ECOLOGY, DIVERSITY, ANTIBIOTIC RESISTANCE OF LACTIC ACID BACTERIA (LAB) ISOLATED FROM PLANT SOURCES AND THEIR ROLE IN NATURAL FERMENTATION OF TEMPOYAK

by

CHUAH LI OON

**Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy**

November 2016

ACKNOWLEDGEMENTS

I owe a debt of gratitude to those who are so generous with their time and expertise. There are so many people whom I deeply respect and whose efforts are amazing. I am no poet I will admit at first, so please forgive me if the rhymes go askew.

Firstly, I would like to attribute my heartfelt gratitude to my supervisor Prof. Dr. Gulam Rusul Rahmat Ali for giving me the opportunity to work with him. His professionalism and dedicated role as a supervisor have provided me the essential guidance throughout my research; his trust in me have helped me grow and improved.

I am also deeply grateful to my co-supervisors Prof. Thong Kwai Lin, Prof. Liong Min Tze and Assoc. Prof. Dr. Rosma Ahmad, for their support, valuable inputs and encouragement in making this research a success. Their caring and easy going characteristics make me feel comfortable to approach them.

I would also extend my sincere gratefulness to my lab mates Ms. Shamila Syuhada Ahamed Kamal for her support and companion throughout my project, and Ms. Ngoi Soo Tein and Mr. Yap Kien Pong for their contribution and sharing of knowledge. I am lucky I get to work with them, and I hope that never alters. Not to be forgotten, my thanks to the lab technicians who have assisted me in the laboratories.

I am deeply grateful to all my family and friends who are there giving me endless love and support when I happened to be down and depressed. Special thanks go to Sze Yee, Yeen Chow, Miao Yin, Hui Cui, Shlrene and Asha, my friends in high places. I am also thankful for the financial support from the Ministry of Higher Education Malaysia through the MyBrain15 (MyPhD) programme, Graduate Assistance Scheme of School of Industrial Technology and also the Fundamental Research Grant Scheme (203/PTEKIND/6711238).

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF APPENDICES

LIST OF ABBREVIATIONS

A	Adenine
ANI	Average Nucleotide Sequence Identity Analysis
APT	All Purpose Tween
ATCC	American Type Culture Collection
AU	Arbitrary unit
BCCM	BCCM/LMG (Belgian Coordinated Collections of Microorganisms
BHI	Brain Heart Infusion
BLIS	bacteriocin-like inhibitory substance
bp	base pair
BPA	Baird Parker Agar
BPW	Buffered peptone water
C	Cytosine
$CaCO_3$	Calcium carbonate
CDS	predict coding sequence
CFS	Cell free supernatant
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DDH	DNA-DNA hybridisation
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DRBC	Dichloran Rose Bengal Chloramphenicol
EDTA	Ethylenediaminetetraacetic acid
FAME	fatty acid methyl ester
FAO	Food and Agriculture Organization
FESEM	Field Emission Scanning Electron Microscope
FLAB	Fructophilic lactic acid bacteria
FYP	Fructose Yeast Peptone
G	Guanine
GRAS	Generally Recognised as Safe

GYP	Glucose Yeast Peptone
JCM	Japan Collection of Microorganisms
kb	kilo base pair
LAB	Lactic acid bacteria
LMG	Laboratory of Microbiology
MAR	Multiple antibiotic resistance
MLS	Macrolides, lincosamides, streptogramins
MLST	Multi-locus sequence typing
MRS	de Man, Rogosa and Sharpe
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
OD	Optical density
OIE	World Organisation for Animal Health
PAGE	Polyacrylamide gel electrophoresis
PCA	Plate count agar
PCR	Polymerase Chain Reaction
PFGE	Pulse-field gel electrophoresis
PMSF	Phenylmethyl sulfonly fluoride
RAPD	Random Amplified Polymorphic DNA
RAST	Rapid Annotation Using Subsystem Technology
RDP	Ribosomal Database Project
RE	Restriction enzyme
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
T	Thymine
TA	Titratable acidity
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TPC	Total plate count
UPGMA	Unweighted pair-group method using arithmetic averages
UV	Ultraviolet

XLD	Xylose lysine deoxycholate
WGS	Whole genome sequencing)
WHO	World Health Organization

EKOLOGI, KEPELBAGAIAN, RINTANGAN ANTIBIOTIK BAKTERIA ASID LAKTIK (LAB) YANG DIPENCILKAN DARIPADA SUMBER TUMBUH-TUMBUHAN DAN PERANAN MEREKA DALAM PENAPAIAN SEMULA JADI TEMPOYAK

