biofilm is widespread among various inert surfaces, such as medical apparatus, water piping system, industrial equipment and food processing facilities (Donlan, 2002). In meat processing industry, conveyer belts and stainless steel surfaces are frequently contaminated with biofilms regardless of treatment with sanitizers and disinfectants (Carpentier & Cerf, 2011; Djordjevic et al., 2002). A significant number of reports showed that *L. monocytogenes* readily formed biofilm in glass, metal, plastic and rubber in food processing industries (Borucki et al., 2003; Doijad et al., 2015; Stepanović et al., 2004). Biofilm formation in food contact surfaces may lead to post-process contamination in food products and eventually cause serious illnesses. Many studies reported the incidences of bacterial biofilms in industrial environments which had persisted for a long time and eventually led to contamination (Gandhi & Chikindas, 2007; Gilmour et al., 2010; Kathariou, 2002; Leong et al., 2017). Cells in biofilm structures are found to be resistant to biocides (e.g. antimicrobials) and stress conditions such as, disinfection, UV, desiccation and freezing (Colagiorgi et al., 2017; Zhou et al., 2012).

Biofilm development is a complex metabolic process and several environmental and virulence factors are associated with the initiation of such molecular mechanism in bacterial cells. The EPS is considered as the hallmark of bacterial biofilm formation and lipoteichoic acid (LTA) is main exopolysaccharide in *L. monocytogenes* biofilms which is responsible for the adhesion in contact surfaces and cohesion in biofilm. A study by Chang et al. (2012) showed that the mutations in genes involved in glycolipid anchor and LTA synthesis significantly reduced the biofilm formation thus indicating that LTA may play major role in biofilm formation in *L. monocytogenes*. In addition to teichoic acid, different proteins, RNA and extracellular DNA are also present in listerial biofilm matrix structure (Colagiorgi et al., 2017).

When *L. monocytogenes* causes infection in the gastrointestinal (GI) tract, the exposure to bile enhances the colonization and biofilm formation. It contains bile salt hydrolase (*bsh*) that confers resistance to bile and gastric acid in the GI tract (Lim et al., 2016). Begley et al. (2009) showed that the exposure to bile significantly increased the initial attachment to plastic surfaces and demonstrated improved biofilm formation. Therefore, the presence of bile during infection may facilitate the colonization and biofilm development in human GI tract and survival in gallbladder. The author also suggested that *L. monocytogenes* can form biofilms on gallstones in a similar mechanism as *Salmonella* spp. Thus, biofilm structure protects the bacterial cells from host immune system and antibiotic therapy. Consequently, planktonic cells are shed continuously from the biofilm EPS and infect healthy host cells again (Begley et al., 2009).

Biofilm formation typically occurs in the following developmental stages: (1) initial attachment of cells to surface, (2) cell aggregation and formation of micro-colonies with the production of EPS, (3) maturation of biofilm with channels for nutrient uptake, and (4) detachment and dispersal of cells (O'Toole et al., 2000). Bacterial cells are capable of

detecting certain environmental constraints that initiate the transition from planktonic state to a sessile state (Davey & O'toole, 2000). Microbial cells in biofilm structures commonly show a reduced growth rate, altered gene transcription, increase production of exopolysaccharide and more importantly a greater resistance to disinfectants and antimicrobial agents (Donlan, 2002; Mereghetti et al., 2000). Detachment of bacterial cells from the biofilm structure, regrowth and dispersal as planktonic cells in the environment could result in contamination in packaged food products. A schematic representation of biofilm development is illustrated in Figure 2.2

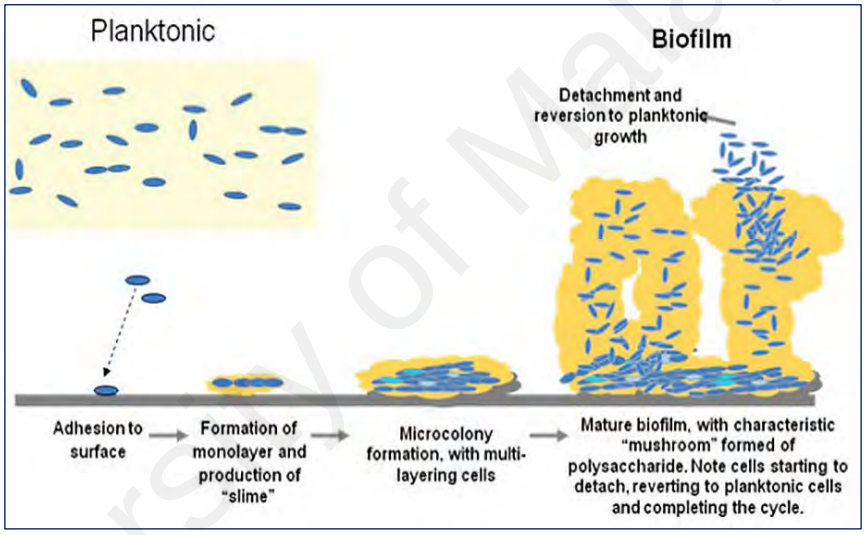

Figure 2.3: Different stages of biofilm formation and development. Diagram adapted with permission from Vasudevan (2014).

2.4.1 Molecular Basis of Biofilm Formation

Molecular analysis of biofilm related genes revealed that these genes are associated with flagellar motility, gene regulation (transcriptional activator), cell surface protein genes (Chang et al., 2012). Earlier study by Lemon et al. (2010) showed that *prfA* gene induces the formation of biofilm in *L. monocytogenes*. Van Der Veen & Abee (2010) reported that Sigma-B factor (*sigB*) contributed to the formation of static and continuous flow biofilms and enhanced the resistance to disinfectants. A recent study by Zetzmann et al. (2016) showed that accessory gene regulator (*agr*) system have major influence on

biofilm formation, virulence and adaptations to environmental conditions. The *agr* operon in *L. monocytogenes* is proven to be homologous to *Staphylococcus aureus* system and it contains the *agrD* encoding protein which stimulates the *agr* gene expression (Zetzmann et al., 2016). The deletion of *agrA* and *agrD* genes resulted in reduced attachment and biofilm formation, therefore suggesting that the *agr* system is indeed involved in the early stages of biofilm formation in *L. monocytogenes* (Rieu et al., 2007). Moreover, a study conducted using transposon mutagenesis approach showed that a gene (*lmo1386*) encoding putative DNA translocase plays important role in biofilm formation (Chang et al., 2013). The molecular determinants involved in the biofilm forming mechanism of *L. monocytogenes* are summarized in Table 2.2 below.

Table 2.2: Genes involved in biofilm formation by *L. monocytogenes*. Adapted from Kocot & Olszewska (2017).

Factor	Role in biofilm formation	Reference
Flagella	Adherence to abiotic surfaces, non-motile cells were defective in biofilm formation	Vatanyoopaisarn et al. (2000)
PrfA	Virulence regulator, controls flagella biosynthesis	Lemon et al. (2010)
LuxS	Inhibition of biofilm formation	Belval et al. (2006)
agrA	Attachment in early stages of biofilm, signal transduction system	Rieu et al. (2007)
hpt	Regulation of starvation response	Taylor et al. (2002)
SecA2	Inactivation promotes cell aggregation, impacts biofilm architecture and induces biofilm formation	Renier et al. (2014)
BapL	Protein required for cell attachment	Jordan et al. (2008)
SigB	Transcriptional regulator of stress response genes, resistance of biofilm cells to disinfectants	Van Der Veen & Abee (2010)
relA	Regulation of starvation response	Taylor et al. (2002)
degU	Adherence to plastic surface, flagella synthesis and biofilm formation	Gueriri et al. (2008)
ami	Initial attachment to abiotic surfaces	Kumar et al. (2009)

Several other proteins that play functional role in biofilm formation in *L. monocytogenes* include Phospholipase (*plcA*), flagellin (*flaA*) (Lemon et al., 2007), a putative penicillin-binding protein (*pbpA*) (Chang et al., 2012), hemolysin listeriolysin O

protein (*hly*) (Price et al., 2018), actin assembly inducing protein (*actA*) (Travier et al., 2013) and a putative cell wall binding protein (*lmo2504*) (Lourenço et al., 2013) and a truncated form of internalin A (*inlA*) protein (Colagiorgi et al., 2016; Franciosa et al., 2009). In contrast, two genes have been reported to show negative impacts on biofilm formation: the *luxS* gene and a putative ABC transporter gene (Sela et al., 2006; Zhu et al., 2008). The LuxS system and the *agr* operon also play major role as signalling system in quorum sensing of Gram-positive bacteria (Garmyn et al., 2009; Riedel et al., 2009; Sela et al., 2006). Furthermore, Piercey et al. (2016) identified nine novel biofilm-related genes using insertional mutagenesis approach that caused increased biofilm formation by *L. monocytogenes* at 15°C typically maintained at the food processing facility.

2.5 Phenotype Microarray Technology

The term 'phenotype' refers to the visible traits or characteristics of an organism. These characteristics could be morphological, physiological, pathological, developmental or biochemical properties which can be monitored and measured by a technical software system (Chen et al., 2014). The process of detecting a microbe based on their different substrate utilization capabilities is known as 'metabolic profiling'. In 1926, Dutch microbiologist Dooren de Jong first profiled an organism by its catabolic enzymes using a large set of chemical components in agar media (Bochner, 2009). Since 1920s, microbiologists had begun to identify and define bacterial species based on their phenotypes that is related to their growth.

In 2001, Bochner and his colleagues developed a novel technique for phenotypic analysis of bacteria, known as Phenotype Microarrays (PMs). PM system is a high-throughput colorimetric assay used to measure the respiration of cells during different growth conditions rather than the expression of genes (Bochner, 2001). Approximately 2000 phenotypic tests can be carried out in wells of twenty 96-well microplates (PM1 -

PM20). The set includes 200 carbon sources, 400 nitrogen sources, 100 phosphorus and sulphur sources, 100 nutrient supplements, 100 osmolytes and pH and a range of chemical inhibitors. Therefore, it allows scientists to detect substrates that stimulate growth and most importantly, substrates that inhibit growth (Bochner et al., 2008). Each well holds a redox indicator, tetrazolium violet dye which turns to purple formazan dye when reduced and remains quantitatively consistent with the respiration of inoculated cells. The color change in PM analysis is highly reproducible and sensitive. The readings can be recorded up to 120 hours and generates kinetic growth curves which are analysed using Biolog Inc. OmniLog^{TM} PM Software (Figure 2.4). In contrast to bacterial growth curves, there is typically no death phase in PMs, as the reduced tetrazolium dye is insoluble. If the bacteria contain the transport system and metabolic pathways, it will utilize the substrate and produce NADH. The electron transport chain takes up the electrons from NADH and passes them to tetrazolium dye. Increases cellular respiration results in the reduction of tetrazolium dye and forms a purple color in the well. Substrates that are strongly metabolized form a dark purple color, while substrates that are weakly metabolized slowly form a light purple color (Bochner, 2003).

Figure 2.4: Biolog Inc. Phenotype Microarray System. Adapted from: (BIOLOG, 2014).

The color intensity and readings in each well can be observed and recorded by OmniLog^{TM} instrument, which includes an incubator and color video camera connected to a computer. The outputs are color coded kinetic graphs generated by the OmniLog^{TM}

software. When two different strains are compared, one is displayed in green, other in red and the overlap in yellow. Therefore, yellow indicates similar phenotypes between two strains, while green or red color suggests more rapid utilization by that strain (Bochner, 2001, 2003).

Phenotype microarray system was designed to examine the phenotypes of a cell from a physiological viewpoint. Physiology is an important tool to achieve the understanding of various subsystems running inside the cell. Some of them are essential to all living forms, while others are specialized functions in differentiated cells and cells that can adapt well under unique environmental conditions (Bochner, 2009). Based on Bochner's study, there are three main advantages to PM assay. Firstly, microbial cells response metabolically by means of respiration. For instance, *Staphylococcus aureus* cannot grow in nitrogen sources, yet it utilizes nitrogen sources and show responses in PM system. Secondly, PM technology can identify one or more catabolic pathways in bacteria. For example, *E. coli* contains a formate dehydrogenase pathway which can be identified in PM; although it was not detected during growth. Lastly, it can quantify the phenotypes of cells that cannot be cultured axenically. Omsland et al. (2009) successfully developed a complex medium for the host-cell free growth of *Coxiella burnetii* using the results of PM assays (Bochner, 2009).

Many published studies reported the successful use of PM technology to determine phenotypic changes in a wide range of microbial species. At present, there are protocols available to examine thousands of bacterial species, yeast and filamentous fungi. PM technology was first used to identify yeast in a species level from a mixed population of yeast and bacteria (Greetham, 2014; McGinnis et al., 1996). A previous study by Datta et al. (2008) showed that *L. monocytogenes* strains associated with invasive listeriosis outbreaks were seemingly acid tolerant, while the strains involved in gastroenteritis

outbreaks were osmotolerant. This speculation was achieved by screening a large collection of listeriosis outbreak strains using PM technology. Several studies reported that temperature fluctuations can trigger the virulence gene regulator PrfA and initiate virulence gene expression in *L. monocytogenes* cells (Bochner, 2009; Ripio et al., 1997; Scortti et al., 2007; Vázquez-Boland et al., 2001). PM analysis also provides an insight to the metabolic pathways that are presumed from whole genome sequencing and likely to discover new genes as well as pseudogene functions from the phenotypic analysis of different bacterial species (Bochner et al., 2008; Zhou et al., 2003). Furthermore, PM assay has been used to study the gene function and to expand gene annotations of microorganisms in order to profile similar bacterial species (Mackie et al., 2014).

The phenotype microarray technique is a semi-high throughput assay that determines the microbial cellular phenotypes. PM approach facilitates the relationship between genotype-phenotype in various microbial species. Moreover, this technique can provide necessary knowledge about the metabolic and physiological properties of individual species or strains and thereby contribute to the knowledge pool that is essential for a comprehensive understanding of the relationship between the whole genome sequence data and functional bacterial biodiversity (Blumenstein et al., 2015). Therefore, Biolog's OmniLog PM Software was used to characterize the metabolic profile of three foodborne *L. monocytogenes* strains.

CHAPTER 3: METHODOLOGY

3.1 Background of the studied bacterial strains

Three Malaysian *L. monocytogenes* strains previously isolated from RTE food samples were selected for this study (Jamali et al., 2013). These three strains were tested since they belong to the serogroups that are associated with human listeriosis. The serogroups are 1/2a, 3a (LM92), 1/2c, 3c (LM41) and 4b, 4d, 4e (LM115) (Table 3.1). A previous study reported that these strains contain virulence genes and belong to the pathogenic serogroups responsible for Listeriosis (Jamali & Thong, 2014). Standard biochemical testing, e.g. Grams-staining, catalase test, oxidase test, SIM, TSI, MR-VP test and species-specific PCR were used to confirm the identity of *L. monocytogenes* strains (Jamali et al., 2013). Among them, LM115 is a multiple drug resistant strain. The strains were preserved in Luria-Bertani (LB) broth with 50% glycerol.

Table 3.1: Background information of *L. monocytogenes* strains used in this study. Adapted from: (Jamali et al., 2013).

Strain	Year	Source	Locality	Serogroup
LM41	2011	Salad with vinegar; RTE	Kuala Lumpur (Chowkit), Malaysia	1/2c, 3c
LM92	2011	Egg tart; RTE	Kuala Lumpur (UM), Malaysia	1/2a, 3a
LM115	2011	Fried fish; RTE	Selangor (Petalling Jaya SS17), Malaysia	4b, 4d, 4e

3.2 Background of Phenotype Microarray System

Biolog's Phenotype Microarray assay included six 96-well PM panels (PM1, PM2A, PM3B, PM6, PM7 and PM8) were used to determine the ability of three *L. monocytogenes* strains to catabolize 190 carbon sources (PM1-PM2A) and 380 nitrogen sources (PM3B, PM6-PM8). The PM microplates consist of 95 substrate-containing wells and a negative control well (A1) without any nutrient sources (Bochner, 2001). Only plates PM6, PM7 and PM8 contain a positive control well (A2: L-glutamine) along with negative control. The principle of the test relies on the redox dye tetrazolium violet to

detect growth (NADH formation) in carbon sources and nitrogen sources (Bochner, 2001). The substrate, dye and nutrients are supplied in each well in a dried-film which is combined by the addition of bacterial culture. The carbon and nitrogen sources and their location in plates PM1, PM2A, PM3B, PM6, PM7 and PM8 are listed in Appendix B.

The phenotype microarray assays were performed as per the manufacturer's protocol (BIOLOG, 2014). Stock cultures of *L. monocytogenes* strains were cultured in LB agar and incubated at 37°C for 24 hours. From the overnight culture, 5 to 6 single colonies were transferred to Biolog inoculating fluid (IF-0a) and the cell density was adjusted to O.D. = 0.1 (Biolog, Hayward, USA). The IF-0a is a buffer salt solution that does not constitute any carbon or nitrogen source but retains the viability of microbial cells (Tang et al., 2010). The Biolog dye 1% dye mix (v/v) was added to the bacterial culture. An aliquot of 100 µL cell suspension was added to the wells of plates PM1, PM2A, PM3B, PM6, PM7 and PM8. The plates were incubated at 37°C for 48 hours in an OmniLog plate reader. The kinetic data for every 15 minutes for each plate were extracted from OmniLog Software for extensive analyses.

3.3 Phenotype Microarray Data Analysis

Phenotype microarray kinetic growth for plates PM1, PM2A, PM3B, PM6, PM7 and PM8 were recorded using OmniLog OL_FM_12 kinetic software (Biolog, USA) and analysed using Microsoft Excel. The OmniLog reader generated a time course curve for tetrazolium color development after incubation at 37°C for 48 hours (Figure 3.1). For each well, the average area under growth and the average slope of time course data were used to measure the threshold value (maximum 100 OmniLog unit, OU) for growth in PM microplates. Cellular phenotypes were determined based on the difference in average area under the growth curve (AUC) values. The well is considered positive if the standardized average area is equal or exceeded the threshold value. Wells with a high

average area value but with a slope close to zero were considered as less significant than wells with a smaller area but increasing signal over time. During data processing, the option of A1 zero (negative control) was selected to subtract the background from each well. Plates were analysed in duplicates and results were checked for errors (Chelvam et al., 2015; Khatri et al., 2013).

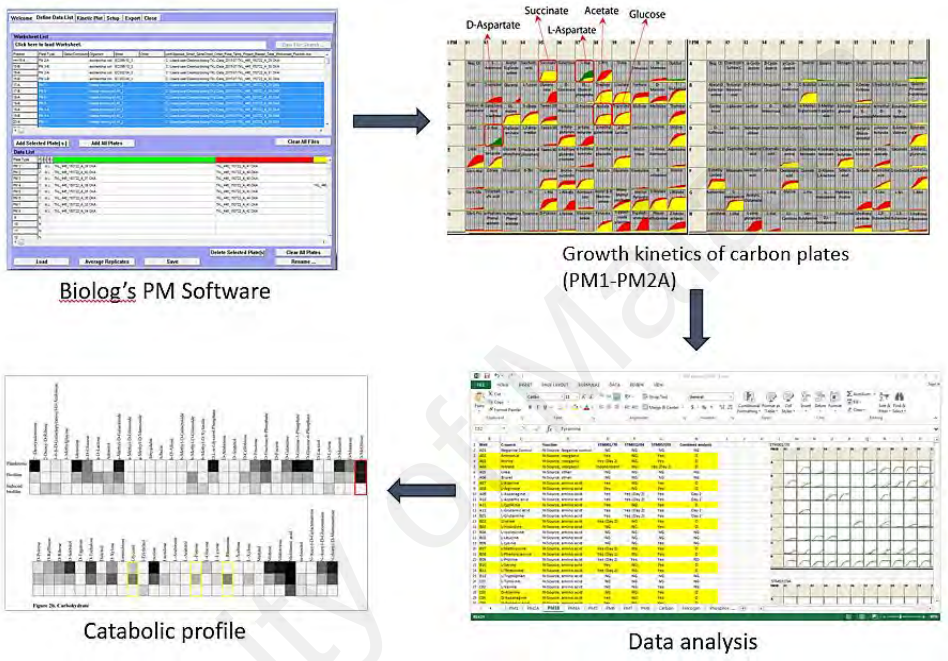

Figure 3.1: Workflow of Phenotype Microarray Data Analysis.

3.3.1 Validation of Phenotype Microarray Analysis

To confirm the PM data, the *L. monocytogenes* strains (LM41, LM92 and LM115) were grown in minimal medium supplemented with carbon and nitrogen substrates that were available in the laboratory. The strains were revived from -20°C glycerol stock and grown in LB broth overnight. Cells were harvested at 6000 rpm for 5 minutes, washed three times with 1x phosphate buffered saline (PBS). Cellular turbidity was adjusted to 10^8 CFU mL^{-1}. Serial dilutions were carried out to ensure the final concentration of cells in each well was standardized to 10^3 CFU mL^{-1}. Working solution (20 mM) for each substrate was prepared by filter sterilization using a 0.22 µ pore size filter. Aliquots of 200 µL inoculum with individual carbon and nitrogen substrates were added to the wells

of sterile 96-well polystyrene microtiter plates and incubated at 37°C for 24 hours. After incubation, cellular turbidity was measured at OD_{600} nm wavelength using a microplate reader (Figure 3.2). The true O.D. readings (OD_{TR}) of each strain were acquired by deducting the negative control ($OD_{control}$) from the sample's O.D. readings (OD_{600}) which is expressed using the formula, $OD_{TR} = OD_{600} - OD_{control}$. Negative control contained minimal medium including the substrates without bacterial culture. This assay was repeated three times and tested in replicates to ensure the reproducibility of the results.

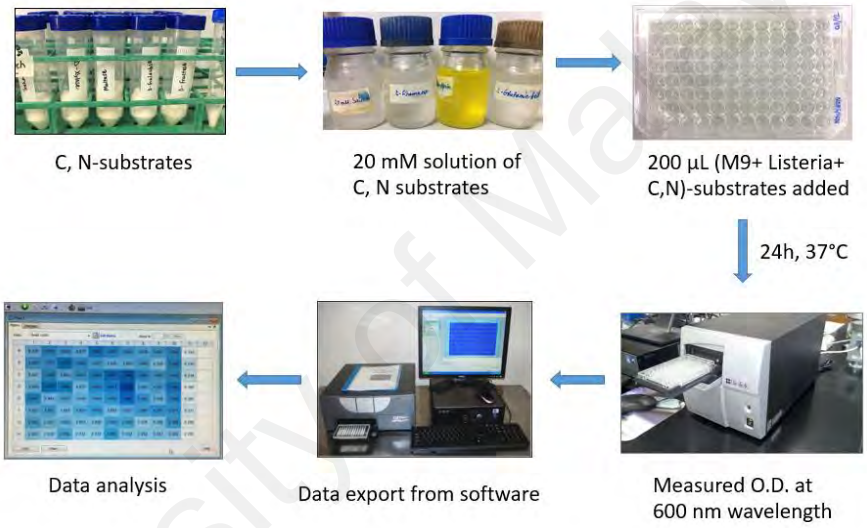

Figure 3.2: Workflow of validation of Phenotype Microarray analysis.

3.4 Biofilm Forming Ability of the *L. monocytogenes* strains

3.4.1 Biofilm Formation in Nutrient-rich Medium

To check for biofilm forming ability, the microtiter plate assay using crystal violet straining method was performed according to the protocol developed by Stepanović et al. (2000) with slight modifications. The studied strains (LM41, LM92 and LM115) were grown in LB broth overnight. Cellular turbidity was adjusted to McFarland standard No.5 which corresponds to 10^8 CFU mL^{-1}. Serial dilutions were carried out to ensure that the final concentration of cells in each well was standardized to 10^3 CFU mL^{-1}. An aliquot of 200 µL bacterial culture was added to the wells of sterile 96-well microtiter plates and

incubated at 37°C for 24 and 48 hours. The inoculated plates were wrapped with parafilm to minimize evaporation. After incubation, the unbound cells were removed by inversion of microtiter plate, followed by vigorous tapping on absorbent paper and washed twice with 1x PBS. Next, the adhered cells were heat-fixed in an oven at 80°C for 30 minutes. Adhered cells were stained by adding 200 µL 0.5% (w/v) crystal violet for 5 minutes. The stain was removed by thorough washing with sterile distilled water (dH_2O) and air dried. Quantification of biofilm cells was performed by addition of 220 µL decoloring solution (ethanol:acetone, 80:20%) (v/v) in each well for 15 minutes (Figure 3.3). The absorption of the eluted stain was measured at 590 nm wavelength using a microplate reader (Epoch, Germany).

Based the $O.D_{590}$ readings and calculation of cut-off O.D. strains were classified into no biofilm producer, weak, moderate and strong biofilm producer. The cut-off O.D. (O.D.c) is defined as three standard deviations (3 × SD) above the mean O.D. ($\mu_{control}$) of negative control, which is expressed as [$O.D.c = \mu_{control} + (3 \times SD)$] (Stepanović et al., 2004; Stepanović et al., 2000). The strains were categorized as follows: non-biofilm producer (0) if $O.D. \leq O.D.c$; weak biofilm producer (+) if $O.D.c < O.D. \leq (2 \times O.D.c)$; moderate biofilm producer (+ +) if $(2 \times O.D.c) < O.D. \leq (4 \times O.D.c)$ and strong biofilm producer (+ + +) if $O.D. > (4 \times O.D.c)$. Negative control contained LB broth only and O.D. readings of negative control were deducted from the background. Biofilm assay was performed in triplicates to ensure the reproducibility of results.

3.4.2 Biofilm Formation in Nutrient-limited Medium

The *L. monocytogenes* strains were grown in LB broth overnight in a shaking water bath (110 rpm) at 37°C. Cells were harvested at 6000 rpm for 5 minutes, washed three times with 1x PBS. Cell density of overnight cell cultures of *L. monocytogenes* strains was adjusted. M9 Minimal Salts Medium was prepared without any carbon or nitrogen

sources and sterilized by autoclaving (121°C for 15 minutes). Aliquots of (200 µL) minimal medium with bacterial cultures were added to wells of 96-well microtiter plates and incubated at 37°C for 24 and 48 hours. After incubation, cell cultures were discarded and washed three times with 220 µL 1x PBS. Adhered cells were heat fixed at 80°C for 30 minutes and stained using 200 µL 0.5% (w/v) crystal violet for 5 minutes. The dye was removed and washed three times with 220 µL 1x PBS. After drying the plates, 200 µL decoloring solution (ethanol:acetone, 80:20%) (v/v) was added to each well to quantify the adhered cells. Absorption of the eluted stain was measured at 590 nm wavelength using a microplate reader. Based the $O.D_{590}$ readings and calculation of O.D.c, strains were classified into no biofilm producer, weak, moderate and strong biofilm producer (Chelvam et al., 2014; Stepanović et al., 2000). Negative control contained M9 medium only and O.D. reading of negative control was deducted. Results were averaged and standard deviation of negative control O.D. reading was calculated for biofilm classification (Stepanović et al., 2000).

3.4.3 Biofilm Formation in Minimal Medium with specific Carbon and Nitrogen substrates

Carbon and nitrogen substrates were selected from the PM analysis and added to the M9 minimal medium to induce biofilm formation in the *L. monocytogenes* strains. The strains were grown in LB broth overnight. Cells were harvested and washed three times with PBS. The cell density of overnight cell cultures of *L. monocytogenes* strains was adjusted. Twenty millimolar (20 mM) solution of each substrate was prepared by membrane filtration method using a 0.22 µ pore size filter. Aliquots of (200 µL) cell cultures with individual carbon or nitrogen substrates were added into the wells of sterile microtiter plates and incubated at 37°C for 24 and 48 hours (Figure 3.3). After incubation, the cultures were discarded. Non-adhered cells were removed by vigorous tapping on absorbent paper and washed three times with 220 µL sterile dH_2O. Adhered cells were

heat fixed at 80°C for 30 minutes. Quantification of biofilm cells was performed by staining of adhered cells with 200 µL 0.5% (w/v) crystal violet and washed twice with sterile dH_2O followed by 220 µL destaining solution (ethanol:acetone, 80:20%) (v/v) for 15 minutes. Absorbance of eluted stain was measured at OD_{590} nm wavelength. Based the $O.D_{590}$ readings and calculation of O.D.c, the strains were classified according to their biofilm forming ability as mentioned before. Negative control contained M9 media with individual substrates. The O.D. readings of negative control were deducted from the sample's readings. Results were averaged and standard deviation of negative control O.D. readings were calculated for biofilm classification (Stepanović et al., 2000). The statistical information of biofilm assay are shown in Appendix C.

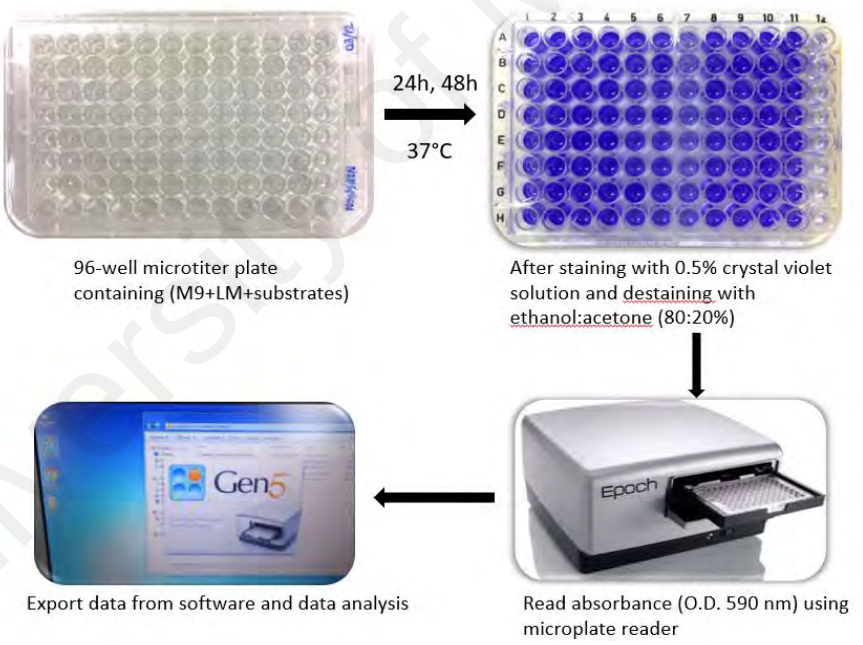

Figure 3.3: An illustration of crystal violet assay to determine the biofilm forming ability of the *L. monocytogenes* strains.

3.5 Use of Bioinformatics tools to identify the Catabolic and Biofilm related Genes in the *L. monocytogenes* strains

Since the aim of this study was to elucidate the genetic basis of the phenotypic characteristics of the studied *L. monocytogenes* strains, multiple bioinformatics tools were used to determine the genes for enzymes associated with the utilization of various substrates and the genes involved in biofilm formation. The draft genome sequences of strains LM41 and LM115 were extracted from previously published study by Lim et al. (2016) for further investigation. The deduced proteins encoded by specific genes were functionally characterized by automated searches in public databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999), KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007) and BlastKOALA (https://www.kegg.jp/blastkoala/) (Kanehisa et al., 2016) genome servers. The BlastKOALA server was used to determine the protein coding genes using the peptide sequences of the studied strains and performed automated search against a dataset of pangenome sequences at species level and then further analysed using the KEGG Pathway database to identify the relevant metabolic pathways. The KEGG database is one of the largest online repositories that focuses on the metabolic pathways present in diverse microorganisms. In addition, similar *L. monocytogenes* strains were identified by megablast search against the National Centre for Biotechnology Information (NCBI) GenBank repository using the genomic data of strains LM41 and LM115 to identify the specific protein coding genes (Lim et al., 2016). The candidate genes potentially involved in the catabolism of specific carbon and nitrogen substrates were confirmed by Nucleotide Basic Local Alignment Search Tool (BLASTn) analysis using 10% cut-off value for each strain. The relevant genes were obtained from the NCBI gene database using the reference strain *L. monocytogenes* EGD-e (GenBank, accession number NC_003210). The reference strain EGD-e (1/2a serotype) is the most vastly studied

and well-characterized animal isolate, which showed genomic similarity with the analysed strains (Glaser et al., 2001; Lim et al., 2016). After the biofilm assay, BLASTn analysis was performed to identify the presence or absence of multiple biofilm relevant genes and compare them with the biofilm forming phenotypes. The workflow and various bioinformatics pipelines used in the genomic analyses are showed in Figure 3.4

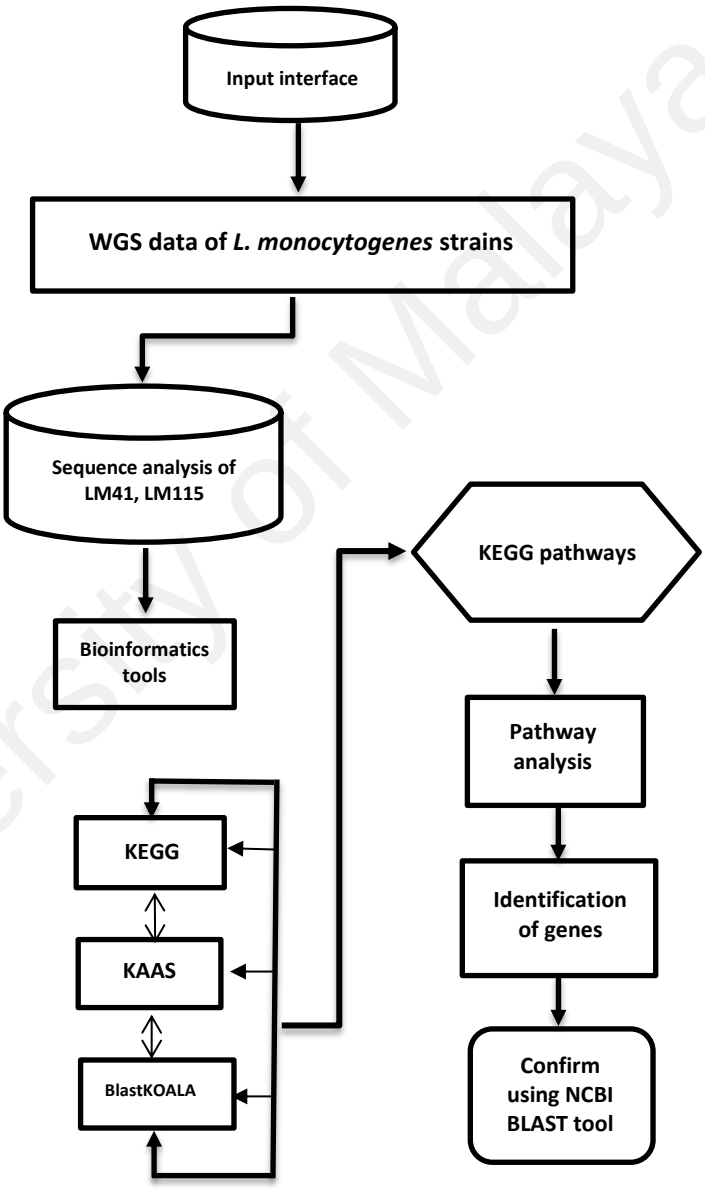

Figure 3.4: Flowchart of bioinformatics analysis of the WGS data of *L. monocytogenes* strains.

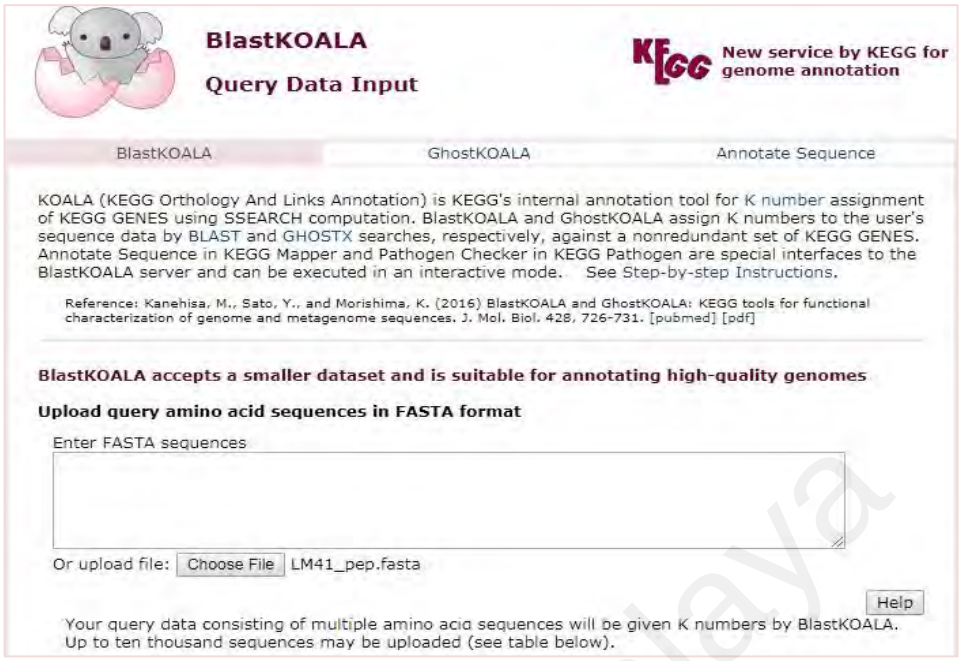

Figure 3.5: The BlastKOALA query data input webpage on the KEGG server set up for amino acid sequence analysis.

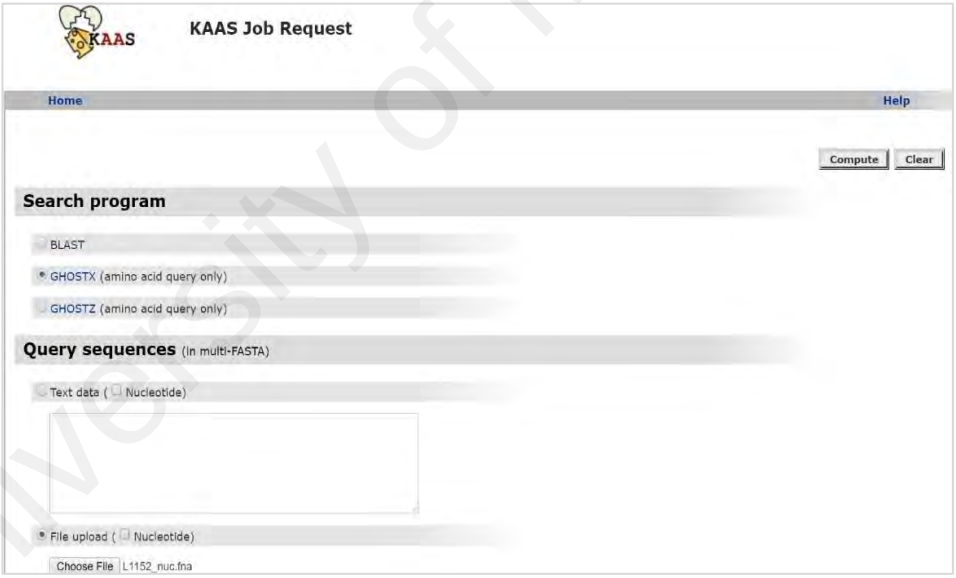

Figure 3.6: The KAAS query data input webpage on the KEGG server set up for nucleotide sequence analysis.

CHAPTER 4: RESULTS

4.1 Phenotype Microarray Analyses

The kinetic growth curves generated by the OmniLog PM Software are represented in Figure 4.1. The analysed strains were able to catabolize different carbon and nitrogen sources. The preferred carbon substrates were: carbohydrates, polymers, nucleosides and the preferred nitrogen substrates were: amino acids, amines, nucleosides and dipeptide nitrogen sources (Table 4.1). Among the 190 carbon substrates tested, only 38 (20%) were catabolized by the strains. The carbon substrates were classified into carbohydrates (n = 26), nucleosides (n = 5), polymers (n = 4), amino acids (n = 2) and amide (n = 1). Out of 38 carbon substrates, only 29 (15.3%) were catabolized by all three strains. However, alcohol, carboxylic acid, fatty acid and esters were not catabolized by any of the three strains (Figure 4.2).

A total of 380 nitrogen substrates were tested in PM3B, PM6, PM7 and PM8 microplates. Out of 380 substrates, total 61 (16%) nitrogen substrates were catabolized by the studied strains. The substrates were categorized into amino acids (n = 6), amines (n = 5), nucleosides (n = 7), heterocyclic organic compounds (n = 3) and peptides (n = 10). Out of the 61 substrates, only 7 (1.8%) substrates were actively metabolized by all three strains (Figure 4.3). These substrates were D-glucosamine, D-mannosamine, N-acetyl-D-glucosamine, cytidine, uridine, xanthosine and uric acid. Strain LM115 utilized only 5 peptides in plate PM3B, whereas strain LM92 utilized only one peptide. The strain LM41 showed a higher respiration rate (10.5%) in plates PM6, PM7 and PM8 containing peptide nitrogen sources, while strains LM92 and LM115 did not respire in any of them (Figure 4.4).

In addition, 24 carbon and nitrogen substrates were tested based on their availability in the laboratory to confirm the PM analysis. There was a correlation between the PM

data and validation test for 24 substrates; although there were 9 substrates that showed discrepancy between the two results (Table 4.2). These substrates were glycine, L-cysteine, D-xylose, D-biotin, riboflavin, thiamine, adenosine, L-glutamic acid and tween 40. Nitrogen substrates, such as glycine, L-cysteine, adenosine and L-glutamic acid were only catabolized by strain LM115, while adenosine was catabolized by strains LM 41 and LM115. On the contrary, the studied strains did not utilize D-xylose, D-biotin, riboflavin, thiamine and tween 40 in PM analysis. Yet, all three strains showed growth in validation experiment (Table 4.2).

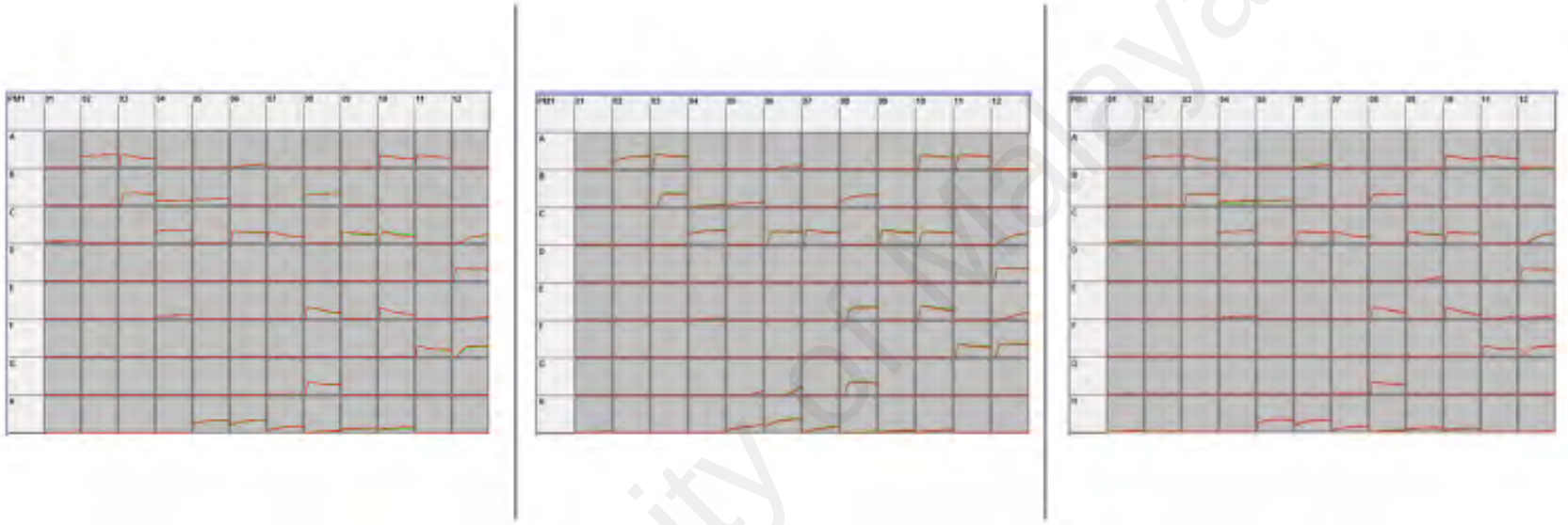

Figure 4.1: Representative of kinetic growth curves of strains LM41, LM92 and LM115 in 95 carbon sources using PM1 microplate (A1 = zero) measured with the Biolog OmniLog PM Software.

Table 4.1: Catabolism of carbon and nitrogen substrates by *L. monocytogenes* strains based on PM analysis.

Carbon substrates		**Number of substrates tested**	**Number of substrates catabolized by all three strains**	**Strain-specific substrate catabolism**			**Number of substrates catabolized by at least two strains**	**Number of substrates not catabolized n(%)**
				LM41	**LM92**	**LM115**		
Sugar and derivatives	Monosaccharide	20	8 (40)	1	0	1	2 (10)	10 (50)
	Disaccharide	9	4 (44)	-	-	-	-	5 (56)
	Oligosaccharide	10	1 (10)	-	-	-	-	9 (90)
	Polysaccharide	6	1 (17)	-	-	-	-	5 (83)
	Sugar alcohol	18	3 (17)	-	-	-	-	15 (83)
	Amino sugar	8	2 (25)	-	-	-	-	6 (75)
	Deoxy sugar	4	-	-	-	-	-	4 (100)
	Aldaric acid	4	-	-	-	-	-	4 (100)
	Aldonic acid	2	-	-	-	-	-	2 (100)
	Uronic acid	8	-	-	-	-	-	8 (100)
	Glycoside	9	2 (22)				-	7 (78)
	Other	1	1 (100)	-	-	-	-	-
Polymers		11	4 (36)	-	-	-	-	7 (64)
Amide		3	-	1	0	0	-	2 (67)
Amine		5	-	-	-	-	-	5 (100)
Amino acid		30	-	2	0	0	-	25 (83)
Nucleic acid	Nucleosides	5	4 (80)	-	-	-	1 (20)	-

Table 4.1, continued.