ABSTRAK

Beberapa tahun kebelakangan ini, bioprospek bakteria asid laktik (LAB) daripada sumber tumbuh-tumbuhan telah menyumbang kepada kepelbagaian LAB dan penemuan aplikasi LAB yang novel dalam industri makanan. Disebabkan LAB memainkan peranan yang penting dalam penapaian makanan, terdapat kebimbangan bahawa unsur genetik yang menyebabkan rintangan yang dikandungi oleh microbiota yang asalnya hadir dalam buah-buahan segar dan makanan tertapai akan disebarkan melalui rantai makanan. Objektif kajian ini adalah untuk mengkaji kepelbagaian dan rintangan antibiotik LAB yang dipencilkan daripada buah-buahan dan bunga-bungaan tropika yang segar, dan juga tempoyak yang dihasilkan melalui penapaian semulajadi; untuk mencirikan "bacteriocin-like substances" (BLIS) yang dihasilkan oleh LAB yang dipencilkan, dan ciri-ciri teknologi yang lain. Kajian ini juga dijalankan untuk menentukan dinamik pelbagai spesies LAB semasa penapaian semulajadi tempoyak. Berdasarkan analisis filogenetik, pencirian fenotip dan perbandingan purata identiti nukleotida (ANI), satu genus novel dengan dua spesies, yang dipunyai oleh keluarga *Streptococcaceae*, telah dikenal pasti. Nama genus baru dan dua spesies telah dicadangkan sebagai *Anthococcus rusulii* (dipencilkan daripada bunga durian dan bunga raya) dan *Anthococcus penangensis* (dipencilkan daripada bunga raya). Ahli LAB dalam genus-genus *Lactobacillus*, *Weissella*, *Lactococcus*, *Leuconostoc* dan *Enterococcus* juga dipencilkan daripada buah-buahan and bunga-bungaan tropika yang segar. Genotyping mempamerkan kepelbagaian genetik yang tinggi dalam

Lactobacillus plantarum dan *Weissella* spp. yang merupakan species dominan yang dipencilkan daripada buah-buahan and bunga-bungaan. Kajian ini juga menonjolkan kebimbangan bahawa unsur genetik yang menyebabkan rintangan yang dikandungi oleh microbiota yang aslinya hadir dalam buah-buahan segar akan disebar melalui rantai makanan. LAB yang mempunyai rintangan terhadap pelbagai antibiotik telah dipencilkan daripada buah-buahan segar. Serupanya, LAB yang mempunyai rintangan terhadap pelbagai antibiotik juga dipencilkan daripada tempoyak. Dalam kajian ini, kajian bioprospek telah dilakukan untuk penemuan metabolit LAB yang dapat digunakan dalam industri. Enam *Lactococcus lactis* subsp. *lactis* menghasilkan BLIS yang mempamerkan kesan antagonistik terhadap *Listeria* spp., *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Streptococcus agalactiae* dan *Streptococcus pyogenes*. Analisis PCR menunjukkan bahawa gen untuk nisin F dan Z wujud. Ciri-ciri teknologi yang dikehendaki (acidolytic and caseinolytic dan toleransi hempedu) telah menunjukkan potensi mereka sebagai probiotik dan dalam penghasilan makanan berfungsi. Semasa penapaian semulajadi tempoyak, ahli-ahli LAB yang berlainan mendominasi pada peringkat penapaian yang berlainan. Susulan dari heterofermentors (*Leuconostoc mesenteroides* dan *Fructobacillus durionis*) ke homofermentor (*Lb. plantarum*) telah diperhatikan. Penapaian tempoyak secara semulajadi tidak menyokong pertumbuhan *Salmonella* spp., *Listeria monocytogenes* dan *Staphylococcus aureus* disebabkan oleh penghasilan asid organik seperti asid laktik, asetik dan propionik, dan pengurangan pH secara mendadak semasa penapaian.