Nitrogen substrates	Number of substrates tested	Number of substrates catabolized by all three strains	Strain-specific substrate catabolism			Number of substrates catabolized by at least two strains	Number of substrates not catabolized n(%)
			LM41	LM92	LM115		
Amino acid	33	-	1	0	5	-	27 (82)
Other	1	-	0	0	1	-	-
Amine	17	3 (18)	0	0	1	1 (6)	12 (71)
Amide	4	-	-	-	-	-	4 (100)
Nucleic Acid	13	3 (23)	1	0	0	3 (23)	6 (46)
Heterocyclic organic compounds	10	1 (10)	0	0	0	2 (20)	7 (70)
Peptide nitrogen sources	294	-	34	1	3	2 (1)	254 (86)

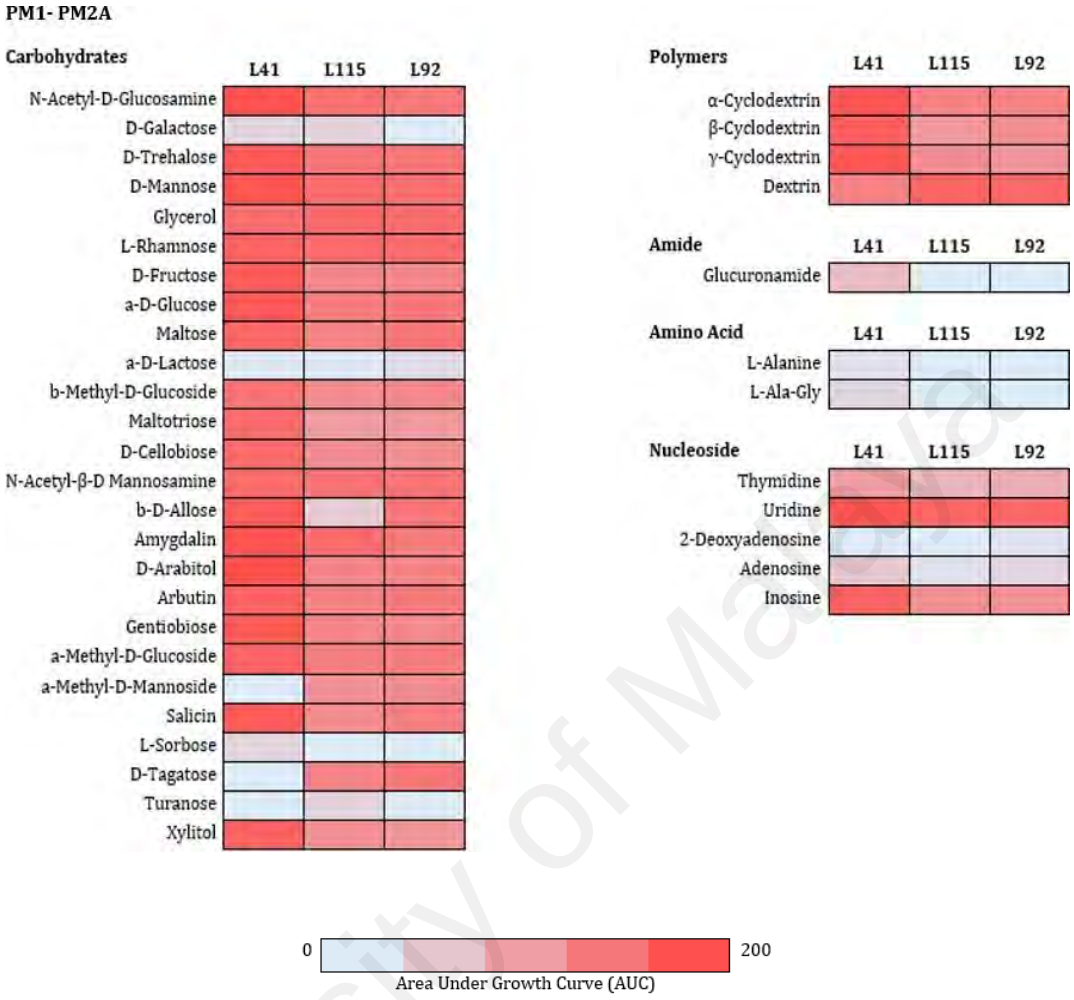

Figure 4.2: The catabolic phenome of *Listeria monocytogenes* strains. Strengths of carbon and nitrogen utilization phenotypes of *L. monocytogenes* strains LM41, LM92 and LM115 were determined using Biolog^{TM} Phenotype Microarray plates PM1, PM2A, PM3B, PM6, PM7 and PM8. The maximal kinetic curve height is expressed as a color scale ranging from 0 (light blue) to 200 (red) area under growth curve (AUC) units. Substrates are considered as 'strongly utilized' if AUC ≥ 180, 'weakly utilized' if AUC ≤ 50 and 'not utilized' if AUC < 10.

Figure 4.2, continued.

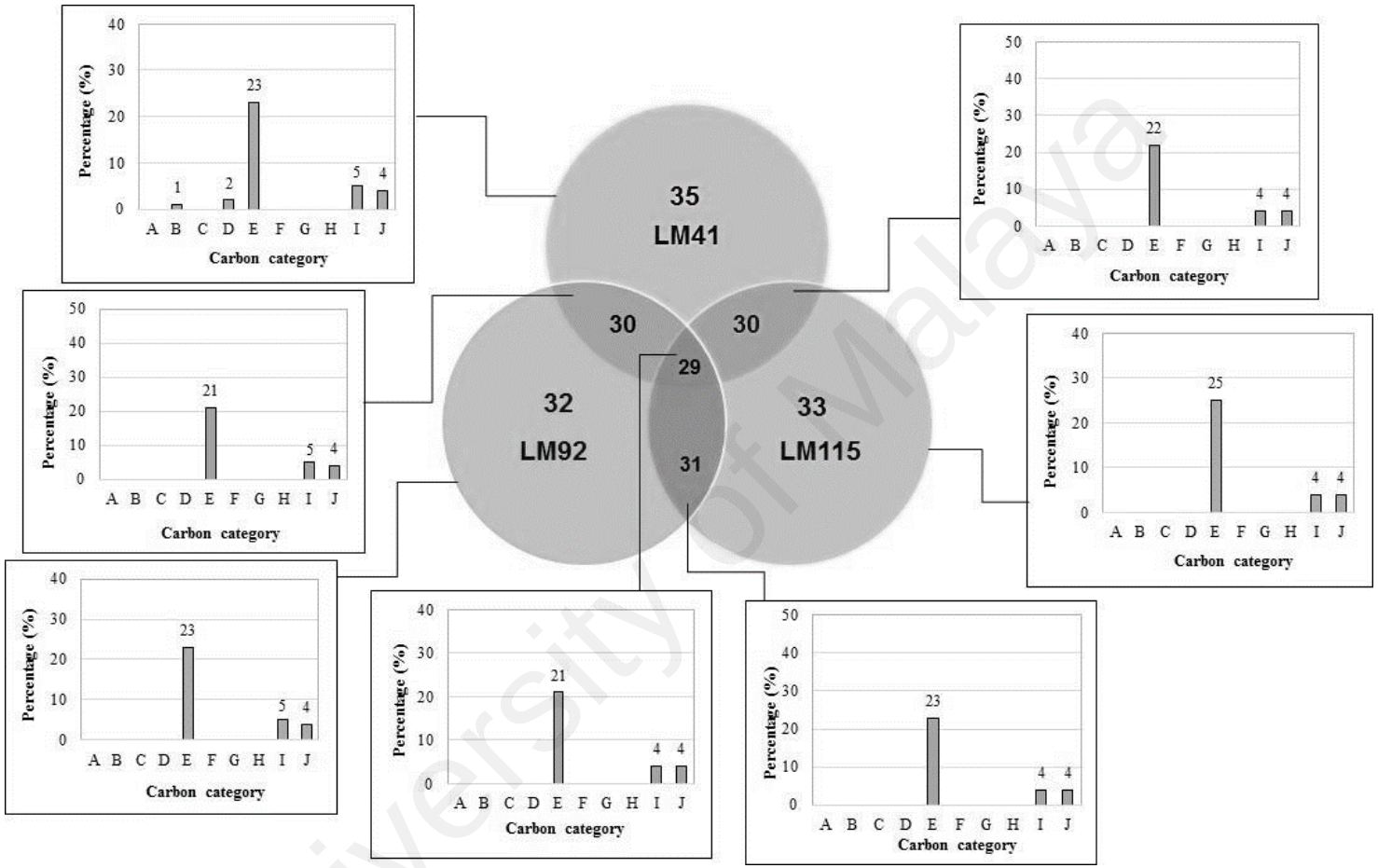

Figure 4.3: Venn diagram showing the carbon catabolic activity of three *L. monocytogenes* strains. A total of 190 carbon substrates are tested. They are categorized as: A: Alcohol, B: Amide, C: Amine, D: Amino acid, E: Carbohydrate, F: Carboxylic acid, G: Ester, H: Fatty acid, I: Nucleotide and J: Polymer. Y-axis indicates the percentage of carbon utilized. X-axis shows the carbon category for each carbon substrate tested. The Venn diagram was obtained based on average growth curve area and the numerals indicate the carbon substrates utilized by three strains, two strains and each strain individually.

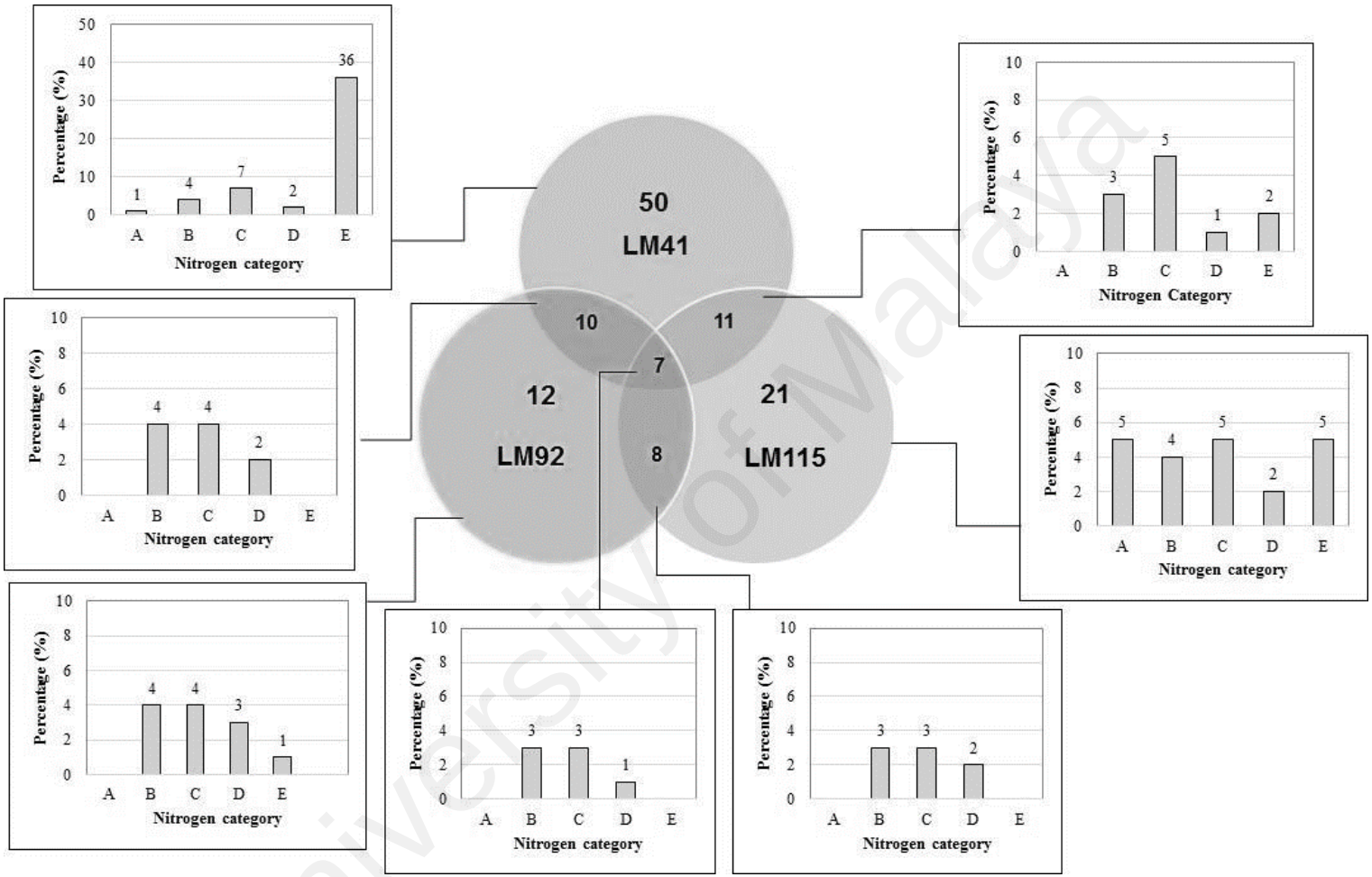

Figure 4.4: Venn diagram showing the nitrogen catabolic activity of three *L. monocytogenes* strains. A total of 380 nitrogen substrates are tested. They are categorized as: A: Amino acid, B: Amine, C: Nucleoside, D: Heterocyclic Organic Compound and E: Dipeptide. Y-axis indicates the percentage of nitrogen utilized. X-axis shows the nitrogen category for each nitrogen substrate tested. The Venn diagram was obtained based on average growth curve area and the numerals indicate the nitrogen substrates utilized bythree strains, two strains and each strain individually.

Table 4.2: Validation of the catabolism result of Phenotype Microarray in standard culture supplemented with carbon or nitrogen substrates.

C- and N-substrates tested	Growth based on PM analysis			Growth in minimal medium supplemented with C- or N-substrates		
	LM41	LM92	LM115	LM41	LM92	LM115
D-Glucose	+	+	+	+	+	+
Glycine	-	-	+	+	+	+
L-Lysine	-	-	-	-	-	-
L-Cysteine	-	-	+	-	-	-
L-Threonine	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+
Tween 80	-	-	-	-	-	-
D-Fructose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
D-Xylose	-	-	-	+	+	+
D-Trehalose	+	+	+	+	+	+
Riboflavin	-	-	-	+	+	+
D-Biotin	-	-	-	+	+	+
Thiamine	-	-	-	+	+	+
Salicin	+	+	+	+	+	+
Adenosine	+	-	+	+	+	+
Thymidine	+	+	+	+	+	+
Cytidine	+	+	+	+	+	+
Uridine	+	+	+	+	+	+
L-Glutamic Acid	-	-	+	+	+	+
L-Rhamnose	+	+	+	+	+	+
Tween 40	-	-	-	+	+	+

'+' indicates presence of growth, '-' indicates absence of growth

4.2 Biofilm forming ability of the *L. monocytogenes* strains

Based on the biofilm classification, all three strains were able to produce strong biofilms in both LB broth and M9 minimal medium after 24 and 48 hours of incubation. Out of the 23 carbon and nitrogen substrates tested based on the PM catabolic profile (Table 4.1) to determine whether these carbon and nitrogen substrates would induce biofilm formation in the studied strains, 15 carbon and nitrogen substrates were found to be able to induce biofilm formation in minimal medium. The substrates were: adenosine, cytidine, uridine, glutamic acid, rhamnose, tween 40, riboflavin, thiamine, biotin, salicin, fructose, maltose, galactose, trehalose and xylose. All three strains formed weak biofilm in threonine and glycerol. Strain LM92 formed moderate biofilm in glycine, lysine, cysteine and glucose; whereas LM41 and LM115 formed weaker biofilms in these substrates. In addition, glucose was not able to induce strong biofilm formation in any of the strains, instead it produced moderate to weak biofilms in minimal medium (Table 4.3). On the contrary, fructose, maltose, galactose, xylose, trehalose, rhamnose, salicin, riboflavin, biotin, thiamine and glutamic acid induced strong biofilm formation in the studied strains (Figure 4.5).

All three strains showed strong to moderate biofilm forming phenotypes when tween 40 and tween 80 were supplemented in the minimal medium; although they did not utilize them as sole carbon sources in PM growth conditions (Table 4.2). Interestingly, none of the strains formed biofilm when thymidine was added to the medium, however they all formed strong biofilms in other nucleosides, such as adenosine, cytidine and uridine. PM analysis indicated an enhanced growth in thymidine, yet it failed to induce biofilm formation in the tested strains (Table 4.3).

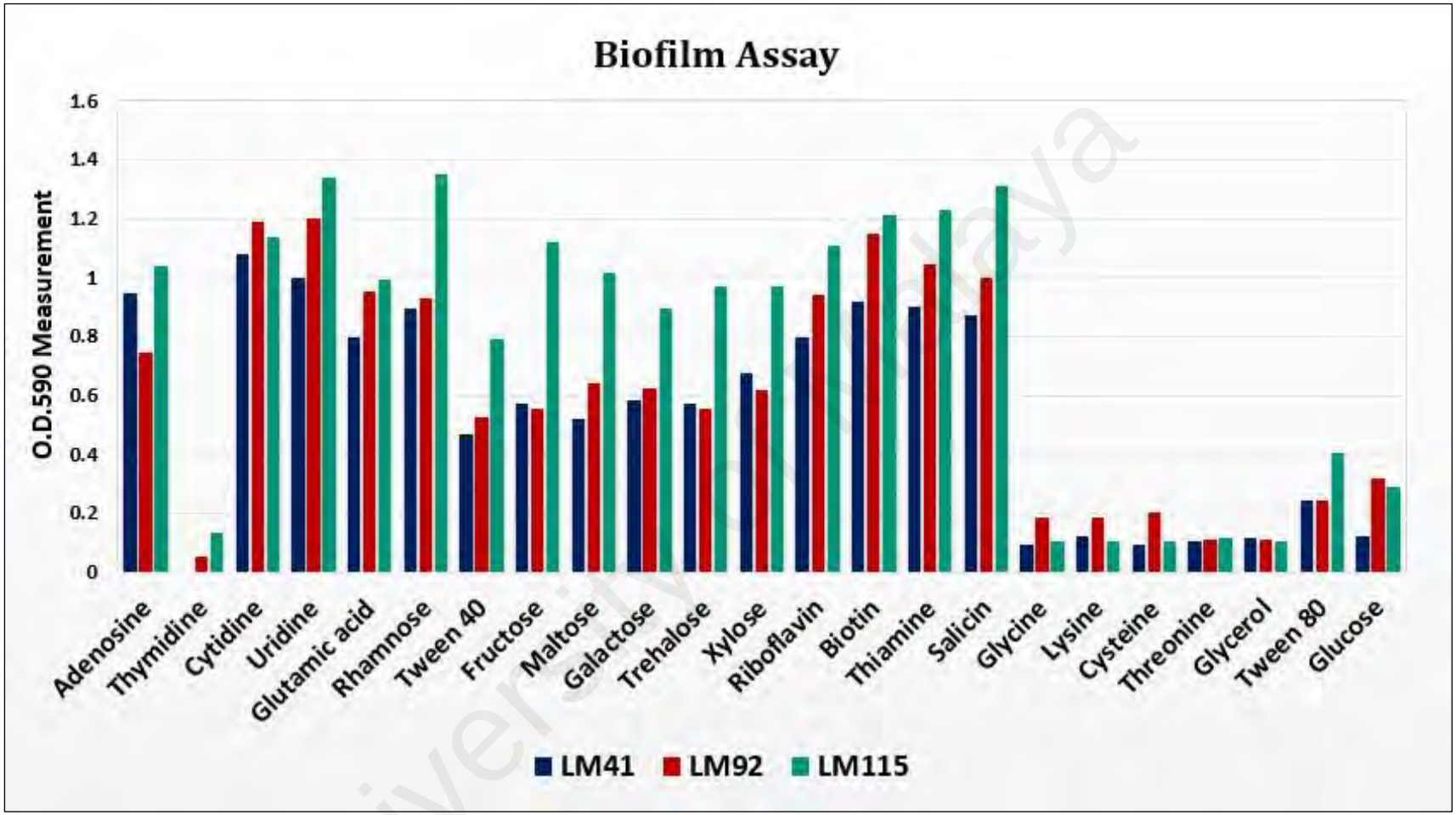

Figure 4.5: Graphical representation of the average optical density (O.D.590) readings of crystal violet assay to determine the biofilm forming ability of three *L. monocytogenes* strains in various carbon and nitrogen substrates.

Table 4.3: The biofilm forming ability of the *L. monocytogenes* strains.

Media and Substrates	Substrate utilization in PM			Biofilm Classification		
	LM41	**LM92**	**LM115**	**LM41**	**LM92**	**LM115**
LB broth	N/A	N/A	N/A	+ + +	+ + +	+ + +
M9 medium only	N/A	N/A	N/A	+ + +	+ + +	+ + +
Adenosine	(+)	(-)	(+)	+ + +	+ + +	+ + +
Thymidine	(+)	(+)	(+)	-	-	-
Cytidine	(+)	(+)	(+)	+ + +	+ + +	+ + +
Uridine	(+)	(+)	(+)	+ + +	+ + +	+ + +
L-Glutamic Acid	(-)	(-)	(+)	+ + +	+ + +	+ + +
L-Rhamnose	(+)	(+)	(+)	+ + +	+ + +	+ + +
Tween 40	(-)	(-)	(-)	+ + +	+ + +	+ + +
Riboflavin	(-)	(-)	(-)	+ + +	+ + +	+ + +
Thiamine	(-)	(-)	(-)	+ + +	+ + +	+ + +
Biotin	(-)	(-)	(-)	+ + +	+ + +	+ + +
Salicin	(+)	(+)	(+)	+ + +	+ + +	+ + +
D-Fructose	(+)	(+)	(+)	+ + +	+ + +	+ + +
Maltose	(+)	(+)	(+)	+ + +	+ + +	+ + +
D-Galactose	(+)	(+)	(+)	+ + +	+ + +	+ + +
D-Trehalose	(+)	(+)	(+)	+ + +	+ + +	+ + +
D-Xylose	(-)	(-)	(-)	+ + +	+ + +	+ + +
Glycine	(-)	(-)	(+)	+	+ +	+
L-Lysine	(-)	(-)	(-)	+	+ +	+
L-Cysteine	(-)	(-)	(+)	+	+ +	+
L-Threonine	(-)	(-)	(-)	+	+	+
Glycerol	(+)	(+)	(+)	+	+	+
Tween 80	(-)	(-)	(-)	+ +	+ +	+ + +
D-Glucose	(+)	(+)	(+)	+	+ +	+ +

[a](+) = positive for growth, (-) = negative for growth, N/A = not applicable

[b]Interpretation based on cut-off O.D. (O.D.c), O.D. ≤ O.D.c = non biofilm producer [-]; O.D.c < O.D.≤ (2 × O.D.c) = weak biofilm producer [+]; (2 ×O.D.c) < O.D. ≤ (4 × O.D.c) = moderate biofilm producer [+ +]; (4 × O.D.c) < O.D. = strong biofilm producer [+ + +]; (Stepanović et al., 2000)

4.3 Identification of genes in the analysed *L. monocytogenes* strains associated with substrate utilization and biofilm formation

The BLASTn analysis showed more than 97% sequence similarity with the reference strain EGD-e and identified 136 genes related to metabolism and biofilm formation in the studied *L. monocytogenes* strains. The online pathway mapping tools (BlastKOALA and KAAS) in KEGG database generated the reconstructed metabolic and enzymatic pathways from which the genes for enzymes responsible for the catabolism of 38 carbon and 61 nitrogen substrates utilized in the PM analysis were identified and presented in Table 4.4 below.