ECOLOGY, DIVERSITY, ANTIBIOTIC RESISTANCE OF LACTIC ACID BACTERIA (LAB) ISOLATED FROM PLANT SOURCES AND THEIR ROLE IN NATURAL FERMENTATION OF TEMPOYAK

ABSTRACT

In recent years, bioprospecting of lactic acid bacteria (LAB) from plant sources contributes to the diversity of LAB and the discovery of novel application of LAB in food industries. As LAB play an important role in food fermentation, there are concern of dissemination of antibiotic resistance determinants harboured by the indigenous microbiota present in fresh fruits and fermented food through the food chain. The objectives of this study were to investigate the diversity and antibiotic resistance of LAB presence in fresh tropical fruits and flowers, as well as tempoyak produced by natural fermentation; to characterise the bacteriocin-like substances (BLIS) produced by these LAB isolates, and other technological properties. This study was also undertaken to determine the dynamics of different LAB species during natural fermentation of tempoyak. Based on phylogenetic analysis, phenotypic characterisation and comparison of average nucleotide identity (ANI), a novel genus with two species belonging to the family *Streptococcaceae*, were identified. The name of the new genus and two species were proposed as *Anthococcus rusulii* (isolated from durian flowers and hibiscus) and *Anthococcus penangensis* (isolated from hibiscus flowers). Members of the genera *Lactobacillus, Weissella, Lactococcus, Leuconostoc,* and *Enterococcus* were also isolated from fresh tropical fruits and flowers. Genotyping using PFGE demonstrated a high polymorphism among the predominant *Lactobacillus plantarum* and *Weissella* spp. isolated from flowers and fruits. The present study also highlighted the concern of dissemination of antibiotic determinants harboured by indigenous microbiota present in fresh fruits through the food chain. Multidrug-

resistant LAB isolates harbouring determinants responsible for multiple antibiotics resistance were isolated from fresh fruits. Similarly, multidrug-resistant LAB isolates were also isolated from tempoyak. In this study, bioprospecting studies was performed in search of LAB metabolites relevant to industry. Six *Lactococcus lactis* subsp. *lactis* isolates produced BLIS which were antagonistic against *Listeria* spp., *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Streptococcus agalactiae* and *Streptococcus pyogenes*. PCR analysis showed the presence of nisin F and Z genes. Desired technological properties (acidifying, caseinolytic activities and bile tolerance) were also observed, suggesting their potential as a probiotic and production of functional foods. During natural fermentation of tempoyak, different members of LAB were predominant at different stages of fermentation. A succession from heterofermentors (*Leuconostoc mesenteroides* and *Fructobacillus durionis*) to homofermentor (*Lb. plantarum*) was observed. Naturally fermented tempoyak did not support the growth of *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* due to the production of organic acids such as lactic, acetic and propionic acids and drastic reduction in pH during the fermentation.

CHAPTER 1

INTRODUCTION

1.1 Background

Lactic acid bacteria (LAB) are a heterogenous group of strictly fermentative, fastidious and acid-tolerant organisms inhabiting nutrient-rich niches. Traditionally, LAB are isolated from dairy products and fermented foods, or alternative sources such as faecal samples and plant sources (Holzapfel and Wood, 2014). Indigenous LAB present both on the external surface and internal tissues of fruits, flowers, leaves and wood, and maybe part of epiphytic microflora present in plants (Martins *et al.*, 2013; Postmaster *et al.*, 1997; Zhang et al., 2010), or introduced by insect vectors (McFrederick *et al.*, 2012). LAB genera commonly isolated from plant sources includes *Lactobacillus*, *Leuconostoc*, *Weissella* and *Enterococcus* (Tyler et al., 2016, Williams et al., 2013, Müller et al., 2001). Prevalence and enumeration of LAB populations in flowers have not been extensively studied (Endo *et al.*, 2009; Endo *et al.*, 2010; Endo *et al.*, 2011b; Kawasaki *et al.*, 2011a; Kawasaki *et al.*, 2011b; Neveling *et al.*, 2012; Vásquez *et al.*, 2012). In raw fruits, LAB constitutes small percentage of the indigenous microbiota and LAB population usually ranged from 2-5 log CFU/g of fruits (Di Cagno *et al*., 2010; 2011; Nyanga *et al.*, 2007). Despite their presence in low numbers on plant tissues (Dias et al., 2015), numerous studies have reported on isolation of LAB with probiotic or desired biotechnological properties from natural plant sources (Tyler et al., 2016, Hwanhlem et al., 2014, Teneva-Angelova and Beshkova, 2015). Isolation of LAB from novel sources is hence, of crucial importance

as this approach helps to explore the natural biodiversity and valuable strains with potential technological properties.