Table 4.4: Identification of genes for the catabolizing enzymes involved in the substrate utilization and biofilm formation by *L. monocytogenes* strains.

C- and N-substrates tested	Growth in PM study			Biofilm forming ability			Genes for enzymes	KEGG ID
	LM41	LM92	LM115	LM41	LM92	LM115		
D-Glucose	+	+	+	W	M	M	Glucokinase	EC: 2.7.1.2
D-Galactose	+	+	+	S	S	S	aldose 1-epimerase	EC: 5.1.3.3
D-Fructose	+	+	+	S	S	S	Fructokinase	EC: 2.7.1.4
							PTS system, fructose-specific IIA component	EC: 2.7.1.202
Maltose	+	+	+	S	S	S	maltose phosphorylase	EC: 2.4.1.8
							maltase-glucoamylase	EC: 3.2.1.20
Maltotriose	+	+	+	n/a	n/a	n/a	maltogenic alpha-amylase	EC: 3.2.1.133
D-Cellobiose	+	+	+	n/a	n/a	n/a	PTS system, cellobiose-specific IIA component	EC: 2.7.1.205
							beta-glucosidase	EC: 3.2.1.21
D-Lactose	+	+	+	n/a	n/a	n/a	PTS system, lactose-specific IIA component	EC: 2.7.1.207
D-Mannose	+	+	+	n/a	n/a	n/a	PTS system, mannose-specific IIA component	EC: 2.7.1.191
D-Trehalose	+	+	+	S	S	S	PTS system, sugar-specific IIA component	EC: 2.7.1.201
L-Rhamnose	+	+	+	S	S	S	L-rhamnose isomerase, rhaA	EC: 5.3.1.14
Glycerol	+	+	+	W	W	W	glycerol kinase	EC: 2.7.1.30
N-Acetyl-D-Glucosamine	+	+	+	n/a	n/a	n/a	N-acetylglucosamine-6-phosphate deacetylase	EC: 3.5.1.25
N-Acetyl-β-D-Mannosamine	+	+	+	n/a	n/a	n/a	unknown	-
a-Methyl-D-Glucoside	+	+	+	n/a	n/a	n/a	unknown	-
b-Methyl-D-Glucoside	+	+	+	n/a	n/a	n/a	beta-glucosidase	EC: 3.2.1.21
a-Methyl-D-Mannoside	+	+	+	n/a	n/a	n/a	PTS system, alpha-glucoside-specific IIB component	-
D-Allose	+	+	+	n/a	n/a	n/a	allose kinase	EC: 2.7.1.55

Table 4.4, continued.

C- and N-substrates tested	Growth in PM			Biofilm forming ability			Genes for enzymes	KEGG ID
	LM41	LM92	LM115	LM41	LM92	LM115		
Amygdalin	+	+	+	n/a	n/a	n/a	unknown	-
Arbutin	+	+	+	n/a	n/a	n/a	PTS system, arbutin-like II component	-
D-Arabitol	+	+	+	n/a	n/a	n/a	PTS system, arabitol-specific IIA component	-
Gentiobiose	+	+	+	n/a	n/a	n/a	beta-glucosidase	EC: 3.2.1.21
Salicin	+	+	+	S	S	S	PTS system, salicin-like II component	-
L-Sorbose	+	+	+	n/a	n/a	n/a	PTS system, sorbose-specific IIA component	EC: 2.7.1.206
D-Tagatose	+	+	+	n/a	n/a	n/a	tagatose kinase	EC: 2.7.2.101
Turanose	+	+	+	n/a	n/a	n/a	unknown	-
Xylitol	+	+	+	n/a	n/a	n/a	unknown	-
Adenosine	+	+	+	S	S	S	purine-nucleoside phosphorylase	EC: 2.4.2.1
Deoxyadenosi-ne	+	-	-	n/a	n/a	n/a	5'-nucleotidase	EC: 3.1.3.5
Cytidine	+	+	+	S	S	S	cytidine deaminase	EC: 3.5.4.5
Guanosine	+	+	+	n/a	n/a	n/a	purine-nucleoside phosphorylase	EC: 2.4.2.1
Inosine	+	+	+	n/a	n/a	n/a	purine-nucleoside phosphorylase	EC: 2.4.2.1
Uridine	+	+	+	S	S	S	uridine kinase	EC: 2.7.1.48
Thymidine	+	+	+	N	N	N	thymidine kinase	EC: 2.7.1.21
Xanthosine	+	+	+	n/a	n/a	n/a	purine-nucleoside phosphorylase	EC: 2.4.2.1
Dextrin	+	+	+	n/a	n/a	n/a	sucrose-isomaltase	EC: 3.2.1.10
a-Cyclodextrin	+	+	+	n/a	n/a	n/a	cyclomaltodextrinase	EC: 3.2.1.54
b-Cyclodextrin	+	+	+	n/a	n/a	n/a	cyclomaltodextrinase	EC: 3.2.1.54
g-Cyclodextrin	+	+	+	n/a	n/a	n/a	cyclomaltodextrinase	EC: 3.2.1.54
Glucurnamide	+	+	+	n/a	n/a	n/a	amidase	EC: 3.5.1.4
L-Alanine	+	-	-	n/a	n/a	n/a	alanine dehydrogenase	EC: 1.4.1.1

Table 4.4, continued.

C- and N-substrates tested	Growth in PM study			Biofilm forming ability			Genes for enzymes	KEGG ID
	LM41	LM92	LM115	LM41	LM92	LM115		
L-alanine	+	-	-	n/a	n/a	n/a	alanine racemase	EC: 5.1.1.1
L-Cysteine	-	-	+	W	M	W	cystathionine beta-lyase	EC: 4.4.1.8
Glycine	-	-	+	W	M	W	glycine dehydrogenase	EC: 1.4.4.2
D-Glutamic acid	-	-	-	n/a	n/a	n/a	glutamine synthetase, glnA	EC: 6.3.1.2
L-Glutamic acid	-	-	-	S	S	S	glutamine synthetase, glnA	EC: 6.3.1.2
Agmatine	+	+	+	n/a	n/a	n/a	agmatine deiminase	EC: 3.5.3.12
D-Glucosamine	+	+	+	n/a	n/a	n/a	glucosamine kinase	EC: 2.7.1.8
D-Galactosamine	+	+	+	n/a	n/a	n/a	PTS system, N-acetylgalactosamine-specific IIA component	-
D-Mannosamine	+	+	+	n/a	n/a	n/a	PTS system	-
Uric acid	+	+	+	n/a	n/a	n/a	Purine metabolism	-
Alloxan	+	+	+	n/a	n/a	n/a	unknown	-
D, L-a-Amino-Caprylic acid	+	+	+	n/a	n/a	n/a	unknown	-
Peptide sources	+	-	-	n/a	n/a	n/a	protease I	EC: 3.5.1.124
							sortase A	EC: 3.4.22.70
							sortase B	EC: 3.4.22.70
							dipeptidase	EC: 3.4.13.19
							aminopeptidase	EC: 3.4.11.-
							signal peptidase I	EC: 3.4.21.89

[a] '+' indicates presence of growth, '-' indicates absence of growth;

[b] S = Strong biofilm former, M = Moderate biofilm former; W = Weak biofilm former; N = Non-biofilm former; n/a = not applicable

4.3.1 Genes involved in Metabolic Activity

4.3.1.1 Genes Responsible for the Utilization of Carbon substrates

Based on the KEGG pathway analysis, both LM41 and LM115 strains carried the essential genes for central carbohydrate metabolism, including glycolysis, pentose phosphate pathway and oxidative phosphorylation (Figure 4.6). However, the genes for Entner-Doudoroff pathway and citrate cycle were partially present, except the metabolic genes for first carbon oxidation (oxaloacetate to 2-oxogluterate) were present (Figure 4.7). The enzyme aconitate hydratase (*citB*) was identified in the genomic analysis, which catalyses the isomerization of citrate to isocitrate in TCA cycle (Chong et al., 2017). The KEGG pathway analysis showed that the genes for enzymes, such as citrate synthase (*citZ*), aconitate hydratase (*citB*), isocitrate dehydrogenase (*citC*), malate dehydrogenase, fumarate hydratase, fumarate reductase, pyruvate dehydrogenase system and oxidases for reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate were present in the analysed strains genomes (Figure 4.7), although carbon sources, e.g. pyruvate, acetate, citrate, isocitrate, succinate, fumarate, malate or α-ketoglutarate did not support the growth of *L. monocytogenes* strains in the PM study. The α-ketoglutarate oxidation system, succinate dehydrogenase, isocitrate lyase and malate synthase were not detected in the genomic analysis, therefore indicating an incomplete TCA cycle in the tested strains which was concordant with previously published studies. All the necessary genes for carbohydrate uptake system, such as the PTS transport system, ABC transport system, inorganic and organic ion transport systems were identified in the studied strains that facilitates the uptake of carbon substrates, e.g. glucose, fructose, maltose, galactose, trehalose, allose, arbutin, cellobiose, rhamnose, gentiobiose and sorbitol (Table 4.4).

The genomic study also identified the metabolic genes for purine and pyrimidine metabolism, including adenine deaminase (*adeC*), thymidylate synthase (*thyA*), adenylate kinase (*adk*) and uridine kinase (*udk*) (Table 4.5). Furthermore, the fatty acid degradation pathway in *L. monocytogenes* strains was incomplete, only acetyl-CoA C-acetyltransferase [EC:2.3.1.9] and alcohol dehydrogenase [EC:1.1.1.1] were detected in the analysed strains (LM41 and LM115) genomes.

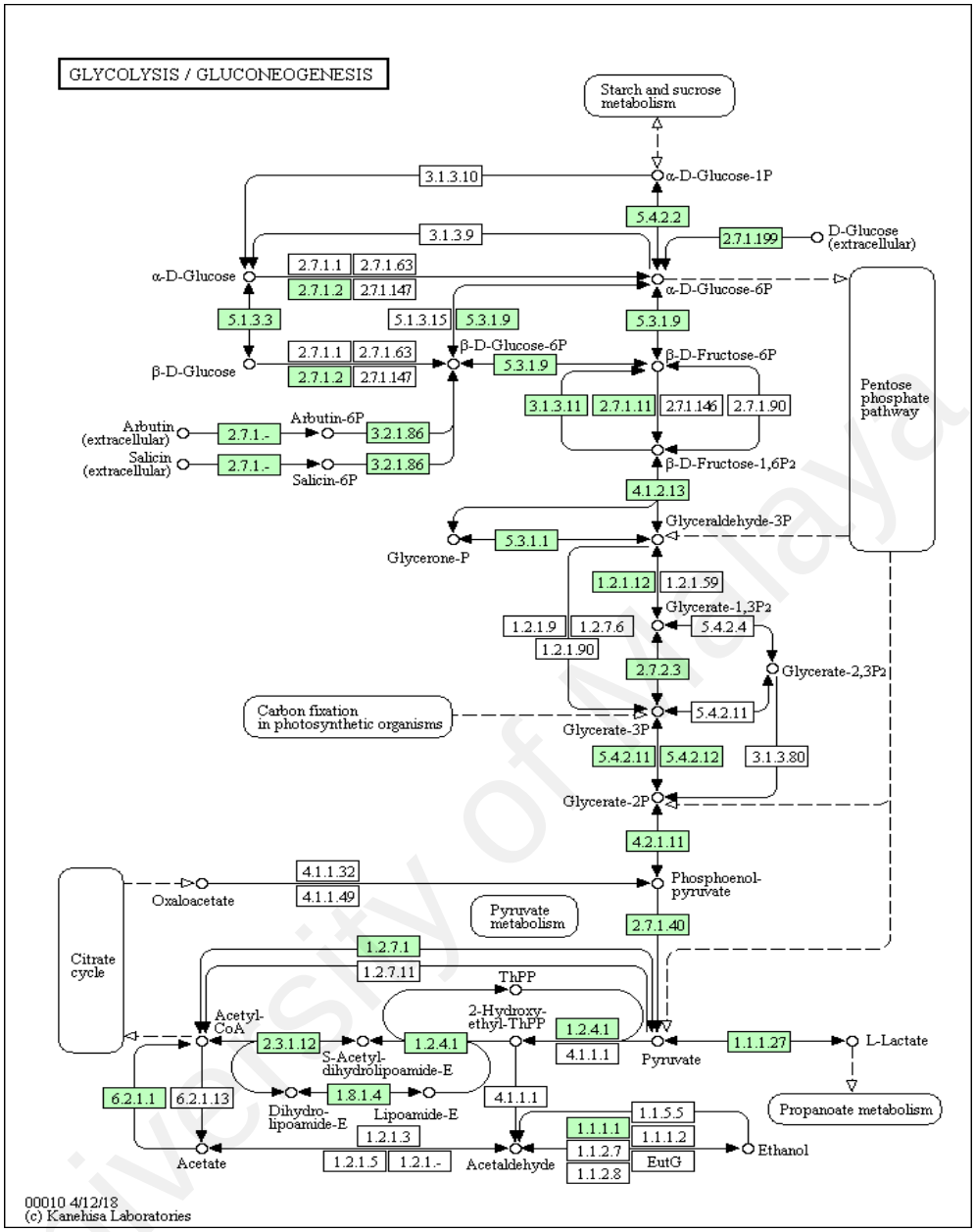

Figure 4.6: A schematic representation of the glycolysis pathway in *L. monocytogenes* developed by the BlastKOALA tool in KEGG Pathway Database. The green boxes indicate the presence of genes for enzymes in strain LM41 genome.

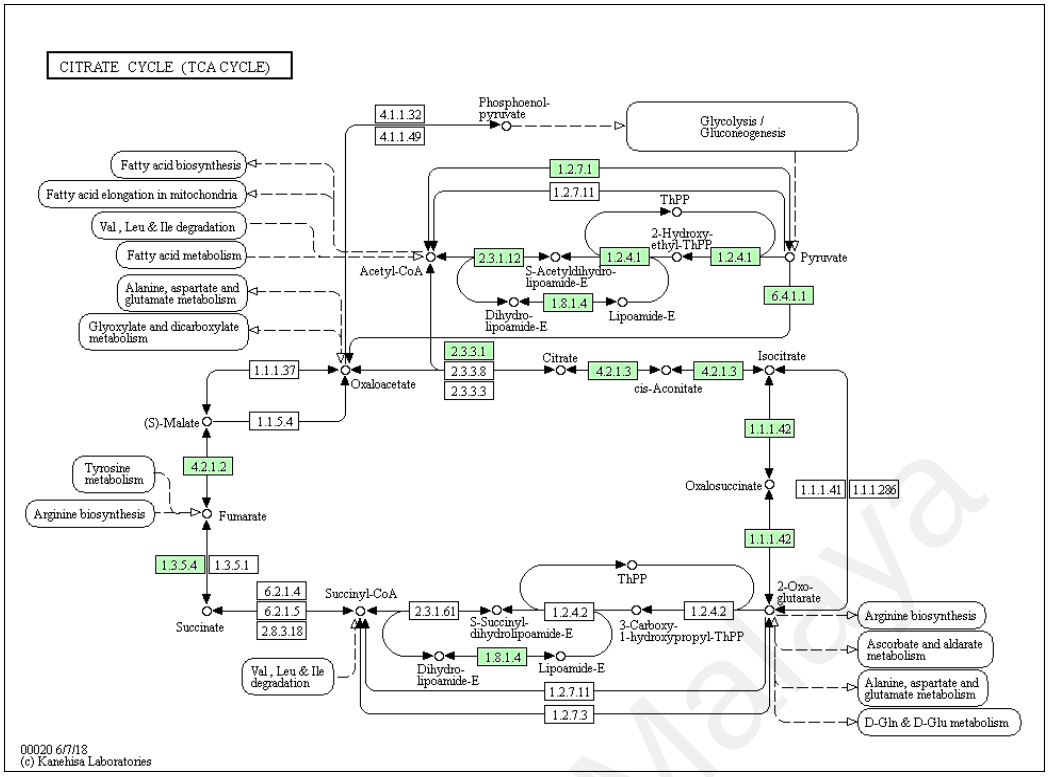

Figure 4.7: A schematic representation of the citrate cycle in *L. monocytogenes* developed by the BlastKOALA tool in KEGG Pathway Database. The green boxes indicate the presence of genes for enzymes in strain LM41 genome.

4.3.1.2 Genes Responsible for the Utilization of Nitrogen substrates

Based on the genomic analysis, the genes involved in the nitrogen metabolism were missing in the analysed strains. In glutamate metabolism, the metabolic genes for enzymes, such as glutamine synthetase [EC:6.3.1.2], glutamate dehydrogenase [EC:1.4.1.4] and glutamate synthase [EC:1.4.1.13 1.4.1.14] were identified that facilitates the conversion of ammonia to L-glutamine and L-glutamine to L-glutamate (Figure 4.8). In addition, enzymes such as carbonic anhydrase [EC:4.2.1.1] and carbamate kinase [EC:2.7.2.2] were identified in the nitrogen metabolism pathway, which catalyses the chemical reaction between ammonia and carbon dioxide. Several amino acid biosynthesis genes for glycine, lysine, cysteine, methionine, serine, threonine, alanine, arginine, proline, histidine and ornithine were detected in this study. The metabolic genes for branched-chain amino acid (valine, leucine and isoleucine) biosynthesis were identified, although the studied strains did not utilize these amino acids in the PM analysis. The KEGG

pathway mapper analysis also identified the genes for proteolytic (proteases, peptidases and sortases) enzymes for peptide, dipeptide, tripeptide and oligopeptide catabolism and transport system (Table 4.4). In addition, the genomic study identified the metabolic genes for vitamins (thiamine, riboflavin and biotin) and cofactor biosynthesis and transport system.

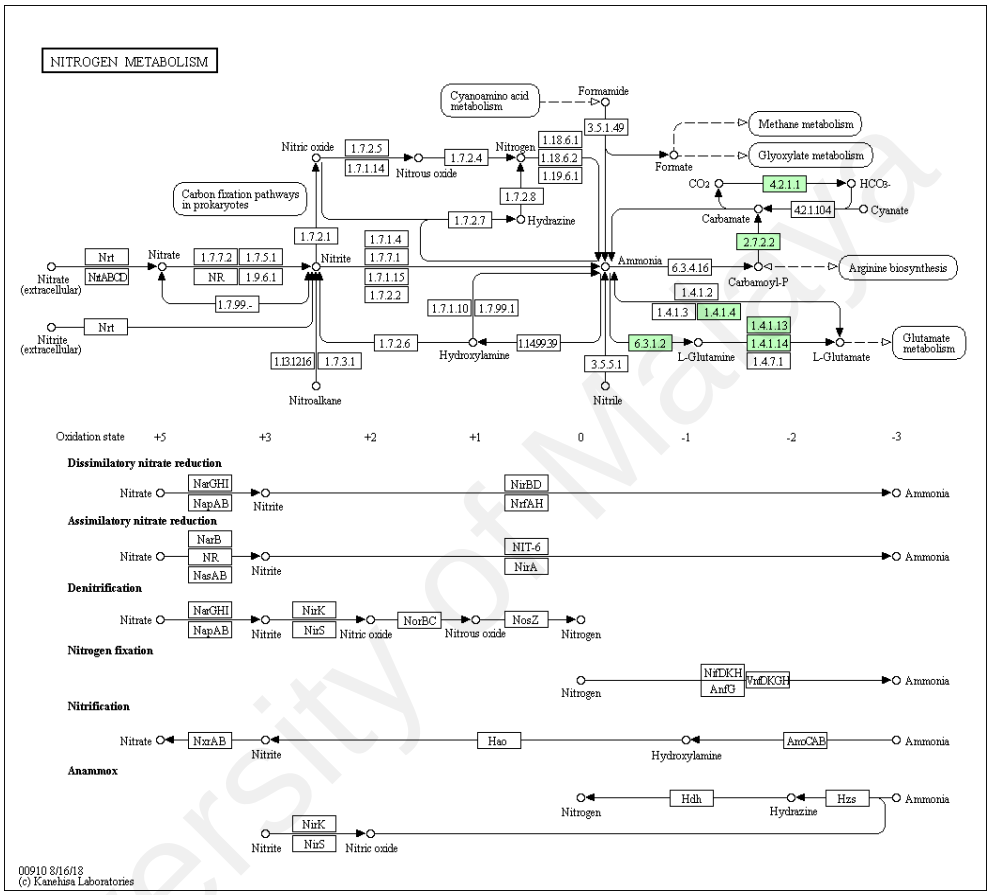

Figure 4.8: A schematic representation of the nitrogen metabolism pathway in *L. monocytogenes* developed by the BlastKOALA tool in KEGG Pathway Database. The green boxes indicate the presence of genes for enzymes in strain LM115 genome.

4.3.2 Genes involved in Biofilm Formation

Based on the BLASTn analysis, a total of 97 biofilm-associated genes were identified in the genomes of *L. monocytogenes* strains, including the genes for virulence (n = 11), stress response (n = 15), flagellar-mediated motility (n = 12), quorum sensing (n = 4), metabolism (n = 29) and gene regulation (n = 26) that are important in the biofilm forming process. The identified genes for surface attachment and cell aggregation include *flaA, motA, degU, agrB, agrD* and *bapL* and the genes required for biofilm maturation include *relA, hpt, prfA, flaA* and *motA*. The distinctive genes encoding the surface proteins InlA and InlB, flagella motor protein (*motA*) and flagella biosynthesis (*flaA, fliQ, fliI, fliD, fliE, flgE* and *fliG*) were identified as well (Figure 4.9). The genomic analysis showed that the regulatory genes associated with cell signaling system, chemotaxis (*cheA, cheR* and *cheY*) and quorum-sensing (*agrBDCA*) were present in the analysed strains genome (Table 4.5). In addition, the genes encoding Lac I family transcriptional regulator (*lmo0734*), cell division suppressor protein (*lmo1303, yneA*), transcriptional regulator (*lmo1262*), peptidoglycan-linked protein (*lmo1666*), Fnr/Crp family transcriptional regulator (*lmo1251*), transcriptional anti-terminator gene (*bglG*), ferritin like protein (*lmo0943*), hexose phosphate transport protein (*uhpT*), RNA polymerase sigma factor (*sigB*), S-ribosylhomocysteine lyase (*lmo1288, luxS*), two-component sensor histidine kinase (*lmo1378, lisK*), competence protein (*lmo1550, comC*), cell wall binding protein (*lmo2504*), secretory proteins (*secA* and *secY*) were identified. The two-component regulatory systems, including cell wall metabolism (VicK-VicR), chemotaxis (CheA-CheY), cell wall stress response (LiaS-LiaR), potassium transport (KdpD-KdpE), phosphate starvation response (PhoR-PhoB) and exoprotein synthesis (AgrC-AgrA) genes were also identified in the genomic analysis that play major roles in the molecular adaptation of *L. monocytogenes* in adverse environment. A detailed list of genes used in the BLAST analysis are mentioned in Appendix D.