Various researchers have reported that the association of certain LAB genera with specific niches is most likely due to adaptation to their ecological niches. Adaptation of LAB to ecological niches may be attributed to the utilisation of relevant carbohydrate sources. For instance, fructophilic LAB (FLAB) adapt to fructose-rich niches via reductive evolution and conservation of genes aid in the fructose metabolism (Kim et al., 2013, Endo et al., 2015). In addition, acquisition or presence of genes conferring bacterial fitness, such as biofilm synthesis, adhesion, bacteriocin and lysozyme, antibiotic resistance, transmembrane protein and phage-related proteins, may also aid in LAB adaptation to various ecological niches (Vásquez et al., 2012, Asenjo et al., 2016b, Butler et al., 2013). Reasearchers are of opinion that interference-based or resource-based competitions with other microorganisms might be key to production of bacteriocin and other antagonistic coumpounds as predation tools, as observed in many LAB (Hibbing et al., 2010, Leisner and Haaber, 2012, Cornforth and Foster, 2013).

Over the past decades, LAB have been extensively utilised in food products for both human and animal nutrition. LAB have traditionally been used for preservation, to enhance nutritional values, flavours and texture of fermented foods (Gerez et al., 2009, Zhang et al., 2008, Steinkraus, 1997). The ability of LAB to prevent the growth of foodborne pathogens and spoilage microorganisms in a multitude of foods may be atributed to the antagonistic properties of LAB metabolites or their end-products such as bacteriocins, organic acids, ethanol, carbon dioxide, hydrogen peroxide, acetoin and diacetyl (Stoianova et al., 2012, Piard and Desmazeaud, 1991, Piard and Desmazeaud, 1992).

In food fermentation, LAB are either added as starter cultures, or, by back-slopping or relying on natural fermentation under conditions favouring the growth of desired microbiota, in more traditional or artisanal productions. Natural fermentation might contribute to the diversity of microflora present in the fermented food products, however, the indigenous microflora is not consistent due to the uncontrolled fermentation process. Researchers have observed an increase in LAB population from the ripe fruit to the fermented fruit pulp during natural fermentation (Nyanga et al., 2007). They are of the opinion that indigenous LAB present in fruits are responsible in natural fermentation. As LAB are present in abundance in many fermented foods, researchers are concern that indigenous LAB in fermented foods may act as reservoir of antibiotic resistant deterrminants which can be potentially transferred through food chain to human gut pathogens (Nawaz et al., 2010, Patel et al., 2012, Gueimonde et al., 2013). In addition, the emergence of antibiotic-resistant LAB strains used as starter culture and probiotics in food industry has highlighted the importance of characterisation of antibiotic resistant profiles and transferable genetic determinants harboured by food-associated LAB (Patel et al., 2012, Hummel et al., 2007).

1.2 Problem statements

LAB inhabit various ecological niches and the ability of LAB to adapt in various ecological niches may contribute to differences in taxonomic attributes and characteristics that confer fitness and their potential application in industries. Bioprospecting of LAB isolates from exotic ecological niches (in this case, tropical flowers, durian and soursop fruits) have not been previously reported. Thus, tropical flowers and fruits could be potential sources for the discovery of novel strains with

taxonomic and industrial importance. In addition to highlighting the importance of bioprospecting, this study also highlights the concerns for dissemination of antibiotic resistance determinants harboured by indigenous microbiota present in fresh fruits and fermented food throughout the food chain, especially when fruits and fermented foods are usually consumed raw or without lethal heat treatment. Moreover, microbial safety of tempoyak which is mainly produced using fallen fruits, is of great concern. Previous studies on tempoyak mainly focused on the physicochemical properties of tempoyak (Neti et al., 2011, Wasnin et al., 2014) and they have concluded that *Lb. plantarum* was predominant in tempoyak (Leisner et al., 2005, Yuliani and Dixon, 2011). The changes in LAB population during tempoyak fermentation and the safety aspects of tempoyak, however, have not been considered in previous studies.

1.3 Objectives

In order to study the biodiversity of LAB present in plant sources and their role in natural fermentation, reliable methods for the identification of LAB to species and strains levels and dynamics of microbial populations occurring during natural fermentation, are of crucial importance. In this study, identification of LAB species was performed by using polyphasic approaches consisting of both phenotypic and genotypic methods for the precise identification of LAB to species and strain levels.