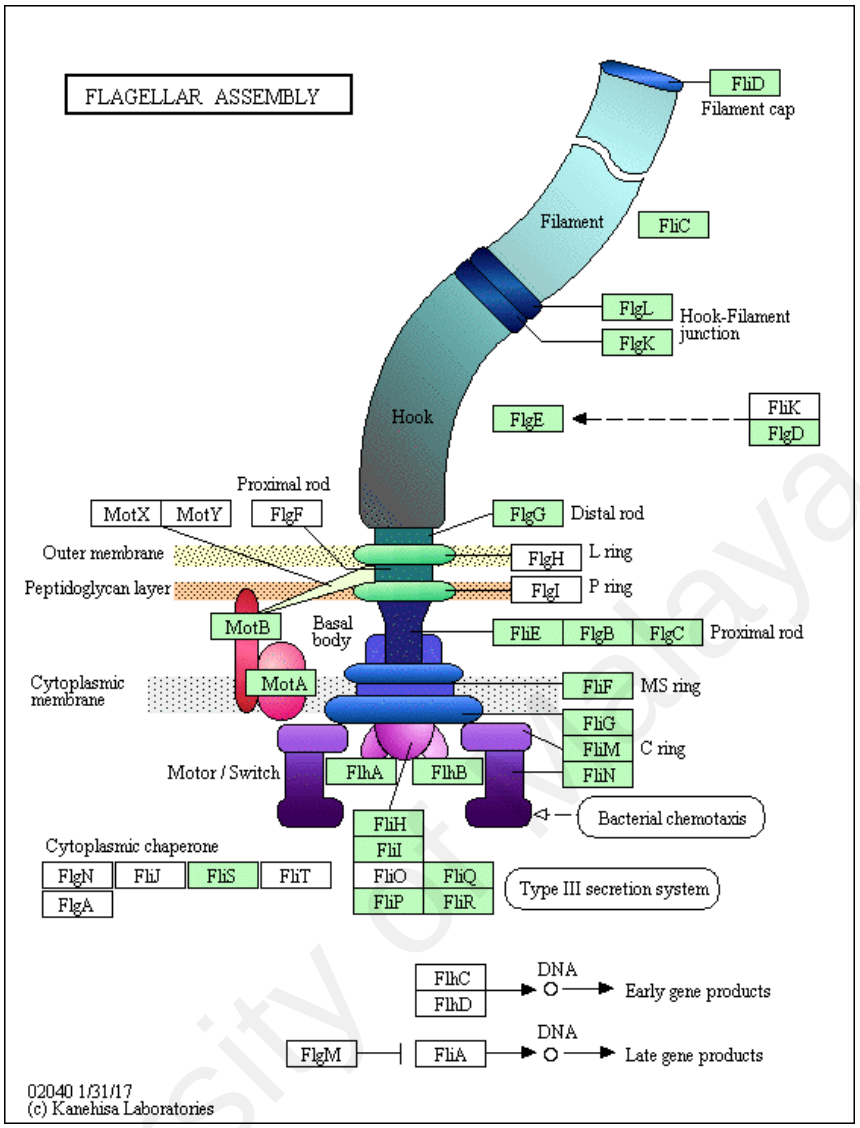

Figure 4.9: A schematic representation of flagella assembly proteins in the reference pathway of *L. monocytogenes* constructed by the BlastKOALA tool in KEGG server. The green color indicates the presence of flagella biosynthesis protein genes in strain LM115 genome.

Table 4.5: The results of BLAST analysis of metabolic and biofilm associated genes in the *L. monocytogenes* strains.

Functional category	Gene locus	Putative function	Strain	
			LM41	LM115
Amino acid transport and metabolism	*lmo1299 (glnA)*	glutamine synthetase	+	+
	lmo1350	glycine dehydrogenase subunit 2	+	+
	lmo2188	oligoendopeptidase	+	+
	lmo1620	dipeptidase	+	+
	lmo1006	aminotransferase	+	+
	lmo0849	amidase	+	+
	lmo1375 (pepT)	tripeptide aminopeptidase	+	+
Carbohydrate transport and metabolism	*lmo1376*	6-phosphogluconate dehydrogenase	+	+
	lmo1305	transketolase	+	+
	lmo1571 (pfkA)	6-phosphofructokinase	+	+
	lmo1566 (citC)	isocitrate dehydrogenase	+	+
	lmo2674	ribose-5-phosphate isomerase B	+	+
	lmo0813	fructokinase	+	+
	lmo2556	fructose-1,6-bisphosphate aldolase	+	+
	lmo1570	pyruvate kinase	+	+
	lmo1567	citrate synthase	+	+
	lmo2477 (galE)	UDP-glucose 4-epimerase	+	+
Nucleotide transport and metabolism	*lmo1874 (thyA)*	thymidylate synthase	+	+
	lmo2758 (guaB)	inosine-5'-monophosphate dehydrogenase	+	+
	lmo1313 (pyrH)	uridylate kinase	+	+
	lmo2611 (adk)	adenylate kinase	+	+
	lmo0055 (purA)	adenylosuccinate synthetase	+	+
	lmo0199	ribose-phosphate pyrophosphokinase	+	+
Flagella (biosynthesis, structure and activity)	*lmo0676 (fliP)*	flagellar biosynthesis protein FliP	+	+
	lmo0690 (flaA)	flagellin	+	+
	lmo0697 (flgE)	flagellar hook protein FlgE	+	+
	lmo0714 (fliG)	flagellar motor switch protein FliG	+	+
	lmo0707 (fliD)	flagellar capping protein FliD	+	+
Motility (chemotaxis activity)	*lmo0685 (motA)*	flagellar motor protein MotA	+	+
	lmo0690 (flaA)	flagellin	+	+
	lmo0716 (fliI)	flagellum-specific ATP synthase	+	+

Table 4.5, continued.

Functional category	Gene locus	Putative function	Strain	
			LM41	LM115
Motility (chemotaxis activity)	*lmo0677 (fliQ)*	flagellar biosynthesis protein FliQ	+	+
	lmo0697 (flgE)	flagellar hook protein FlgE	+	+
	lmo0692 (cheA)	two-component sensor histidine kinase	+	+
	lmo0691 (cheY)	Chemotaxis response regulator CheY	+	+
	lmo0683	Chemotaxis protein CheR	+	+
Other biofilm related genes	*lmo0435 (bapL)*	cell attachment	+	+
	lmo1523 (relA)	cell aggregation	+	+
	lmo0048 (agrB)	cell accumulation	+	+
	lmo0049 (agrD)	cell accumulation	+	+
	lmo0050 (agrC)	initial attachment	+	+
	lmo0051 (agrA)	initial attachment	+	+
	lmo2558 (ami)	initial attachment	+	+
	lmo2515 (degU)	adherence, flagella synthesis and biofilm formation	+	+
	lmo2510 (secA)	influence biofilm formation	+	+
	lmo0200 (prfA)	regulates flagella synthesis and biofilm formation	+	+
	lmo1288 (luxS)	dispersion of cells from mature biofilm	+	+

'+' indicates the presence of gene

CHAPTER 5: DISCUSSION

5.1 Catabolic Activity of *L. monocytogenes* strains

The aim of this study was to determine the metabolic capability of three foodborne *L. monocytogenes* in various carbon and nitrogen substrates. Based on the PM analysis, the carbon catabolism activity of the *L. monocytogenes* strains was extremely low, which was only 15 - 20% out of 190 carbon substrates tested (Table 4.1). Other studies showed that pathogenic organisms, such as *Acinetobacter baumannii*, *Bacillus subtilis* and *Escherichia coli* were able to catabolize a higher number of carbohydrate and amino acid sources (Bren et al., 2016; Farrugia et al., 2013). Therefore the studied *L. monocytogenes* strains do not require multiple nutrient sources in the medium for growth. The foodborne strains mainly utilized carbohydrates, polymers and nucleotides and the choice of carbon sources were rather similar among the three strains (Figure 4.2). The nitrogen substrate utilization was even lower than the carbon substrate utilization. The strains catabolized only 16% out of 380 nitrogen substrates tested, where 13% was utilized by LM41 strain itself. The studied strains catabolized several nitrogen substrates individually that were amino acids, amines, peptides and nucleosides (Figure 4.3). For instance, 7 (2%) out of 380 nitrogen sources were utilized by all strains, while strain LM41 utilized 10% of the dipeptide nitrogen sources, the other two strains (LM92 and LM115) were unable to utilize the peptide sources in plates PM6, PM7 and PM8. Nevertheless, LM115 strain catabolized 5 dipeptides and LM92 strain catabolized only 1 dipeptide out of 10 peptide nitrogen sources in plate PM3B (Figure 4.3). The genomic analysis of two *L. monocytogenes* strains showed that the strains carry a number of carbohydrate, amino acid, fatty acid and nucleotide metabolizing genes and therefore, these foodborne strains should be able to metabolize a wide range of carbohydrate and amino acid compounds (Lim et al., 2016). The limited carbon and nitrogen catabolic activity of the studied strains suggests the presence of incomplete catabolic pathways involved in their metabolism.

However, based on the KEGG pathway analysis the strains contained several genes for amino acid and carbohydrate uptake and degradation in their genomes, yet they did not utilize those substrates in the PM analysis. This could be due to the low concentration of carbon or nitrogen sources supplied in the PM microplates that is not sufficient for the optimum growth of *L. monocytogenes* strains.

In this study, *L. monocytogenes* strains showed limited growth activity in D-glucose, while an increased growth rate was observed in L-glutamic acid, fructose, maltose, galactose and plant-derived carbon sources, e.g. arbutin, D-xylose, salicin and sorbitol in the PM analysis. Conceivably, glucose is not the sole carbon source for their optimum growth. *L. monocytogenes* has a complex phosphotransferase system mediated glucose transport system where it catabolizes fructose, mannose, cellobiose as sole carbon sources when grown in a defined minimal liquid medium and was able to utilize branched-chain amino acids (Tsai & Hodgson, 2003). In this study, none of the strain was able to grow on ammonia, nitrates, nitrites, urea and uric acid since they lack the genes for nitrate and nitrite reductases (Haber et al., 2017) and as a result they only catabolized organic nitrogen sources (Table 4.1). Additionally, glycine, xylose, riboflavin, thiamine, biotin and tween 40 supported the bacterial growth in validation experiment, while none of them represent growth in PM analysis (Table 4.2). This could be due to the low concentration of substrates in the PM microplates. Furthermore, vitamins such as riboflavin, biotin and thiamine may not be compulsory for growth since they did not improve the growth, hence *L. monocytogenes* growth seemed to be unaffected by the presence of these vitamins in minimal medium.

The optimum growth of *L. monocytogenes* strains was observed in carbohydrate sources, such as fructose, galactose, rhamnose, xylose, trehalose and maltose and amino acid sources, including L-glutamic acid, glycine, salicin and L-cysteine (not methionine),

regardless of their distinct serogroups (Table 3.1). Previous study by Premaratne et al. (1991) showed that glucose and glutamine are the main sources of carbon and nitrogen for *L. monocytogenes* Scott A strain growth. However, glucose can be replaced by several other sugars and glutamine can be replaced by other essential amino acids, such as leucine, isoleucine, valine, arginine, methionine, tryptophan, histidine and cysteine. Moreover, growth was stimulated by ferric citrate, thiamine, biotin, riboflavin and lipoic acid (Premaratne et al., 1991). On the contrary, Tsai & Hodgson (2003) demonstrated that two essential amino acids, e.g. methionine and cysteine as primary nitrogen sources and glucose, fructose, mannose and glycerol as primary carbon sources for *L. monocytogenes* 10403S strain growth. The findings of these two studies stipulate that the growth and behaviour of *L. monocytogenes* differ between serotypes, since strains Scott A and 10403S belong to serotypes 4b and 1/2a, respectively. Therefore, the carbon and nitrogen catabolism of *L. monocytogenes* cannot be generalized by strain variations.

In the PM validation test, the selected *L. monocytogenes* strains efficiently utilized glycerol as the sole carbon source in liquid minimal medium and PM assay. Glycerol is typically phosphorylated to glycerol phosphate by an enzyme glycerol kinase (*glpK*) and glycerol uptake is facilitated by glycerol-3-phosphate dehydrogenase (*glpD*) (Joseph et al., 2008). This non-PTS substrate also have the ability to enhance the expression of the central virulence gene regulator PrfA, when it is used as the sole carbon source in minimal medium (Mertins et al., 2007). In human body, glycerol is rapidly absorbed by the small intestine and distributed in the extracellular spaces of stomach lining (Chelvam et al., 2015). Glycerol is also abundantly found in liver and kidneys, therefore it may promote the colonization and proliferation of *L. monocytogenes* in hepatic cells and kidneys. Furthermore, purine and pyrimidine sources were rapidly catabolized by many bacteria as sole carbon and energy sources (Schuch et al., 1999; Tozzi et al., 2006). The studied *L. monocytogenes* strains were able to utilize adenosine, thymidine, cytidine, uridine and 2-

deoxyadenosine in the PM analysis. Human blood serum is rich in amino acids and nucleosides, thereby indicating that active metabolism of nucleosides supports the rapid proliferation of *L. monocytogenes* cells during invasive infection in blood and lymph nodes which might lead to severe cases of listeriosis.

The KEGG pathway analysis showed that the studied strains contained a non-cyclic citric acid pathway which is divided into an oxidative chain (citrate synthase, aconitate hydratase and isocitrate dehydrogenase) and a reductive chain (malate dehydrogenase, fumarate hydratase and fumarate reductase) primarily involved in biosynthesis, rather than energy production, and the result was consistent with previous study (Trivett & Meyer, 1971). On the contrary, none of the tested strains were able to utilize alcohol, carboxylic acids, esters and fatty acids tested in the PM study. It could be due to the absence of genes for enzymes for fatty acid and lipid metabolism, as the genes for glyoxylate shunts are missing in *L. monocytogenes* genome (Joseph & Goebel, 2007). The analysed strains were able to catabolize Tween 40 when it was added to minimal medium, although genomic analysis showed that none of the strain contained the enzyme lipase involved in the breakdown of detergents (Tween 40 or Tween 80) into their respective fatty acids. Additional transcriptomic and proteomic analyses are required to validate such findings. The ability to utilize fatty acids indicates that the *L. monocytogenes* strains can survive and colonize in gastrointestinal tract where fatty acid is present in high concentrations and therefore confirming their pathogenic nature (Scaria et al., 2015).

5.2 Biofilm forming ability among the *L. monocytogenes* strains

5.2.1 Effects of nutrient media on biofilm formation

In this study, all three tested strains were able to form strong biofilm in LB broth and minimal media without any nutrients. The accessibility of nutrient sources greatly influences the biofilm formation by bacterial cells. A number of studies found that *L. monocytogenes* formed increased biofilm in nutrient-limited medium compared to nutrient-rich medium (Borucki et al., 2003; Djordjevic, 2002; Kadam et al., 2013; Nowak et al., 2015). The inadequate nutrient sources in minimal medium causes stress condition in *L. monocytogenes* cells which results in persistent biofilm structures. It has been reported in many studies that a multitude of factors influence the biofilm formation in *L. monocytogenes*, including temperature, incubation time, strain background, medium and adhesion surface (Borucki et al., 2003; Colagiorgi et al., 2016; Doijad et al., 2015; Harvey et al., 2007; Reis-Teixeira et al., 2017). Environmental stress also induces biofilm formation as a result of extreme heat, low pH, high oxygen levels, osmotic pressure and limited nutrient access in the environment (Nowak et al., 2015). Many studies showed that *L. monocytogenes* produced better biofilm in nutrient-poor medium; while they have increased growth rate in nutrient-rich medium (Doijad et al., 2015; Tsai & Hodgson, 2003; Zhou et al., 2012). Previous findings also reported that listerial growth in nutrient rich media, such as Brain Heart Infusion broth or Tryptone Soy broth do not shift the cells from planktonic to biofilm state, while growth in chemically defined medium, such as modified Welshimer's broth stimulates the adherence to different abiotic surfaces and biofilm formation (Pilchová et al., 2014). Nevertheless, strong biofilm formation in both nutrient-rich and nutrient-limited media suggests that the studied strains exploit an unusual molecular mechanism to produce biofilms in a nutrient-poor condition.

5.2.2 Effects of various substrates on biofilm formation

In this study, polysorbates (Tween 40 and Tween 80) induced strong to moderate biofilm formation in the tested strains in nutrient-limited medium. Polysorbates are non-ionic surfactants often emulsified in the sanitizers to clean the surfaces and equipment in food production facility (Fagerlund et al., 2017). *Listeria monocytogenes* typically enters the food processing facility through the environment, contaminated raw materials and food handlers (Muhterem-Uyar et al., 2015). Organic residues in meat processing facilities and contaminated slicing materials are the major sources of *L. monocytogenes* contamination, since they serve as adhesion surfaces for bacterial accumulation and biofilm formation (Simões et al., 2010; Srey et al., 2013). Biofilms are reported to be least susceptible to antimicrobial treatments than their planktonic counterparts (Omar et al., 2017; Thomas et al., 2012). Previous studies showed that foodborne *L. monocytogenes* isolates were resistant to cleaning and sanitization and survive after the disinfection process in various niches that are difficult to clean (Chaitiemwong et al., 2010; Gandhi & Chikindas, 2007). Recent studies by Møretrø et al. (2017) and Dutta et al. (2013) reported that *L. monocytogenes* showed increased resistance towards quaternary ammonium compounds (QAC), such as Benzalkonium chloride due to the presence of QAC resistance genes, *qacH* and *bcrABC* in their genome. Furthermore, *L. monocytogenes* can survive as biofilms in the surface water residues containing residual QAC after disinfection, thereby conferring growth advantage to bacterial cells (Møretrø et al., 2017). Therefore, the ability to form biofilm using polysorbates stipulates that the studied strains possess the QAC resistance genes in their genomes and presence of such genes were confirmed in the genomic analysis.

Several monosaccharides and disaccharides, including D-glucose, D-fructose, maltose, D-galactose, D-xylose, D-trehalose and L-rhamnose were also incorporated in

the biofilm assay. All induced strong biofilm formation, except D-glucose in which the studied *L. monocytogenes* strains did not grow well and produced moderate to weak biofilm in minimal medium. This could be a consequence of carbon catabolite repression (CCR). The carbohydrate content of the biofilm matrix of *L. monocytogenes* has been extensively studied by other researchers (Colagiorgi et al., 2016). It has been speculated in many studies that carbohydrate metabolism might play an important role during the biofilm formation by Gram-positive bacteria. Shi & Zhu (2009) reported that two enzymes crucial in carbohydrate metabolism, namely pyruvate dehydrogenase (PdhD) and 6-phosphofructokinase (PfkA) were significantly elevated in biofilm cells, therefore biofilm development influences the central carbon metabolism. Both of these enzymes were identified in the studied strains. Furthermore, biofilm formation was inhibited in few species of *Enterobacteriaceae* in the presence of glucose due to catabolite repression. The CCR is a physiological phenomenon observed in many Gram-positive bacteria through which they regulate the uptake of a preferred carbon source when more than one carbon sources are available in a medium (Brückner et al., 2002; Pillai et al., 2004).

In this study, L-amino acids including L-cysteine, L-lysine and L-threonine produced weak biofilm in minimal medium. Only LM92 strain formed moderate biofilm in L-cysteine and L- lysine. L-amino acids produced weaker biofilm, whereas L-glutamic acid produced stronger biofilm in the tested strains. Previous studies showed that cysteine and threonine are two essential amino acids when *L. monocytogenes* are grown in a defined minimal medium. In addition, L-glutamic acid plays vital role in amino acid biosynthesis. Since L-glutamine is a primary nitrogen source in listerial growth, the synthesis of glutamine from glutamate is an important part of amino acid metabolism in *L. monocytogenes* (Kaspar et al., 2014).

The addition of purine and pyrimidine ribonucleosides (adenosine, cytidine and uridine) in minimal medium induced strong biofilm formation in the tested strains. Previous studies showed that *E. coli* and *Salmonella enterica* utilized ribonucleosides as carbon and energy sources and during the colonization in gastrointestinal tract (Andino & Hanning, 2015; Polzin et al., 2013). However, thymidine did not induce biofilm formation in the studied strains (Table 4.3), although an increased growth rate was observed when it was added to minimal medium (Table 4.2). Therefore, thymidine may not be the preferred nutrient source in biofilm stage, since it showed inhibitory effects during biofilm formation. Further experimental analysis should be taken into consideration regarding the use of thymidine as an anti-biofilm approach to reduce biofilm formation in food processing industries.

The presence of some vitamins, e.g. riboflavin, biotin and thiamine also facilitated growth and induced strong biofilm formation in the studied strains. Jarvis et al. (2016) reported that both riboflavin and biotin were essential for *L. monocytogenes* optimum growth, while thiamine showed stimulatory effect but not a requisite for growth. Tsai & Hodgson (2003) also observed an increased growth rate of *L. monocytogenes* in a defined liquid medium, containing riboflavin, biotin, thiamine and lipoic acid. On the contrary, both glycerol and glycine induced weak biofilm formation in this study. Although glycine is the simplest and essential amino acid found in the environment, it was not a preferred nitrogen source by the tested strains during biofilm formation. Moreover, glycerol is commonly used as a food preservative agent in food processing industries. The ability to produce biofilms in glycerol thus suggests that the foodborne strains can cross the food preservation barrier and contaminate finished food products. This study also showed that carbon source, such as salicin can induce strong biofilm formation in minimal medium by all strains. Salicin is a plant-derived beta-glucoside which is usually found in the barks of

trees and plants. The ability to utilize salicin hence indicates that the studied strains can cause infection in plants and produce biofilms.

5.2.3 Effects of surface material on biofilm formation

In this study, polystyrene microtiter plates were chosen as an abiotic surface to allow biofilm formation by the foodborne strains. Polystyrene is one of the most widely used plastics in commercial food packaging and processing equipment used in medical and industrial settings. *L. monocytogenes* strains exhibited strong adhesion to polystyrene plates in this study and thereby corroborating the similar findings observed in previous studies (Djordjevic et al., 2002; Stepanović et al., 2004). Several studies also reported that persistent biofilm structures are often formed on steel and glass surfaces and the corners in food processing facilities that are difficult to clean (Borucki et al., 2003; Doijad et al., 2015).