The objectives of this study are:

a) To determine the diversity of LAB isolated from fresh tropical fruits and flowers, and their antibiotic resistance profile.
b) To identify and characterise the novel LAB species isolated from fresh flowers.

c) To characterise the antimicrobial properties of bacteriocin-like substances (BLIS) and technological properties of *Lactococcus lactis* isolated from fresh tropical flowers and fruits.

d) To examine the biodiversity of LAB present in naturally fermented tempoyak and to determine the presence of multidrug-resistant LAB isolates isolated at different stages of tempoyak fermentation.

e) To elucidate the microbial changes occurring during natural fermentation of tempoyak and the microbial safety of the naturally fermented tempoyak.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic acid bacteria (LAB)

LAB are described as Gram-positive, acid tolerant, of non-aerobic habit, nonsporing, nonrespiring cocci or rods that produce lactic acid as the major end product during the fermentation of carbohydrates. Typically, they are non-motile and do not reduce nitrate (Hammes and Hertel, 2006). LAB are fastidious organisms since they require complex nutrients as their growth factors, such as carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins. They are strictly fermentative since they have no respiratory system and rely mostly on substrate level phosphorylation during sugar(s) fermentation to generate and provide energy (Saier et al., 1996, Stiles and Holzapfel, 1997). LAB carry out lactic acid fermentation and were among the first microorganisms to be used in food manufacturing (Konings et al., 2000). The most common LAB genera in food fermentations are *Lactobacillus*, *Lactococcus, Leuconostoc, Bifidobacterium, Enterococcus, Oenococcus, Pediococcus*, *Streptococcus, Tetragenococcus, Weissella* and *Carnobacterium*. It is assumed that most representatives of this group do not pose any health risk to man, and hence they were granted as "Generally Recognized as Safe (GRAS)" organisms (Dekker et al., 2009, Chouraqui et al., 2008, Zhou et al., 2000).

2.1.1 Ecology and biodiversity of LAB

LAB is a highly diverse group which diversity is contributed by variable characteristics such as morphology, carbohydrate metabolism, tolerance to oxygen. The genus *Lactobacillus* is by far (September 2016) the largest genus in LAB. Consists of 221 species of valid standing nomenclature in the genus, *Lactobacillus* spp. greatly contributes to the biodiversity of LAB. LAB inhabit various nutrient-rich environments such as fermented foods, plant materials, dairy products, and gastrointestinal tract of animals (Hammes and Hertel, 2006). Similarly, members of *Pediococcus* inhabit a variety of ecological niches. Owing to the inability to utilise lactose in some members of the genus *Pediococcus*, their presence, however, in dairy sources is restricted (Holzapfel et al., 2009). As for *Lactococcus*, they are best known as dairy-associated LAB species, though *Lactococcus* spp. has been previously isolated from plant sources. However, isolation of *Lactococcus* spp. from faecal or soil samples is rare (Tuber, 2009). Association of certain LAB genera with specific niches is most likely due to adaptation to their ecological niches (Section 2.2). The various species of LAB used as starter cultures in a variety of foods in presented in Table 2.1. Information provided in Table 2.1 emphasises the significance of LAB in the food industry and also on the diversity of LAB.

Table 2.1 Diversity of LAB in different traditional fermented foods

Food sources	LAB species isolated	
Dairy products		
Yogurt	*Strep. thermophiles*, *Lactobacillus* (*Lb. delbrueckii subsp. bulgaricus*, *Lb. acidophilus*, *Lb. casei*, *Lb. rhamnosus*, *Lb. gasseri*, *Lb. johnsonii*), *Bifidobacterium* spp.	(Angelakis et al., 2011, Tamime, 2007)
Cheese	*Lactococcus* (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*), *Lactobacillus* (*Lb. delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. casei*, *Lb. salivarius*, *Lb. plantarum*), *Strep. thermophiles*, *Enterococcus* (*Ent. faecium*, *Ent. durans*), *Leuconostoc* spp.	(Parente and Cogan, 2004, Quigley et al., 2011)
Kefir	*Leuconostoc* (*Leu. mesenteroides*, *Leu. lactis*), *Lactobacillus* (*Lb. helveticus*, *Lb. kefiranofaciens*, *Lb. kefiri*, *Lb. casei*, *Lb. plantarum*), *Lc. lactis*	(Zhou et al., 2009, Gao et al., 2012)
Vegetables and fruits		
Sauerkraut	*Leuconostoc* (*Leu. mesenteroides, Leu. citreum, Leu. argentinum*), *Lactobacillus* (*Lb. plantarum, Lb. curvatus, Lb. brevis, Lb. paraplantarum, Lb. coryniformis*), *Pediococcus pentasaceus, Weissella* sp.	(Plengvidhya et al., 2007)
Kimchi	*Leuconostoc* (*Leu. mesenteroides, Leu. citreum, Leu. carnosum, Leu. gasicomitatum, Leu. inhae, Leu. gelidum, Leu. kimchii, Leu. lactis, Leu. hozapfelii*), *Lactobacillus* (*Lb. sakei, Lb. plantarum, Lb. curvatus*), *Weissella* (*W. koreensis, W. cibaria, W. soli, and W. confusa*)	(Cho et al., 2006, Jeong et al., 2013)
Tempoyak	*Lactobacillus* (*Lb. plantarum, Lb. brevis, Lb. mali, Lb. fermentum, Lb. durianis, Lb. corynebacterium, Lb. fersantum, Lb. casei*), *Fructobacillus durionis*, *Weissella* paramesenteroides, *P. acidilactici*, *Leu. mesenteroides*	(Endo and Okada, 2008, Leisner et al., 2002, Leisner et al., 2001, Leisner et al., 2005, Wirawati, 2002, Mohd Adnan and Tan, 2007, Ekowati, 1998)

Table 2.1 (continue)

Food sources	LAB species isolated	
Cereal foods		
Sourdough	*Lactobacillus* (*Lb. plantarum, Lb. fermentum, Lb. paralimentarius, Lb. sanfranciscensis, Lb. amylovorus, Lb. frumenti, Lb. casei, Lb. helveticus, Lb. pontis, Lb. reuteri, Lb. panis, Lb. zymae*)	(De Vuyst et al., 2009, Viiard et al., 2016)
Masa Agria (fermented maize dough)	*Lactobacillus* (*Lb. plantarum, Lb. fermentum, Lb. gallinarum, Lb. helveticus, Lb. delbrueckii, Lb. siliginis, Lb. curvatus, Lb. vaccinostercus*), *Leu. citreum*, *Weissella* (*W. confusa*, *W. beninensis*, *W. fabaria*, *W. fabalis*), *Lc. lactis*	(Chaves-Lopez et al., 2016)
Cassava and legumes		
Sour cassava starch	*Leu. citreum, Leu. mesenteroides, Lb. plantarum, Lc. lactis*	(Lacerda et al., 2011)
Miso	*Pediococcus acidilactici*, *Tetragenococcus halophilus*, *W. confusa*, *Lb. fructivorans*	(Takumi et al., 2003)
Chinese soy sauce	*Weissella* (*W. cibaria, W. kimchi, W. salipiscis*), *Lactobacillus* (*Lb. fermentum, Lb. plantarum, Lb. iners*), *Streptococcus thermophilus*, *Tetragenococcus halophilus*, *Lactococcus, Enterococcus, Pediococcus. Leuconostoc* spp.	(Sulaiman et al., 2014, Tanaka et al., 2012)
Meat and seafood		
Alheira	*Lactobacillus* (*Lb. plantarum, Lb. paraplantarum, Lb. brevis, Lb. rhamnosus, Lb. sakei, Lb. zeae, Lb. paracasei*), *Enterococcus* (*Ent. faecalis, Ent. faecium*), *Pediococcus* (*P. pentosaceus*, *P. acidilactici*), *Weissella* (*W. cibaria, W. viridescens*), *Leu. mesenteroides*	(Albano et al., 2009)
Budu	*Lb. plantarum, Lb. delbrueckii*), *P. pentosaceus, P. acidilactici, Lc. lactis*	(Sim et al., 2015)

Adopted from (Tamang et al., 2016)

2.1.2 Phylogenetics and systematics of LAB

In general, LAB are constituted of a genetically diverse or heterogeneous group of bacteria (Temmerman et al., 2004) with low guanine plus cytosine (G+C) contents varying from 34 to 53 mol %, encompassing rod- and coccoid-shaped bacteria. Albeit LAB are not a strictly defined taxonomic grouping, the term LAB includes a number of phylogenetically-related genera with several biochemical and ecological features in common. Phylogenetically, LAB consist of member belongs to the *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Streptococcaceae* (Holzapfel and Wood, 2014). The genus *Bifidobacterium* is unrelated to LAB phylogenetically and genetically in the context of its high G+C content in deoxyribonucleic acid (DNA), using a unique metabolic pathway for sugar metabolism. However, *Bifidobacterium* species are often considered to be LAB on account of the probiotic features they play by living in the gastrointestinal tract of human and animals (Holzapfel and Wood, 2014).