5.3 Genetic Factors influencing the Biofilm Formation in the studied *L. monocytogenes* strains

In this study, the *agr* gene cluster (*agrBDCA*) was identified in the genome of foodborne *L. monocytogenes* strains. In natural environment, bacterial cells use cell signaling molecules for cell-to-cell communication in population by a phenomenon known as quorum sensing (QS) system. Microbial cells secret autoinducing molecules to recruit planktonic cells from the surrounding environment prior to surface attachment. Once initial attachment is achieved, bacterial cells produce extracellular polymeric substances and gradually mature into persistent biofilm structure in the environmental surface (Colagiorgi et al., 2016; Srey et al., 2013). While the adherent cell population in a biofilm structure increases in size, the QS molecules (small peptides) induces a number changes in the expression of specific genes in order to facilitate mature biofilm formation or dissociation of planktonic cells in the environment (Omar et al., 2017). In Gram-

positive bacteria, QS system is mediated by autoinducing peptides (AIPs) as signaling molecules. The AIPs are synthesized by the *agr* locus in *Staphylococci* and *L. monocytogenes* (Omar et al., 2017). The *agr* locus is also responsible for virulence factors including biofilm formation in *L. monocytogenes* (Rieu et al., 2007). Furthermore, the *luxS* QS system is present in many Gram-positive bacteria which is primarily associated with bacterial metabolism and gene regulation (Vendeville et al., 2005). Kong et al. (2006) reported that the *agr* operon enhances biofilm dissociation by up-regulating the expression of detergent like peptides, while the *luxS* gene decreases cell-to-cell adhesion by down-regulating the expression of exopolysaccharide involved in biofilm attachment. As a result, biofilm dispersion sheds free planktonic cells in the environment and causes biofilm-associated infections in humans and animals (Kong et al., 2006). Previous studies also showed that both *agr* and *luxS* mutants form thicker biofilm than the wild-type strains (Vuong et al., 2000; Xu et al., 2006). The *agr* locus consists of four genes, namely *agrB, agrD, agrC* and *agrA.* The *agrB* and *agrD* genes are involved in the production of autoinducing molecules called autoinducers, whereas *agrA* and *agrC* regulate two-component signal transduction system (acts as sensor kinases). Initial attachment of planktonic cells to polystyrene surface is achieved by the *agr* system due to the up regulation of attachment molecules (autolysin family) and down regulation of detachment molecules. Therefore, *agr* system plays central role in microbial biofilm formation (Kong et al., 2006).

Comparative genomic analysis also showed that both LM41 and LM115 strains carry the flagellar-associated protein gene (*lmo0690*) in their genome and showed increased adherence to polystyrene surfaces. The gene expressions in biofilm cells are different than the planktonic cells. There are several molecular and genetic factors that influence the bacterial biofilm formation. Studies found that flagella or flagellin protein (FlaA) play important roles in surface attachment and biofilm formation in *L. monocytogenes* (Lemon et al., 2007). Flagellum is composed of flagellin monomer encoded by *flaA* gene

(Colagiorgi et al., 2016). Previous studies showed that *L. monocytogenes* has a temperature-dependent motility, due to the expression of flagellin between 20 to 25ºC, while reduced expression at 37ºC (Bierne & Cossart, 2007; Peel et al., 1988). Response regulator DegU and transcriptional activator PrfA regulates flagella biosynthesis at 25ºC (Knudsen et al., 2004; Mauder et al., 2008; Zhou et al., 2011). Lemon et al. (2010) reported that PrfA plays major role in biofilm development as the PrfA mutants formed defective biofilms compared to the wild-type strains and the regulation by PrfA was temperature-dependent. Since PrfA regulates the expression of virulence genes and genes related to biofilm formation, Lemon et al. (2010) suggested that it may act as a global regulator of *L. monocytogenes* lifestyle. Interestingly, the expression of flagellin protein was down-regulated in mature biofilm cells, thereby indicating that FlaA protein is essential in initial attachment and its activity is suppressed during biofilm development (Shi & Zhu, 2009). Furthermore, Lemon et al. (2007) previously reported that flagellum-mediated motility showed significant impact on the initial attachment and biofilm formation by *L. monocytogenes* and it acts as adhesins on abiotic surfaces which is motility dependent under optimized conditions (Lemon et al., 2007).

The genome data also showed that the presence of *relA* and *hpt* genes in the analysed strains contributed to strong biofilm forming phenotype in minimal medium without additional nutrient sources. Taylor et al. (2002) demonstrated that *relA* and *hpt* mutants were unable to form mature biofilm after surface adhesion. The RelA and Hpt proteins are associated with the biosynthesis of signal molecule (p)ppGpp (guanosine pentaphosphate), which is involved in the physiological adaptation of *Listeria* cells to stringent conditions. The (p)ppGpp plays a significant role in nutrient starvation and stringent response which is essential in bacterial growth, survival and biofilm development (Shi & Zhu, 2009; Taylor et al., 2002). Furthermore, Alonso et al. (2014) reported the identification of 38 genetic loci that are important in biofilm forming process.

Among them, D-alanylation pathway genes *dltABCD* and phosphate-sensing two component system *phoPR* were subjected to deletion analysis. The deletion of these two loci resulted in lesser biofilm formation by the mutant strains compared to their wild type strains. Therefore, both *dltABCD* and *phoPR* loci play crucial roles in biofilm forming process of *L. monocytogenes*. In this study, the genetic loci *dltABCD* and *phoPR* were identified in the analysed strains and showed 100% sequence similarity with the reference strain EGD-e in BLASTn analysis.

The genomic analysis identified two surface binding protein genes (*inlA* and *bapL*) in the tested strains that are important in surface attachment and biofilm formation. Many studies reported that *L. monocytogenes* has more than 120 surface proteins which facilitate the bacterial cells to invade host cell or survive in adverse conditions (Colagiorgi et al., 2016; Desvaux et al., 2010). Internalin A (InlA) is one of the major surface binding protein involved in host cell invasion and adherence to abiotic surfaces (Franciosa et al., 2009; Gilmartin et al., 2016). Franciosa et al. (2009) found that a truncated form of InlA was able to significantly increase biofilm formation in *L. monocytogenes* strains. In this study, the presence of InlA protein gene (*lmo0433*) was confirmed by BLASTn analysis. Additionally, biofilm-associated surface protein (Bap) plays important role in adhesion and biofilm development in many Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, Enterococcus faecalis* and *Salmonella enterica* serovar Enteritidis (Cucarella et al., 2001; Latasa et al., 2005; Toledo-Arana et al., 2001). In *L. monocytogenes*, a Bap-like surface protein designated as *bapL* (*lmo0435*), which is homologous to the Bap-protein found in *S. aureus* has shown significance in surface adherence. According to the findings by Jordan et al. (2008), both *bapL*-positive strains and *bapL*-negative strains showed attachment to abiotic surfaces, thereby indicating that *bapL* is not crucial for biofilm formation in all *L. monocytogenes* strains. A comparative study of biofilm and planktonic exoproteomes by Lourenço et al. (2013) showed that

Phospholipase A (*plcA*), flagellin (*flaA*), actin assembly inducing protein (*actA*), a putative penicillin-binding protein (*pbpA*) and a cell wall binding protein (*lmo2504*) were present in higher abundance in the biofilm state. All the above-mentioned genes were also identified in the genomic analysis.

The secretory protein genes (*secA* and *secY*) were identified in the analysed strains genomes. The bacterial secretion system or secretory proteins influence the biofilm development in many Gram-positive bacteria, including *L. monocytogenes* (Renier et al., 2011). The inhibition of SecA2 protein export pathway reduced cell adhesion but showed increased cellular accumulation and biofilm formation (Renier et al., 2014). Furthermore, Salazar et al. (2013) reported that Crp/Fnr family transcriptional factor (*lmo0753*) had significant impact on *L. monocytogenes* attachment and biofilm formation in fresh produce and showed structural similarity with the central virulence gene regulator PrfA. The gene *lmo0753* was also identified in the present study.

5.4 Limitations of the Study

PM approach effectively screened the substrate utilization and characterized the metabolic profile of the bacterial strains. However, the interpretation of PM data can be challenging since methodological limitations are multifaceted. For instance, PM4A plate contains inorganic and organic sulphur and phosphorus compounds. Since nitrate and sulphate reductases are absent in *L. monocytogenes*, the studied strains were unable to reduce the sulphur sources. In addition, there were some false positive results in plate PM5 which contains nutrient supplements. This could be due to the reduction of tetrazolium violet dye during the measurement of absorbance. Therefore, PM data for plates PM4 and PM5 were considered invalid and excluded from the analysis. The genomic study identified the genes related to catabolism and biofilm formation of the *L. monocytogenes* strains. However, some catabolic genes were missing in the genomic

analysis, since the analysed nucleotide and amino acid sequences belonged to a draft genome. Moreover, the presence of the studied genes could not be confirmed due to inadequate knowledge regarding the expression of these genes in biofilm state. Therefore, a transcriptomic study of intermediate and mature stages of biofilm development should be performed to determine the gene expression profiles of the studied strains. In future work, a multi-disciplinary approach towards transcriptomics, proteomics and metabolomics analyses are required to identify and characterize the exact molecular mechanism and regulatory pathways involved in *L. monocytogenes* biofilm formation.

CHAPTER 6: CONCLUSION

In conclusion, based on the phenotype microarray analysis the catabolic activity of the studied strains was considerably limited. The strain LM41 was able to utilize a wide range of carbohydrates and amino acid sources, while strains LM92 and LM115 were able to catabolize fewer nitrogen sources, including amino acids, amides and peptides. Therefore, the studied *L. monocytogenes* do not require additional nutrient sources for optimal growth.

Secondly, this study determined the biofilm forming ability of the foodborne *L. monocytogenes* strains. All three strains were strong biofilm producers even in the absence of nutrients. The biofilm forming ability thus facilitates the survival in adverse environmental conditions which may lead to persistence and subsequent contamination in food that results in disease transmission in humans.

Lastly, the genomic study identified the specific genes (flagella assembly, motility, surface protein genes, chemotaxis, quorum sensing and stress response genes) involved in the biofilm formation of *L. monocytogenes* strains and the genes for enzymes responsible for the catabolism of various carbon and nitrogen substrates. Therefore, the phenomic and genomic data provided a basis for understanding the impacts of biofilm formation on the virulence potential of *L. monocytogenes* and can be used to develop anti-biofilm strategies to control biofilm development in food manufacturing environment to ensure food safety and consumer health.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. **Sharar, N. S.,** Chai, L. C. & Thong, K. L. (2018). Catabolic activity and biofilm formation of foodborne *Listeria monocytogenes* strains. *Journal of Consumer Protection and Food Safety, 13*(3), 289-298.

PAPERS PRESENTED

1. **Sharar, N. S.,** Chai, L. C. & Thong, K. L. (2017). *Phenotype Microarray Analysis and Biofilm Study of selected Listeria monocytogenes strains from Ready-to-Eat food*. Paper presented at the 22nd Biological Sciences Graduate Congress, 19-21st December 2017, National University of Singapore, Singapore.

2. **Sharar, N. S.,** Chai, L. C. & Thong, K. L. (2018*). Biofilm Formation by a Foodborne Pathogen Listeria monocytogenes*. Paper presented at the Malaysian Society for Microbiology Postgraduate Seminar, 9th October 2018, Universiti Teknologi MARA, Selangor, Malaysia.

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RESEARCH ARTICLE

Catabolic activity and biofilm formation of foodborne *Listeria monocytogenes* strains

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Abstract
Listeria monocytogenes is a major foodborne pathogen causing increased morbidity worldwide. It forms resistant biofilm structures in food processing facilities after sanitization, consequently creating a public health concern. Many studies on the metabolism and transmission of *L. monocytogenes* has provided insights into its intracellular infection process, however there is limited understanding on the substrate utilization of the bacteria. Therefore, the main objective of this study was to investigate the carbon and nitrogen substrate catabolism and the biofilm forming potential of 3 Malaysian *L. monocytogenes* strains (LM41, LM92 and LM115) previously isolated from ready-to-eat foods. Biolog Phenotype Microarray (PM) system was used to study the catabolic activity of the foodborne strains in 190 carbon and 380 nitrogen sources. PM analysis showed that the carbon and nitrogen catabolic activity of *L. monocytogenes* strains were considerably limited and these strains utilised Tween 40 and Tween 80, which are commonly used for the sanitation in food and meat processing industries. Furthermore, all 3 strains showed strong biofilm forming potential in nutrient-rich and nutrient-limited media, irrespective of the serogroups. The data generated could be utilised to develop alternative measure to inhibit biofilm formation in *L. monocytogenes* in the food processing environment.

Keywords Biofilm · Foodborne pathogen · *Listeria monocytogenes* · Phenotype microarray · Ready-to-eat food

1 Introduction

Listeria monocytogenes is a facultative anaerobic, non-sporulating, Gram-positive rod shaped bacterium that is commonly found in the soil, plant material and raw and processed food products. It can survive and grow over a wide range of environmental conditions such as refrigeration temperatures (0–4 °C), low pH (2–4) and high salt concentration (10% NaCl), thus making them very hard to control (Doijad et al. 2015; Zhou et al. 2012). It is an emerging foodborne pathogen and the causative agent of human listeriosis. Infection is typically acquired through the ingestion of contaminated food products and the common site of infection is the intestinal epithelium. *L. monocytogenes* infection has several clinical conditions, including meningitis, encephalitis, gastroenteritis, septicemia, abortions, convulsions and causes high mortality (20–30%) rates among pregnant women, neonates, elderly and immune-compromised patients (Lomonaco et al. 2015). *Listeria monocytogenes* is accountable for the majority of deaths caused by food-borne epidemics in Europe and USA (Lomonaco et al. 2015). The largest listeriosis outbreak in U.S. history occurred in 2011, due to the consumption of cantaloupe from a single farm that caused 147 illnesses, 33 deaths, and 1 miscarriage in 28 states (CDC 2012). In Malaysia, foodborne *L. monocytogenes* had been detected in raw and ready-to-eat (RTE) foods and the majority of incidences resulted from the contamination of *L. monocytogenes* in various street-side foods, salads, vegetables, raw and processed deli meats and fish products (Jamali et al. 2013; Jeyaletchumi et al. 2012; Marian et al. 2012; Ponniah et al. 2010; Wong et al. 2011). Listeriosis is not a notifiable disease in Malaysia, hence official report on foodborne listeriosis is lacking.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00003-018-1169-6) contains supplementary material, which is available to authorized users.

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APPENDICES

APPENDIX A

List of media, buffers and chemicals used in this study.

Luria-Bertani (LB) agar

Tryptone	4 g
Yeast extract	2 g
NaCl	4 g
Bacto® Agar	6 g
Distilled water up to	400 mL

Autoclave at 121°C for 15 minutes.

LB broth

Tryptone	2 g
Yeast extract	1 g
NaCl	2 g
Distilled water up to	200 mL

Autoclave at 121°C for 15 minutes.

M9 minimal salts medium (M9MM)

1x M9 minimal salts medium	4.52 g
Distilled water up to	400 mL

Autoclave at 121°C for 15 minutes.

1 M $MgSO_4$	800 µL
1 M $CaCl_2$	40 µL

Add $MgSO_4$ and $CaCl_2$ solution in sterile M9 minimal medium and mix thoroughly.

1 M Magnesium sulphate (MW = 120.366 g/mol)

Dissolve 12 g $MgSO_4$ in 100 mL distilled water. Autoclave at 121°C for 15 minutes.

1 M Calcium chloride (MW = 110.98 g/mol)

Dissolve 11.1 g $CaCl_2$ in 100 mL distilled water. Autoclave at 121°C for 15 minutes.

1x Phosphate buffered solution (PBS)

Dissolve 10 g 10x PBS in 100 mL distilled water

Add 900 mL distilled water up to 1 litre

Autoclave at 121°C for 15 minutes.

0.5% Crystal Violet solution

Crystal violet powder	0.5 g
Methanol	20 mL
Distilled water up to	80 mL

Filter sterilize the solution using 0.22 µ filter.

80:20 Ethanol: Acetone solution

Ethanol	80 mL
Acetone	20 mL

Preparation of C- and N-substrate solutions

20 mM carbon and nitrogen substrate solutions were prepared by filter sterilization using 0.22 µ filter and stored at 4°C.

1. D-Glucose (MW = 180.2 g/mol)

Dissolve 0.36 g D-glucose in 100 mL distilled water.

2. Glycine (MW = 75.07 g/mol)

Dissolve 0.15 g glycine in 100 mL distilled water

3. L-Lysine (MW = 146.19 g/mol)

Dissolve 0.292 g L-lysine in 100 mL distilled water

4. L-Cysteine (MW = 240.3 g/mol)

Dissolve 0.48 g L-cysteine in 100 mL distilled water

5. L-Threonine (MW = 119.12 g/mol)

Dissolve 0.24 g L-threonine in 100 mL distilled water

6. Glycerol (MW = 92.1 g/mol)

Dissolve 0.18 g glycerol in 100 mL distilled water

7. Mannitol (MW = 182.17 g/mol)

Dissolve 0.364 g mannitol in 100 mL distilled water

8. Tween 80 (MW = 1310 g/mol)

Add 20 ml Tween 80 solution in 80 mL sterile distilled water and mix thoroughly. Then, add 2 mL solution in 98 mL distilled water and mix again.

9. Maltose (MW = 360.32 g/mol)

Dissolve 0.721 g maltose in 100 mL distilled water

10. D-Fructose (MW = 180.16 g/mol)

Dissolve 0.36 g D-fructose in 100 mL distilled water

11. D-Galactose (MW = 180.2 g/mol)

Dissolve 0.36 g D-galactose in 100 mL distilled water

12. D-Trehalose (MW = 342.296 g/mol)

Dissolve 0.685 g D-trehalose in 100 mL distilled water

13. D-Xylose (MW =150.13 g/mol)

Dissolve 0.3 g D-xylose in 100 mL distilled water

14. Riboflavin (MW = 376.36 g/mol)

Dissolve 0.753 g riboflavin in 100 mL distilled water

15. Biotin (MW = 244.31 g/mol)

Dissolve 0.489 g biotin in 100 mL distilled water

16. Thiamine (MW = 265.35 g/mol)

Dissolve 0.531 g thiamine in 100 mL distilled water

17. Salicin (MW = 286.28 g/mol)

Dissolve 0.573 g salicin in 100 mL distilled water

18. Adenosine (MW = 267.25 g/mol)

Dissolve 0.535 g adenosine in 50 mL distilled water

19. Thymidine (MW = 242.23 g/mol)

Dissolve 0.484 g thymidine in 50 mL distilled water

20. Cytidine (MW = 243.217 g/mol)

Dissolve 0.490 g cytidine in 50 mL distilled water

21. Uridine (MW = 244.2 g/mol)

Dissolve 0.488 g uridine in 50 mL distilled water

22. L-Glutamic Acid (MW = 147.13 g/mol)

Dissolve 0.294 g L-glutamic acid in 50 mL distilled water

23. L-Rhamnose (MW = 164.16 g/mol)

Dissolve 0.303 g L-rhamnose in 50 mL distilled water

24. Tween 40 (MW = 620.865 g/mol)

Add 20 ml Tween 40 solution in 80 mL sterile distilled water and mix thoroughly. Then, add 2 mL solution in 98 mL distilled water and mix well.

APPENDIX B

List of all the conditions in PM microplates PM1, PM2A, PM3B, PM6, PM7 and PM8.

Plate	Location	Chemical name	Function
PM1	A01	Negative Control	C-Source, negative control
PM1	A02	L-Arabinose	C-Source, carbohydrate
PM1	A03	N-Acetyl-D-Glucosamine	C-Source, carbohydrate
PM1	A04	D-Saccharic acid	C-Source, carboxylic acid
PM1	A05	Succinic acid	C-Source, carboxylic acid
PM1	A06	D-Galactose	C-Source, carbohydrate
PM1	A07	L-Aspartic acid	C-Source, amino acid
PM1	A08	L-Proline	C-Source, amino acid
PM1	A09	D-Alanine	C-Source, amino acid
PM1	A10	D-Trehalose	C-Source, carbohydrate
PM1	A11	D-Mannose	C-Source, carbohydrate
PM1	A12	Dulcitol	C-Source, carbohydrate
PM1	B01	D-Serine	C-Source, amino acid
PM1	B02	D-Sorbitol	C-Source, carbohydrate
PM1	B03	Glycerol	C-Source, carbohydrate
PM1	B04	L-Fucose	C-Source, carbohydrate
PM1	B05	D-Glucuronic acid	C-Source, carboxylic acid
PM1	B06	D-Gluconic acid	C-Source, carboxylic acid
PM1	B07	D, L-a-Glycerol Phosphate	C-Source, carbohydrate
PM1	B08	D-Xylose	C-Source, carbohydrate
PM1	B09	L-Lactic acid	C-Source, carboxylic acid
PM1	B10	Formic acid	C-Source, carboxylic acid
PM1	B11	D-Mannitol	C-Source, carbohydrate
PM1	B12	L-Glutamic acid	C-Source, amino acid
PM1	C01	D-Glucose-6-Phosphate	C-Source, carbohydrate
PM1	C02	D-Galactonic acid-g-Lactone	C-Source, carboxylic acid
PM1	C03	D, L-Malic acid	C-Source, carboxylic acid
PM1	C04	D-Ribose	C-Source, carbohydrate
PM1	C05	Tween 20	C-Source, fatty acid
PM1	C06	L-Rhamnose	C-Source, carbohydrate
PM1	C07	D-Fructose	C-Source, carbohydrate
PM1	C08	Acetic acid	C-Source, carboxylic acid
PM1	C09	a-D-Glucose	C-Source, carbohydrate
PM1	C10	Maltose	C-Source, carbohydrate
PM1	C11	D-Melibiose	C-Source, carbohydrate
PM1	C12	Thymidine	C-Source, carbohydrate
PM1	D01	L-Asparagine	C-Source, amino acid
PM1	D02	D-Aspartic acid	C-Source, amino acid
PM1	D03	D-Glucosaminic acid	C-Source, carboxylic acid
PM1	D04	1,2-Propanediol	C-Source, alcohol

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM1	D05	Tween 40	C-Source, fatty acid
PM1	D06	a-Ketoglutaric acid	C-Source, carboxylic acid
PM1	D07	a-Ketobutyric acid	C-Source, carboxylic acid
PM1	D08	a-Methyl-D-Galactoside	C-Source, carbohydrate
PM1	D09	a-D-Lactose	C-Source, carbohydrate
PM1	D10	Lactulose	C-Source, carbohydrate
PM1	D11	Sucrose	C-Source, carbohydrate
PM1	D12	Uridine	C-Source, carbohydrate
PM1	E01	L-Glutamine	C-Source, amino acid
PM1	E02	m-Tartaric acid	C-Source, carboxylic acid
PM1	E03	D-Glucose-1-Phosphate	C-Source, carbohydrate
PM1	E04	D-Fructose-6-Phosphate	C-Source, carbohydrate
PM1	E05	Tween 80	C-Source, fatty acid
PM1	E06	a-Hydroxyglutaric acid-g-Lactone	C-Source, carboxylic acid
PM1	E07	a-Hydroxybutyric acid	C-Source, carboxylic acid
PM1	E08	b-Methyl-D-Glucoside	C-Source, carbohydrate
PM1	E09	Adonitol	C-Source, carbohydrate
PM1	E10	Maltotriose	C-Source, carbohydrate
PM1	E11	2-Deoxyadenosine	C-Source, carbohydrate
PM1	E12	Adenosine	C-Source, carbohydrate
PM1	F01	Gly-Asp	C-Source, amino acid
PM1	F02	Citric acid	C-Source, carboxylic acid
PM1	F03	m-Inositol	C-Source, carbohydrate
PM1	F04	D-Threonine	C-Source, amino acid
PM1	F05	Fumaric acid	C-Source, carboxylic acid
PM1	F06	Bromosuccinic acid	C-Source, carboxylic acid
PM1	F07	Propionic acid	C-Source, carboxylic acid
PM1	F08	Mucic acid	C-Source, carboxylic acid
PM1	F09	Glycolic acid	C-Source, carboxylic acid
PM1	F10	Glyoxylic acid	C-Source, carboxylic acid
PM1	F11	D-Cellobiose	C-Source, carbohydrate
PM1	F12	Inosine	C-Source, carbohydrate
PM1	G01	Glycyl-L-Glutamic acid	C-Source, amino acid
PM1	G02	Tricarballylic acid	C-Source, carboxylic acid
PM1	G03	L-Serine	C-Source, amino acid
PM1	G04	L-Threonine	C-Source, amino acid
PM1	G05	L-Alanine	C-Source, amino acid
PM1	G06	L-Ala-Gly	C-Source, amino acid
PM1	G07	Acetoacetic acid	C-Source, carboxylic acid
PM1	G08	N-Acetyl-β-D-Mannosamine	C-Source, carbohydrate
PM1	G09	Mono-Methylsuccinate	C-Source, carboxylic acid
PM1	G10	Methylpyruvate	C-Source, ester
PM1	G11	D-Malic acid	C-Source, carboxylic acid