The heterogeneity of genera recognised as members of LAB leads to the need of accurate identification and characterisation of LAB. Although conventional phenotypical identification has been proven to be useful for certain LAB, it is limited in terms of its discriminating ability, accuracy and ambiguity of some techniques (Stackebrandt et al., 2002, Tindall et al., 2010). As the number of LAB are ever increasing and the realization that phenotypic identification is not reliable, microbiologists have resorted molecular techniques for accurate and rapid identification of LAB (Tindall et al., 2010, Chun and Rainey, 2014). Genotypic approaches eliminate or reduce variations due to complex growth conditions of LAB and also allow for increase degree of discriminatory power from species to strain level,

and thus giving rise to a much higher taxonomic resolution for LAB (Chun and Rainey, 2014, Temmerman et al., 2004). However, by employing only genotypic approaches, it is unlikely or impossible to know the general growth characteristics of the identified LAB, especially novel species leading to incomplete description profile of the novel LAB. By using polyphasic approach in LAB systematics, predominant features of one method can compensate the shortcomings of another method.

2.1.3 Phenotypic characterisation of LAB

Traditionally, LAB have been classified on the basis of phenotypic properties such as morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, and fermentation of various carbohydrates (Holzapfel et al., 2001). Based on their hexose catabolism, LAB can be divided into two major groups, the homofermentative and heterofermentative LAB. Homofermentative LAB produces lactic acid from glucose in almost stoichiometric quantities, but in the presence of limited nutrient availability, formic and acetic acids, and ethanol are also being produced. Heterofermentative LAB ferments glucose into lactic acid, ethanol, CO_2, and in some cases, acetic acid (Piard and Desmazeaud, 1991). In heterofermentative LAB, lactic acid comprises about 70% of the catabolic end-products. Recently, the term “fructophilic” has been assigned to describe a specific group of LAB which has preference towards fructose, and also possess unique characteristics which are different from commonly described LAB (section 2.2). These unique characteristics are most likely attributed to the habitat of these fructose-loving LAB species. As these organisms inhabitat fructose-rich environments, they have evolved and adapted in fructose-rich environment (Endo et al., 2015).

2.1.4 Genotypic identification and characterisation of LAB

Current advances in molecular biology provides a bewildering range of nucleic-acid-based assays for rapid and accurate identification and characterisation. Sequencing of the 16S rRNA gene is integral in microbial taxonomy and identification to genus/species level (Janda and Abbott, 2007, Armougom, 2009). DNA-DNA hybridisation (DDH) developed by McCarthy and Bolton (1963) has been the cornerstone for differenting closely-related species. Both methods are particularly useful in detection of misclassification among named species and establishment of phylogenetic associations (Kostinek et al., 2005, Dellaglio et al., 2006, Endo and Okada, 2008). Organisms with a low character match to any described species are not classifiable and thus its phylogenetic position should be re-evaluated to obtain taxon-specific characters (Stackebrandt and Goebel, 1994). *The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less* ΔT_m (Tindall et al., 2010, Wayne et al., 1987). Stackebrandt and Goebel (1994) asserted that 97% 16S rDNA sequence similarity [or more recently, 98.7 to 99% was recommended (Stackebrandt and Ebers, 2006)] as a cut-off value, which corresponded to DNA reassociation value of 70%, for species delineation. In addition, Stackebrandt et al. (2002) recommended that sequencing of housekeeping genes offers great promise to genomically differentiate closely-related species. It has been proposed that *RecA* gene could be used as a phylogenetic marker (Eisen, 1995), and satisfactory results had been obtained in various bacterial genera, including lactobacilli (Torriani et al., 2001).

Probiotic ability or technological properties of LAB are often strain-specific. Genotypic techniques have been extensively employed to determine the genetic

relatedness of food-associated LAB, most prevalently in studying microbial diversity or monitoring starter culture dynamics during fermentation (Di Cagno et al., 2014, Blana et al., 2014). Various molecular typing methods have been developed based on DNA hybridisation, restriction enzyme analysis, PCR or direct sequencing of DNA, with the latter possessing the highest accuracy. Molecular typing methods such as random amplified polymorphic DNA (RAPD)-PCR and pulse-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and whole genome sequencing (WGS) are extensively used (Moisan and Moineau, 2012, Agaliya and Jeevaratnam, 2013, Blana et al., 2014, Bull et al., 2014), in determining genetic diversity or variability. Even though RAPD lacks reproducibility, it is appreciated because it is simple, cheap, rapid, and considerably accurate for the typing of LAB strains (Sabat et al., 2013). MLST allows for unambiguous typing and superior taxonomic resolution due to the availability of an internationally standardized nomenclature. PFGE is regarded as the gold standard for molecular typing owing to its excellent discriminatory index (Michael et al., 2006, Jensen et al., 2009, Picozzi et al., 2010). In addition, WGS is priced for its high accuracy as shown by excellent correlation between clusters delineated based on WGS and DNA-DNA hybridisation studies (Goris et al., 2007).