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM1	G12	L-Malic acid	C-Source, carboxylic acid
PM1	H01	Gly-Pro	C-Source, amino acid
PM1	H02	p-Hydroxyphenyl Acetic acid	C-Source, carboxylic acid
PM1	H03	m-Hydroxyphenyl Acetic acid	C-Source, carboxylic acid
PM1	H04	Tyramine	C-Source, amine
PM1	H05	D-Psicose	C-Source, carbohydrate
PM1	H06	L-Lyxose	C-Source, carbohydrate
PM1	H07	Glucuronamide	C-Source, amide
PM1	H08	Pyruvic acid	C-Source, carboxylic acid
PM1	H09	L-Galactonic acid-g-Lactone	C-Source, carboxylic acid
PM1	H10	D-Galacturonic acid	C-Source, carboxylic acid
PM1	H11	Phenylethylamine	C-Source, amine
PM1	H12	2-Aminoethanol	C-Source, alcohol
PM2A	A01	Negative Control	C-Source, negative control
PM2A	A02	Chondroitin Sulfate C	C-Source, polymer
PM2A	A03	a-Cyclodextrin	C-Source, polymer
PM2A	A04	b-Cyclodextrin	C-Source, polymer
PM2A	A05	g-Cyclodextrin	C-Source, polymer
PM2A	A06	Dextrin	C-Source, polymer
PM2A	A07	Gelatin	C-Source, polymer
PM2A	A08	Glycogen	C-Source, polymer
PM2A	A09	Inulin	C-Source, polymer
PM2A	A10	Laminarin	C-Source, polymer
PM2A	A11	Mannan	C-Source, polymer
PM2A	A12	Pectin	C-Source, polymer
PM2A	B01	N-Acetyl-D-Galactosamine	C-Source, carbohydrate
PM2A	B02	N-Acetyl-Neuraminic acid	C-Source, carboxylic acid
PM2A	B03	b-D-Allose	C-Source, carbohydrate
PM2A	B04	Amygdalin	C-Source, carbohydrate
PM2A	B05	D-Arabinose	C-Source, carbohydrate
PM2A	B06	D-Arabitol	C-Source, carbohydrate
PM2A	B07	L-Arabitol	C-Source, carbohydrate
PM2A	B08	Arbutin	C-Source, carbohydrate
PM2A	B09	2-Deoxy-D-Ribose	C-Source, carbohydrate
PM2A	B10	i-Erythritol	C-Source, carbohydrate
PM2A	B11	D-Fucose	C-Source, carbohydrate
PM2A	B12	3-b-D-Galactopyranosyl-D-Arabinose	C-Source, carbohydrate
PM2A	C01	Gentiobiose	C-Source, carbohydrate
PM2A	C02	L-Glucose	C-Source, carbohydrate
PM2A	C03	D-Lactitol	C-Source, carbohydrate
PM2A	C04	D-Melezitose	C-Source, carbohydrate
PM2A	C05	Maltitol	C-Source, carbohydrate
PM2A	C06	a-Methyl-D-Glucoside	C-Source, carbohydrate

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM2A	C07	b-Methyl-D-Galactoside	C-Source, carbohydrate
PM2A	C08	3-Methylglucose	C-Source, carbohydrate
PM2A	C09	b-Methyl-D-Glucuronic acid	C-Source, carboxylic acid
PM2A	C10	a-Methyl-D-Mannoside	C-Source, carbohydrate
PM2A	C11	b-Methyl-D-Xyloside	C-Source, carbohydrate
PM2A	C12	Palatinose	C-Source, carbohydrate
PM2A	D01	D-Raffinose	C-Source, carbohydrate
PM2A	D02	Salicin	C-Source, carbohydrate
PM2A	D03	Sedoheptulosan	C-Source, carbohydrate
PM2A	D04	L-Sorbose	C-Source, carbohydrate
PM2A	D05	Stachyose	C-Source, carbohydrate
PM2A	D06	D-Tagatose	C-Source, carbohydrate
PM2A	D07	Turanose	C-Source, carbohydrate
PM2A	D08	Xylitol	C-Source, carbohydrate
PM2A	D09	N-Acetyl-D-Glucosaminitol	C-Source, carbohydrate
PM2A	D10	g-Amino-N-Butyric acid	C-Source, carboxylic acid
PM2A	D11	d-Amino Valeric acid	C-Source, carboxylic acid
PM2A	D12	Butyric acid	C-Source, carboxylic acid
PM2A	E01	Capric acid	C-Source, carboxylic acid
PM2A	E02	Caproic acid	C-Source, carboxylic acid
PM2A	E03	Citraconic acid	C-Source, carboxylic acid
PM2A	E04	Citramalic acid	C-Source, carboxylic acid
PM2A	E05	D-Glucosamine	C-Source, carbohydrate
PM2A	E06	2-Hydroxybenzoic acid	C-Source, carboxylic acid
PM2A	E07	4-Hydroxybenzoic acid	C-Source, carboxylic acid
PM2A	E08	b-Hydroxybutyric acid	C-Source, carboxylic acid
PM2A	E09	g-Hydroxybutyric acid	C-Source, carboxylic acid
PM2A	E10	a-Keto-Valeric acid	C-Source, carboxylic acid
PM2A	E11	Itaconic acid	C-Source, carboxylic acid
PM2A	E12	5-Keto-D-Gluconic acid	C-Source, carboxylic acid
PM2A	F01	D-Lactic Acid Methyl Ester	C-Source, ester
PM2A	F02	Malonic acid	C-Source, carboxylic acid
PM2A	F03	Melibionic acid	C-Source, carbohydrate
PM2A	F04	Oxalic acid	C-Source, carboxylic acid
PM2A	F05	Oxalomalic acid	C-Source, carboxylic acid
PM2A	F06	Quinic acid	C-Source, carboxylic acid
PM2A	F07	D-Ribono-1,4-Lactone	C-Source, carboxylic acid
PM2A	F08	Sebacic acid	C-Source, carboxylic acid
PM2A	F09	Sorbic acid	C-Source, carboxylic acid
PM2A	F10	Succinamic acid	C-Source, carboxylic acid
PM2A	F11	D-Tartaric acid	C-Source, carboxylic acid
PM2A	F12	L-Tartaric acid	C-Source, carboxylic acid
PM2A	G01	Acetamide	C-Source, amide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM2A	G02	L-Alaninamide	C-Source, amide
PM2A	G03	N-Acetyl-L-Glutamic acid	C-Source, amino acid
PM2A	G04	L-Arginine	C-Source, amino acid
PM2A	G05	Glycine	C-Source, amino acid
PM2A	G06	L-Histidine	C-Source, amino acid
PM2A	G07	L-Homoserine	C-Source, amino acid
PM2A	G08	Hydroxy-L-Proline	C-Source, amino acid
PM2A	G09	L-Isoleucine	C-Source, amino acid
PM2A	G10	L-Leucine	C-Source, amino acid
PM2A	G11	L-Lysine	C-Source, amino acid
PM2A	G12	L-Methionine	C-Source, amino acid
PM2A	H01	L-Ornithine	C-Source, amino acid
PM2A	H02	L-Phenylalanine	C-Source, amino acid
PM2A	H03	L-Pyroglutamic acid	C-Source, amino acid
PM2A	H04	L-Valine	C-Source, amino acid
PM2A	H05	D,L-Carnitine	C-Source, carboxylic acid
PM2A	H06	Sec-Butylamine	C-Source, amine
PM2A	H07	D,L-Octopamine	C-Source, amine
PM2A	H08	Putrescine	C-Source, amine
PM2A	H09	Dihydroxyacetone	C-Source, alcohol
PM2A	H10	2,3-Butanediol	C-Source, alcohol
PM2A	H11	2,3-Butanone	C-Source, alcohol
PM2A	H12	3-Hydroxy-2-butanone	C-Source, alcohol
PM3B	A01	Negative Control	N-Source, Negative control
PM3B	A02	Ammonia	N-Source, inorganic
PM3B	A03	Nitrite	N-Source, inorganic
PM3B	A04	Nitrate	N-Source, inorganic
PM3B	A05	Urea	N-Source, other
PM3B	A06	Biuret	N-Source, other
PM3B	A07	L-Alanine	N-Source, amino acid
PM3B	A08	L-Arginine	N-Source, amino acid
PM3B	A09	L-Asparagine	N-Source, amino acid
PM3B	A10	L-Aspartic acid	N-Source, amino acid
PM3B	A11	L-Cysteine	N-Source, amino acid
PM3B	A12	L-Glutamic acid	N-Source, amino acid
PM3B	B01	L-Glutamine	N-Source, amino acid
PM3B	B02	Glycine	N-Source, amino acid
PM3B	B03	L-Histidine	N-Source, amino acid
PM3B	B04	L-Isoleucine	N-Source, amino acid
PM3B	B05	L-Leucine	N-Source, amino acid
PM3B	B06	L-Lysine	N-Source, amino acid
PM3B	B07	L-Methionine	N-Source, amino acid
PM3B	B08	L-Phenylalanine	N-Source, amino acid

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM3B	B09	L-Proline	N-Source, amino acid
PM3B	B10	L-Serine	N-Source, amino acid
PM3B	B11	L-Threonine	N-Source, amino acid
PM3B	B12	L-Tryptophan	N-Source, amino acid
PM3B	C01	L-Tyrosine	N-Source, amino acid
PM3B	C02	L-Valine	N-Source, amino acid
PM3B	C03	D-Alanine	N-Source, amino acid
PM3B	C04	D-Asparagine	N-Source, amino acid
PM3B	C05	D-Aspartic acid	N-Source, amino acid
PM3B	C06	D-Glutamic acid	N-Source, amino acid
PM3B	C07	D-Lysine	N-Source, amino acid
PM3B	C08	D-Serine	N-Source, amino acid
PM3B	C09	D-Valine	N-Source, amino acid
PM3B	C10	L-Citrulline	N-Source, amino acid
PM3B	C11	L-Homoserine	N-Source, amino acid
PM3B	C12	L-Ornithine	N-Source, amino acid
PM3B	D01	N-Acetyl-L-Glutamic acid	N-Source, amino acid
PM3B	D02	N-Phthaloyl-L-Glutamic acid	N-Source, amino acid
PM3B	D03	L-Pyroglutamic acid	N-Source, amino acid
PM3B	D04	Hydroxylamine	N-Source, other
PM3B	D05	Methylamine	N-Source, other
PM3B	D06	N-Amylamine	N-Source, other
PM3B	D07	N-Butylamine	N-Source, other
PM3B	D08	Ethylamine	N-Source, other
PM3B	D09	Ethanolamine	N-Source, other
PM3B	D10	Ethylenediamine	N-Source, other
PM3B	D11	Putrescine	N-Source, other
PM3B	D12	Agmatine	N-Source, other
PM3B	E01	Histamine	N-Source, other
PM3B	E02	b-Phenylethylamine	N-Source, other
PM3B	E03	Tyramine	N-Source, other
PM3B	E04	Acetamide	N-Source, other
PM3B	E05	Formamide	N-Source, other
PM3B	E06	Glucuronamide	N-Source, other
PM3B	E07	D,L-Lactamide	N-Source, other
PM3B	E08	D-Glucosamine	N-Source, other
PM3B	E09	D-Galactosamine	N-Source, other
PM3B	E10	D-Mannosamine	N-Source, other
PM3B	E11	N-Acetyl-D-Glucosamine	N-Source, other
PM3B	E12	N-Acetyl-D- Galactosamine	N-Source, other
PM3B	F01	N-Acetyl-D-Mannosamine	N-Source, other
PM3B	F02	Adenine	N-Source, other
PM3B	F03	Adenosine	N-Source, other

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM3B	F04	Cytidine	N-Source, other
PM3B	F05	Cytosine	N-Source, other
PM3B	F06	Guanine	N-Source, other
PM3B	F07	Guanosine	N-Source, other
PM3B	F08	Thymine	N-Source, other
PM3B	F09	Thymidine	N-Source, other
PM3B	F10	Uracil	N-Source, other
PM3B	F11	Uridine	N-Source, other
PM3B	F12	Inosine	N-Source, other
PM3B	G01	Xanthine	N-Source, other
PM3B	G02	Xanthosine	N-Source, other
PM3B	G03	Uric acid	N-Source, other
PM3B	G04	Alloxan	N-Source, other
PM3B	G05	Allantoin	N-Source, other
PM3B	G06	Parabanic acid	N-Source, other
PM3B	G07	D, L-a-Amino-N-Butyric acid	N-Source, other
PM3B	G08	g-Amino-N-Butyric acid	N-Source, other
PM3B	G09	e-Amino-N-Caproic acid	N-Source, other
PM3B	G10	D, L-a-Amino-Caprylic acid	N-Source, other
PM3B	G11	d-Amino-N-Valeric acid	N-Source, other
PM3B	G12	a-Amino-N-Valeric acid	N-Source, other
PM3B	H01	Ala-Asp	N-Source, peptide
PM3B	H02	Ala-Gln	N-Source, peptide
PM3B	H03	Ala-Glu	N-Source, peptide
PM3B	H04	Ala-Gly	N-Source, peptide
PM3B	H05	Ala-His	N-Source, peptide
PM3B	H06	Ala-Leu	N-Source, peptide
PM3B	H07	Ala-Thr	N-Source, peptide
PM3B	H08	Gly-Asn	N-Source, peptide
PM3B	H09	Gly-Gln	N-Source, peptide
PM3B	H10	Gly-Glu	N-Source, peptide
PM3B	H11	Gly-Met	N-Source, peptide
PM3B	H12	Met-Ala	N-Source, peptide
PM6	A01	Negative Control	N-Source, Negative control
PM6	A02	L-Glutamine	N-Source, amino acid, Positive control
PM6	A03	Ala-Ala	N-Source, peptide
PM6	A04	Ala-Arg	N-Source, peptide
PM6	A05	Ala-Asn	N-Source, peptide
PM6	A06	Ala-Glu	N-Source, peptide
PM6	A07	Ala-Gly	N-Source, peptide
PM6	A08	Ala-His	N-Source, peptide
PM6	A09	Ala-Leu	N-Source, peptide
PM6	A10	Ala-Lys	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM6	A11	Ala-Phe	N-Source, peptide
PM6	A12	Ala-Pro	N-Source, peptide
PM6	B01	Ala-Ser	N-Source, peptide
PM6	B02	Ala-Thr	N-Source, peptide
PM6	B03	Ala-Trp	N-Source, peptide
PM6	B04	Ala-Tyr	N-Source, peptide
PM6	B05	Arg-Ala	N-Source, peptide
PM6	B06	Arg-Arg	N-Source, peptide
PM6	B07	Arg-Asp	N-Source, peptide
PM6	B08	Arg-Gln	N-Source, peptide
PM6	B09	Arg-Glu	N-Source, peptide
PM6	B10	Arg-Ile	N-Source, peptide
PM6	B11	Arg-Leu	N-Source, peptide
PM6	B12	Arg-Lys	N-Source, peptide
PM6	C01	Arg-Met	N-Source, peptide
PM6	C02	Arg-Phe	N-Source, peptide
PM6	C03	Arg-Ser	N-Source, peptide
PM6	C04	Arg-Trp	N-Source, peptide
PM6	C05	Arg-Tyr	N-Source, peptide
PM6	C06	Arg-Val	N-Source, peptide
PM6	C07	Asn-Glu	N-Source, peptide
PM6	C08	Asn-Val	N-Source, peptide
PM6	C09	Asp-Asp	N-Source, peptide
PM6	C10	Asp-Glu	N-Source, peptide
PM6	C11	Asp-Leu	N-Source, peptide
PM6	C12	Asp-Lys	N-Source, peptide
PM6	D01	Asp-Phe	N-Source, peptide
PM6	D02	Asp-Trp	N-Source, peptide
PM6	D03	Asp-Val	N-Source, peptide
PM6	D04	Cys-Gly	N-Source, peptide
PM6	D05	Gln-Gln	N-Source, peptide
PM6	D06	Gln-Gly	N-Source, peptide
PM6	D07	Glu-Asp	N-Source, peptide
PM6	D08	Glu-Glu	N-Source, peptide
PM6	D09	Glu-Gly	N-Source, peptide
PM6	D10	Glu-Ser	N-Source, peptide
PM6	D11	Glu-Trp	N-Source, peptide
PM6	D12	Glu-Tyr	N-Source, peptide
PM6	E01	Glu-Val	N-Source, peptide
PM6	E02	Gly-Ala	N-Source, peptide
PM6	E03	Gly-Arg	N-Source, peptide
PM6	E04	Gly-Cys	N-Source, peptide
PM6	E05	Gly-Gly	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM6	E06	Gly-His	N-Source, peptide
PM6	E07	Gly-Leu	N-Source, peptide
PM6	E08	Gly-Lys	N-Source, peptide
PM6	E09	Gly-Met	N-Source, peptide
PM6	E10	Gly-Phe	N-Source, peptide
PM6	E11	Gly-Pro	N-Source, peptide
PM6	E12	Gly-Ser	N-Source, peptide
PM6	F01	Gly-Thr	N-Source, peptide
PM6	F02	Gly-Trp	N-Source, peptide
PM6	F03	Gly-Tyr	N-Source, peptide
PM6	F04	Gly-Val	N-Source, peptide
PM6	F05	His-Asp	N-Source, peptide
PM6	F06	His-Gly	N-Source, peptide
PM6	F07	His-Leu	N-Source, peptide
PM6	F08	His-Lys	N-Source, peptide
PM6	F09	His-Met	N-Source, peptide
PM6	F10	His-Pro	N-Source, peptide
PM6	F11	His-Ser	N-Source, peptide
PM6	F12	His-Trp	N-Source, peptide
PM6	G01	His-Tyr	N-Source, peptide
PM6	G02	His-Val	N-Source, peptide
PM6	G03	Ile-Ala	N-Source, peptide
PM6	G04	Ile-Arg	N-Source, peptide
PM6	G05	Ile-Gln	N-Source, peptide
PM6	G06	Ile-Gly	N-Source, peptide
PM6	G07	Ile-His	N-Source, peptide
PM6	G08	Ile-Ile	N-Source, peptide
PM6	G09	Ile-Met	N-Source, peptide
PM6	G10	Ile-Phe	N-Source, peptide
PM6	G11	Ile-Pro	N-Source, peptide
PM6	G12	Ile-Ser	N-Source, peptide
PM6	H01	Ile-Trp	N-Source, peptide
PM6	H02	Ile-Tyr	N-Source, peptide
PM6	H03	Ile-Val	N-Source, peptide
PM6	H04	Leu-Ala	N-Source, peptide
PM6	H05	Leu-Arg	N-Source, peptide
PM6	H06	Leu-Asp	N-Source, peptide
PM6	H07	Leu-Glu	N-Source, peptide
PM6	H08	Leu-Gly	N-Source, peptide
PM6	H09	Leu-Ile	N-Source, peptide
PM6	H10	Leu-Leu	N-Source, peptide
PM6	H11	Leu-Met	N-Source, peptide
PM6	H12	Leu-Phe	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM7	A01	Negative Control	N-Source, Negative control
PM7	A02	L-Glutamine	N-Source, amino acid, Positive control
PM7	A03	Leu-Ser	N-Source, peptide
PM7	A04	Leu-Trp	N-Source, peptide
PM7	A05	Leu-Val	N-Source, peptide
PM7	A06	Lys-Ala	N-Source, peptide
PM7	A07	Lys-Arg	N-Source, peptide
PM7	A08	Lys-Glu	N-Source, peptide
PM7	A09	Lys-Ile	N-Source, peptide
PM7	A10	Lys-Leu	N-Source, peptide
PM7	A11	Lys-Lys	N-Source, peptide
PM7	A12	Lys-Phe	N-Source, peptide
PM7	B01	Lys-Pro	N-Source, peptide
PM7	B02	Lys-Ser	N-Source, peptide
PM7	B03	Lys-Thr	N-Source, peptide
PM7	B04	Lys-Trp	N-Source, peptide
PM7	B05	Lys-Tyr	N-Source, peptide
PM7	B06	Lys-Val	N-Source, peptide
PM7	B07	Met-Arg	N-Source, peptide
PM7	B08	Met-Asp	N-Source, peptide
PM7	B09	Met-Gln	N-Source, peptide
PM7	B10	Met-Glu	N-Source, peptide
PM7	B11	Met-Gly	N-Source, peptide
PM7	B12	Met-His	N-Source, peptide
PM7	C01	Met-Ile	N-Source, peptide
PM7	C02	Met-Leu	N-Source, peptide
PM7	C03	Met-Lys	N-Source, peptide
PM7	C04	Met-Met	N-Source, peptide
PM7	C05	Met-Phe	N-Source, peptide
PM7	C06	Met-Pro	N-Source, peptide
PM7	C07	Met-Trp	N-Source, peptide
PM7	C08	Met-Val	N-Source, peptide
PM7	C09	Phe-Ala	N-Source, peptide
PM7	C10	Phe-Gly	N-Source, peptide
PM7	C11	Phe-Ile	N-Source, peptide
PM7	C12	Phe-Phe	N-Source, peptide
PM7	D01	Phe-Pro	N-Source, peptide
PM7	D02	Phe-Ser	N-Source, peptide
PM7	D03	Phe-Trp	N-Source, peptide
PM7	D04	Pro-Ala	N-Source, peptide
PM7	D05	Pro-Asp	N-Source, peptide
PM7	D06	Pro-Gln	N-Source, peptide
PM7	D07	Pro-Gly	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM7	D08	Pro-Hyp	N-Source, peptide
PM7	D09	Pro-Leu	N-Source, peptide
PM7	D10	Pro-Phe	N-Source, peptide
PM7	D11	Pro-Pro	N-Source, peptide
PM7	D12	Pro-Tyr	N-Source, peptide
PM7	E01	Ser-Ala	N-Source, peptide
PM7	E02	Ser-Gly	N-Source, peptide
PM7	E03	Ser-His	N-Source, peptide
PM7	E04	Ser-Leu	N-Source, peptide
PM7	E05	Ser-Met	N-Source, peptide
PM7	E06	Ser-Phe	N-Source, peptide
PM7	E07	Ser-Pro	N-Source, peptide
PM7	E08	Ser-Ser	N-Source, peptide
PM7	E09	Ser-Tyr	N-Source, peptide
PM7	E10	Ser-Val	N-Source, peptide
PM7	E11	Thr-Ala	N-Source, peptide
PM7	E12	Thr-Arg	N-Source, peptide
PM7	F01	Thr-Glu	N-Source, peptide
PM7	F02	Thr-Gly	N-Source, peptide
PM7	F03	Thr-Leu	N-Source, peptide
PM7	F04	Thr-Met	N-Source, peptide
PM7	F05	Thr-Pro	N-Source, peptide
PM7	F06	Trp-Ala	N-Source, peptide
PM7	F07	Trp-Arg	N-Source, peptide
PM7	F08	Trp-Asp	N-Source, peptide
PM7	F09	Trp-Glu	N-Source, peptide
PM7	F10	Trp-Gly	N-Source, peptide
PM7	F11	Trp-Leu	N-Source, peptide
PM7	F12	Trp-Lys	N-Source, peptide
PM7	G01	Trp-Phe	N-Source, peptide
PM7	G02	Trp-Ser	N-Source, peptide
PM7	G03	Trp-Trp	N-Source, peptide
PM7	G04	Trp-Tyr	N-Source, peptide
PM7	G05	Tyr-Ala	N-Source, peptide
PM7	G06	Tyr-Gln	N-Source, peptide
PM7	G07	Tyr-Glu	N-Source, peptide
PM7	G08	Tyr-Gly	N-Source, peptide
PM7	G09	Tyr-His	N-Source, peptide
PM7	G10	Tyr-Leu	N-Source, peptide
PM7	G11	Tyr-Lys	N-Source, peptide
PM7	G12	Tyr-Phe	N-Source, peptide
PM7	H01	Tyr-Trp	N-Source, peptide
PM7	H02	Tyr-Tyr	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM7	H03	Val-Arg	N-Source, peptide
PM7	H04	Val-Asn	N-Source, peptide
PM7	H05	Val-Asp	N-Source, peptide
PM7	H06	Val-Gly	N-Source, peptide
PM7	H07	Val-His	N-Source, peptide
PM7	H08	Val-Ile	N-Source, peptide
PM7	H09	Val-Leu	N-Source, peptide
PM7	H10	Val-Tyr	N-Source, peptide
PM7	H11	Val-Val	N-Source, peptide
PM7	H12	g-Glu-Gly	N-Source, peptide
PM8	A01	Negative Control	N-Source, Negative control
PM8	A02	L-Glutamine	N-Source, amino acid, Positive control
PM8	A03	Ala-Asp	N-Source, peptide
PM8	A04	Ala-Gln	N-Source, peptide
PM8	A05	Ala-Ile	N-Source, peptide
PM8	A06	Ala-Met	N-Source, peptide
PM8	A07	Ala-Val	N-Source, peptide
PM8	A08	Asp-Ala	N-Source, peptide
PM8	A09	Asp-Gln	N-Source, peptide
PM8	A10	Asp-Gly	N-Source, peptide
PM8	A11	Glu-Ala	N-Source, peptide
PM8	A12	Gly-Asn	N-Source, peptide
PM8	B01	Gly-Asp	N-Source, peptide
PM8	B02	Gly-Ile	N-Source, peptide
PM8	B03	His-Ala	N-Source, peptide
PM8	B04	His-Glu	N-Source, peptide
PM8	B05	His-His	N-Source, peptide
PM8	B06	Ile-Asn	N-Source, peptide
PM8	B07	Ile-Leu	N-Source, peptide
PM8	B08	Leu-Asn	N-Source, peptide
PM8	B09	Leu-His	N-Source, peptide
PM8	B10	Leu-Pro	N-Source, peptide
PM8	B11	Leu-Tyr	N-Source, peptide
PM8	B12	Lys-Asp	N-Source, peptide
PM8	C01	Lys-Gly	N-Source, peptide
PM8	C02	Lys-Met	N-Source, peptide
PM8	C03	Met-Thr	N-Source, peptide
PM8	C04	Met-Tyr	N-Source, peptide
PM8	C05	Phe-Asp	N-Source, peptide
PM8	C06	Phe-Glu	N-Source, peptide
PM8	C07	Gln-Glu	N-Source, peptide
PM8	C08	Phe-Met	N-Source, peptide
PM8	C09	Phe-Tyr	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM8	C10	Phe-Val	N-Source, peptide
PM8	C11	Pro-Arg	N-Source, peptide
PM8	C12	Pro-Asn	N-Source, peptide
PM8	D01	Pro-Glu	N-Source, peptide
PM8	D02	Pro-lle	N-Source, peptide
PM8	D03	Pro-Lys	N-Source, peptide
PM8	D04	Pro-Ser	N-Source, peptide
PM8	D05	Pro-Trp	N-Source, peptide
PM8	D06	Pro-Val	N-Source, peptide
PM8	D07	Ser-Asn	N-Source, peptide
PM8	D08	Ser-Asp	N-Source, peptide
PM8	D09	Ser-Gln	N-Source, peptide
PM8	D10	Ser-Glu	N-Source, peptide
PM8	D11	Thr-Asp	N-Source, peptide
PM8	D12	Thr-Gln	N-Source, peptide
PM8	E01	Thr-Phe	N-Source, peptide
PM8	E02	Thr-Ser	N-Source, peptide
PM8	E03	Trp-Val	N-Source, peptide
PM8	E04	Tyr-Ile	N-Source, peptide
PM8	E05	Tyr-Val	N-Source, peptide
PM8	E06	Val-Ala	N-Source, peptide
PM8	E07	Val-Gln	N-Source, peptide
PM8	E08	Val-Glu	N-Source, peptide
PM8	E09	Val-Lys	N-Source, peptide
PM8	E10	Val-Met	N-Source, peptide
PM8	E11	Val-Phe	N-Source, peptide
PM8	E12	Val-Pro	N-Source, peptide
PM8	F01	Val-Ser	N-Source, peptide
PM8	F02	b-Ala-Ala	N-Source, peptide
PM8	F03	b-Ala-Gly	N-Source, peptide
PM8	F04	b-Ala-His	N-Source, peptide
PM8	F05	Met-b-Ala	N-Source, peptide
PM8	F06	b-Ala-Phe	N-Source, peptide
PM8	F07	D-Ala-D-Ala	N-Source, peptide
PM8	F08	D-Ala-Gly	N-Source, peptide
PM8	F09	D-Ala-Leu	N-Source, peptide
PM8	F10	D-Leu-D-Leu	N-Source, peptide
PM8	F11	D-Leu-Gly	N-Source, peptide
PM8	F12	D-Leu-Tyr	N-Source, peptide
PM8	G01	g-Glu-Gly	N-Source, peptide
PM8	G02	g-D-Glu-Gly	N-Source, peptide
PM8	G03	Gly-D-Ala	N-Source, peptide
PM8	G04	Gly-D-Asp	N-Source, peptide

APPENDIX B, continued.

Plate	Location	Chemical name	Function
PM8	G05	Gly-D-Ser	N-Source, peptide
PM8	G06	Gly-D-Thr	N-Source, peptide
PM8	G07	Gly-D-Val	N-Source, peptide
PM8	G08	Leu-b-Ala	N-Source, peptide
PM8	G09	Leu-D-Leu	N-Source, peptide
PM8	G10	Phe-b-Ala	N-Source, peptide
PM8	G11	Ala-Ala-Ala	N-Source, peptide
PM8	G12	D-Ala-Gly-Gly	N-Source, peptide
PM8	H01	Gly-Gly-Ala	N-Source, peptide
PM8	H02	Gly-Gly-D-Leu	N-Source, peptide
PM8	H03	Gly-Gly-Gly	N-Source, peptide
PM8	H04	Gly-Gly-Ile	N-Source, peptide
PM8	H05	Gly-Gly-Leu	N-Source, peptide
PM8	H06	Gly-Gly-Phe	N-Source, peptide
PM8	H07	Val-Tyr-Val	N-Source, peptide
PM8	H08	Gly-Phe-Phe	N-Source, peptide
PM8	H09	Leu-Gly-Gly	N-Source, peptide
PM8	H10	Leu-Leu-Leu	N-Source, peptide
PM8	H11	Phe-Gly-Gly	N-Source, peptide
PM8	H12	Tyr-Gly-Gly	N-Source, peptide

APPENDIX C

Average OD readings of crystal violet assay to determine the biofilm forming potential of three *L. monocytogenes* strains.

Controls (M9+Substrates)	O.D.c.					Average O.D.		
	Average O.D.	SD	μ + (2 × SD)	2 × O.D.c	4 × O.D.c	LM41	LM92	LM115
Adenosine	0.17	0.026	0.22	0.44	0.89	1.12	0.92	1.21
Thymidine	1.43	0.116	1.66	3.32	6.65	1.18	1.48	1.57
Cytidine	0.13	0.009	0.15	0.30	0.61	1.21	1.32	1.27
Uridine	0.14	0.021	0.18	0.36	0.72	1.13	1.34	1.47
L-Glutamic Acid	0.13	0.008	0.15	0.30	0.59	0.93	1.09	1.12
L-Rhamnose	0.13	0.010	0.15	0.30	0.59	1.03	1.06	1.48
Tween 40	0.11	0.016	0.15	0.29	0.58	0.58	0.64	0.90
D-Fructose	0.12	0.007	0.13	0.27	0.53	0.69	0.68	1.24
Maltose	0.11	0.005	0.12	0.25	0.49	0.64	0.75	1.13
D-Galactose	0.12	0.012	0.15	0.30	0.59	0.71	0.75	1.02
D-Trehalose	0.11	0.008	0.13	0.26	0.52	0.68	0.67	1.08
D-Xylose	0.12	0.018	0.15	0.31	0.61	0.79	0.74	1.09
Riboflavin	0.18	0.032	0.24	0.48	0.97	0.97	1.12	1.29
Biotin	0.13	0.025	0.18	0.37	0.73	1.05	1.28	1.34
Thiamine	0.13	0.020	0.17	0.33	0.66	1.03	1.17	1.36
Salicin	0.14	0.020	0.18	0.35	0.71	1.01	1.14	1.45
Glycine	0.13	0.012	0.15	0.31	0.62	0.22	0.32	0.23
L-Lysine	0.12	0.009	0.14	0.28	0.56	0.24	0.31	0.23
L-Cysteine	0.12	0.017	0.16	0.32	0.63	0.22	0.32	0.23
L-Threonine	0.13	0.007	0.15	0.29	0.58	0.24	0.24	0.25
Glycerol	0.12	0.009	0.14	0.28	0.56	0.24	0.23	0.23
Tween 80	0.09	0.003	0.09	0.19	0.38	0.34	0.33	0.50
D-Glucose	0.13	0.019	0.16	0.33	0.66	0.25	0.45	0.42
LB broth	0.11	0.012	0.13	0.26	0.53	1.01	0.75	0.84
M9 medium	0.12	0.011	0.14	0.28	0.56	0.59	0.59	0.99

[a]O.D.c. = cut-off O.D., SD = Standard Deviation, μ = Mean; (Stepanović et al., 2000)

APPENDIX D

List of genes used in BLAST study.

Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Biosynthesis	*lmo1641*	*citB*	Aconitate hydratase	EGD-e	Alonso et al. (2014)
	lmo1566	*citC*	Isocitrate dehydrogenase	EGD-e	
	lmo1567	*citZ*	Citrate synthase	EGD-e	
	lmo0974	*dltA*	D-alanine poly(phosphoribitol) ligase subunit I	EGD-e	
	lmo0973	*dltB*	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid	EGD-e	
	lmo0972	*dltC*	D-alanine poly(phosphoribitol) ligase subunit II	EGD-e	
	lmo0971	*dltD*	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid	EGD-e	
	lmo0956	-	N-acetylglucosamine-6P-phosphate deacetylase	EGD-e	
	lmo0823	-	Oxidoreductase	EGD-e	
	lmo1305	*tkt*	Transketolase	EGD-e	
	lmo1370	-	Butyrate kinase	EGD-e	
	lmo2547	*hom*	Homoserine dehydrogenase	EGD-e	
	lmo2457	*tpiA*	Triosephosphate isomerase	EGD-e	
	lmo2477	*galE*	UDP-glucose 4-epimerase	EGD-e	
	lmo1765	*purH*	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltranferase	EGD-e	
	lmo1766	*purN*	Phosphoribosylglycinamide formyltransferase	EGD-e	
	lmo1768	*purF*	Amidophosphoribosyltransferase	EGD-e	
	lmo1770	*purL*	Phosphoribosylformylglycinamide synthase I	EGD-e	
	lmo1771	*purS*	Phosphoribosylformylglycinamide synthase subunit	EGD-e	

APPENDIX D, continued.

Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Biosynthesis	*lmo1774*	*purK*	Phosphoribosylaminoimidazolecarboxylase ATPase subunit	EGD-e	Ouyang et al. (2012)
	lmo1775	*purE*	Phosphoribosylaminoimidazolecarboxylase catalytic subunit	EGD-e	
	lmo1874	*thyA*	Thymidylate synthase	EGD-e	
	lmo1892	*pbpA*	Penicillin-binding protein 2A	EGD-e	
	lmo2554	-	Galactosyltransferase	EGD-e	
	lmo2555	-	N-acetylglucosaminyl-phosphatidylinositol biosynthesis protein	EGD-e	
Gene Regulation	*lmo1218*	-	rRNA methylase	EGD-e	Alonso et al. (2014)
	lmo1250	-	Antibiotic resistance protein	EGD-e	
	lmo1251	-	Fnr/Crp family transcriptional regulator	EGD-e	
	lmo1262	-	Transcriptional regulator	EGD-e	
	lmo1267	*tig*	Trigger factor	EGD-e	
	lmo1269	-	Signal peptidase type I	EGD-e	
	lmo1378	*lisK*	Two component sensor histidine kinase	EGD-e	
	lmo1386	-	DNA translocase	EGD-e	Chang et al. (2012)
	lmo1481	*holA*	DNA polymerase III subunit delta	EGD-e	
	lmo1496	*greA*	Transcription elongation factor GreA	EGD-e	
	lmo1550	*comC*	Competence protein ComC	EGD-e	
	lmo1565	*polA*	DNA polymerase I	EGD-e	Alonso et al. (2014)
	lmo1811	-	ATP-dependent DNA helicase RecG	EGD-e	
	lmo1844	*lspA*	Lipoprotein signal peptidase	EGD-e	
	lmo1878	-	Manganese transport transcriptional regulator	EGD-e	
	lmo2365	-	RofA family transcriptional regulator	EGD-e	
	lmo2501	*phoP*	Two component response phosphate regulator	EGD-e	
	lmo2500	*phoR*	Two component sensor histidine kinase	EGD-e	

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Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Gene regulation	*lmo2515*	*degU*	Two component response regulator DegU	EGD-e	Kumar et al. (2009)
	lmo2611	*adk*	Adenylate kinase	EGD-e	
	lmo2802	*gidB*	16S rRNA methyltransferase GidB	EGD-e	
	lmo0246	*nusG*	Transcription antitermination protein NusG	EGD-e	Van der Veen & Abee (2011)
	lmo0258	*rpoB*	DNA directed RNA polymerase subunit beta	EGD-e	
	lmo0371	-	GntR family transcriptional regulator	EGD-e	Ouyang et al. (2012)
	lmo0443	-	LytR family transcriptional regulator	EGD-e	
	lmo0734	-	LacI family transcriptional regulator	EGD-e	
Metabolism	*lmo0107*	-	ABC transporter ATP-binding protein	EGD-e	Chang et al. (2012)
	lmo0239	*cysS*	Cysteinyl-tRNA synthetase	EGD-e	
	lmo0372	-	Beta-glucosidase	EGD-e	
	lmo0529	-	Glucosaminyltransferase	EGD-e	
	lmo0538	-	N-acyl-L-amino acid amidohydrolase	EGD-e	
	lmo0539	-	Tagatose 1,6-diphosphate aldolase	EGD-e	
	lmo0541	-	ABC transporter substrate-binding protein	EGD-e	
	lmo0542	-	PTS sorbitol transporter subunit IIA	EGD-e	
	lmo0570	*hisJ*	Histidinol phosphatase	EGD-e	
	lmo0631	-	PTS fructose transporter subunit IIA	EGD-e	
	lmo1174	*eutA*	Ethanolamine utilization protein EutA	EGD-e	Sela et al. (2006)
	lmo1288	*luxS*	S-ribosylhomocysteine lyase	EGD-e	
	lmo1354	-	Aminopeptidase P	EGD-e	Friedman et al. (2017)
	lmo1497	*udk*	Uridine kinase	EGD-e	
	lmo1599	*ccpA*	Catabolite control protein A	EGD-e	

APPENDIX D, continued.

Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Metabolism	*lmo1600*	*aroA*	Bifunctional 3-deoxy-7-phosphoheptulose synthase/ chorismate mutase	EGD-e	Alonso et al. (2014)
	lmo1632	*trpG*	Anthranilate synthase subunit beta	EGD-e	
	lmo1719	-	PTS lichenan transporter subunit IIA	EGD-e	
	lmo1809	*plsX*	Glycerol-3-phosphate acyltransferase PlsX	EGD-e	
	lmo1915	-	Malate dehydrogenase	EGD-e	Deutscher et al. (2014)
	lmo1916	-	Peptidase	EGD-e	
	lmo2205	-	Phosphoglyceromutase	EGD-e	
	lmo2497	-	Phosphate ABC transporter permease	EGD-e	
	lmo2529	*atpD*	ATP synthase F0F1 subunit beta	EGD-e	
	lmo2534	*atpE*	ATP synthase F0F1 subunit C	EGD-e	
	lmo2535	*atpB*	ATP synthase F0F1 subunit A	EGD-e	
	lmo2651	-	PTS mannitol transporter subunit IIA	EGD-e	
	lmo2667	-	PTS galacticol transporter subunit IIA	EGD-e	
	lmo2749	-	Glutamine amidotransferase	EGD-e	
Motility	*lmo0685*	*motA*	Flagellar motor protein MotA	EGD-e	Chang et al. (2012)
	lmo0690	*flaA*	Flagellin protein	EGD-e	
	lmo0677	*fliQ*	Flagellar biosynthesis protein FliQ	EGD-e	
	lmo0680	*flhA*	Flagellar biosynthesis protein	EGD-e	
	lmo0682	*flgG*	Flagellar basal body rod protein FlgG	EGD-e	
	lmo0683	-	Chemotaxis protein CheR	EGD-e	Behari et al. (1998)
	lmo0691	*cheY*	Chemotaxis response regulator CheY	EGD-e	
	lmo0692	*cheA*	two-component sensor histidine kinase CheA	EGD-e	
	lmo0697	*flgE*	Flagellar hook protein FlgE	EGD-e	Chang et al. (2012)
	lmo0707	*fliD*	Flagellar capping protein FliD	EGD-e	

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Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Motility	*lmo0714*	*fliG*	Flagellar motor switch protein FliG	EGD-e	Chang et al. (2012)
	lmo0716	*fliI*	Flagellum-specific ATP synthase	EGD-e	
Virulence	*lmo0200*	*prfA*	Listeriolysin positive regulatory protein	EGD-e	Lemon et al. (2010)
	lmo0201	*plcA*	Phosphatidylinositol-specific phospholipase c	EGD-e	Meloni et al. (2012)
	lmo0202	*hly*	Listeriolysin O precursor	EGD-e	
	lmo0203	*mpl*	Zinc metalloproteinase precursor	EGD-e	
	lmo0204	*actA*	Actin-assembly inducing protein precursor	EGD-e	Travier et al. (2013)
	lmo0205	*plcB*	Phospholipase C	EGD-e	Meloni et al. (2012)
	lmo0433	*inlA*	Internalin A	EGD-e	Franciosa et al. (2009)
	lmo0434	*inlB*	Internalin B	EGD-e	
	lmo0582	*iap*	Invasion associated secreted endopeptidase	EGD-e	Monk et al. (2004)
	lmo2558	*ami*	Autolysin, amidase	EGD-e	Kumar et al. (2009)
Stress response	*lmo0895*	*sigB*	RNA polymerase sigma factor SigB	EGD-e	Van der Veen & Abee (2010)
	lmo0942	-	Heat shock protein 90	EGD-e	Wilson et al. (2006)
	lmo0943	*fri*	Non-heme iron-binding ferritin	EGD-e	Liu et al. (2002)
	lmo2206	*clpB*	Clp protease subunit B	EGD-e	
	lmo2068	*groEL*	Molecular chaperone	EGD-e	
	lmo2478	*trxB*	Thioredoxin reductase	EGD-e	
	lmo2461	*sigL*	RNA polymerase factor sigma-54	EGD-e	
	lmo0501	*bglG*	Transcriptional anti-terminator BglG	EGD-e	

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Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Stress response	*lmo0292*	*htrA*	Heat-shock protein htrA serine protease	EGD-e	Wilson et al. (2006)
	lmo0571	-	Methyltransferase	EGD-e	
	lmo1303	-	Cell division suppressor	EGD-e	Liu et al. (2002); Sue et al. (2012)
	lmo1439	*sod*	Superoxide dismutase	EGD-e	
	lmo1508	-	Histidine kinase	EGD-e	
	lmo1523	*relA*	(p)ppGpp synthetase	EGD-e	Taylor et al. (2002)
	lmo2785	*kat*	Catalase	EGD-e	Alonso et al. (2014)
Quorum Sensing	*lmo0048*	*agrB*	Sensor histidine kinase AgrB	EGD-e	Rieu et al. (2007)
	lmo0049	*agrD*	-	EGD-e	
	lmo0050	*agrC*	Histidine kinase	EGD-e	
	lmo0051	*agrA*	Response regulator	EGD-e	
LPXTG surface proteins	*lmo0929*	-	Sortase	EGD-e	Chang et al. (2012)
	lmo1666	-	Peptidoglycan-linked protein	EGD-e	-
Cell wall associated protein	*lmo0435*	*bapL*	Peptidoglycan binding protein	EGD-e	Jordan et al. (2008)
	lmo2504	-	Cell wall-binding protein	EGD-e	Lorenco et al. (2013)
Cell surface alterations	*lmo0721*		Fibronectin-binding protein	EGD-e	Liu et al. (2002)
Cell division associated protein	*lmo2505*	*spl*	Peptidoglycan lytic protein P45	EGD-e	Zhou et al. (2012)
	lmo2506	*ftsX*	Cell division protein FtsX	EGD-e	-

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Functional Group	Locus Tag	Gene symbol	Gene description	Reference strain	Reference
Protein translocation	*lmo2510*	*secA*	Preprotein translocase subunit SecA	EGD-e	Renier et al. (2014)
	lmo2612	*secY*	Preprotein translocase subunit SecY	EGD-e	Durack et al. (2015)
Unknown	*lmo1918*	-	Hypothetical protein	EGD-e	Ouyang et al. (2012)
	lmo2056	-	Hypothetical protein	EGD-e	
	lmo2402	-	Hypothetical protein	EGD-e	
	lmo2487	-	Hypothetical protein	EGD-e	
	lmo2553	-	Hypothetical protein	EGD-e	
	lmo2566	-	Hypothetical protein	EGD-e	