The factors most important for achieving the desired results must be considered before choosing a molecular technique. Methods that are accurate in reflecting genetic variation, and are rapid and inexpensive are necessary. Identification of LAB by sequencing of the 16S rRNA and housekeeping genes are necessary for accurate identification. As the costs of WGS continue to decline (Service, 2006), WGS should be widely used in species delineation and typing of LAB strains for better understanding of key aspects of LAB.

2.2 Niche-specific adaptations of LAB

LAB are highly adaptable bacteria (van Reenen and Dicks, 2011). During adaptation and evolution of LAB, reduction of genome sizes are known to occur (Pfeiler and Klaenhammer, 2007). The relatively small size of commonly described LAB genomes, 1.7-3.3 megabase pairs (Mbp), consisting of numerous genes ranging from 1,600 to 3,000 (van Reenen and Dicks, 2011), is usually attributed to genes loss (Makarova et al., 2006) caused by their continuous adaptation to nutrient-rich environments. One example of such adaptation is fructophilic LAB (FLAB), which underwent reductive evolution during adaptation to fructose-rich niches. According to Chelo et al. (2010), the estimated genome sizes in *Fructobacillus* spp. (*F. fructosus*, *F. ficulneus* and *F. pseudoficulneus*) are in the range of 1.41-1.55 Mbp, which are smaller compared to other LAB. Genomic analysis conducted by Tamarit et al. (2015) on *Lb. kunkeei* revealed an extreme reduction in genomic size due to loss of 509 genes encoding various proteins compared to its common ancestor and closely-related *Lb. sanfrancisensis* during evolution.

Conserved genes present in LAB may also aid in the adaptation of these organisms to less complex environment containing abundance of specific simple sugars. Genes encoding the phosphotransferase (PTS) proteins are highly conserved in many LAB species such as *Lactococcus lactis* (Aleksandrzak-Piekarczyk et al., 2011), *Oenococcus oeni* (Jamal et al., 2013) and *Lactobacillus bulgaricus* (Leong-Morgenthaler et al., 1991). PTS is regarded as a predominant pathway for carbohydrate uptake in LAB, and is also best known for its efficiency in the transportation of lactose (Aleksandrzak-Piekarczyk et al., 2011). Analysis of a draft genome of fructophilic *Lb. florum* revealed that the genes encoding enzymes essential for fructose metabolism are

highly conserved when compared to the closely-related *Lactobacillus sanfranciscensis* (Kim et al., 2013). In addition, adaptation of LAB to ecological niches may also be attributed to the acquisition of plasmids which enhanced the utilisation of relevant carbohydrates. For instance, Flórez and Mayo (2015) revealed that the adaptation of *Lactococcus garvieae* in a dairy environment was due to the presence of plasmid which complements lactose assimilation. They also reported that similar plasmid with complete nucleotide identity was present in *Lactococcus lactis*.

It is important to note that adaptation by LAB to various ecological niches might include a variety of other parameters rather than the mere ability to utilise relevant carbohydrates. Acquisition or presence of genes conferring bacterial fitness, such as biofilm synthesis, adhesion, bacteriocin and lysozyme, antibiotic resistance, transmembrane protein and phage-related proteins, has been reported in *Lb. kunkeei* (Asenjo et al., 2016a, Butler et al., 2013, Vásquez et al., 2012). It is also important to consider that interference-based or resource-based competitions with other microorganisms might be parameters to consider (Hibbing et al., 2010). Flowers and fruits as well as fermented foods can also be considered as rich sources of nutrients which might limit the relevance of such competitions. Another hypothesis is the production of antagonistic compounds as predation tools to obtain nutrients from lysed target cells as observed in many lactic acid bacteria (Leisner and Haaber, 2012).

2.2.1 Fructophilic LAB (FLAB)

The term “fructophilic LAB” (FLAB) designate a group of LAB with a preference for fructose as carbohydrate substrate. FLAB consist of all members of the genus *Fructobacillus* within the family of *Leuconostoceae* (*F. fructosus*, *F. ficulneus*,

F. durionis, *F. pseudoficulneus* and *F. tropaeoli*) (Endo et al., 2012) and six members of *Lactobacillus* within the order of *Lactobacillales* (*Lb. kunkeei*, *Lb. florum*, *Lb. plantarum*, *Lb. fabifermentans*, *Lb. cacaonum* and *Lb. brevis*) (Endo et al., 2012, Edwards et al., 1998, Neveling et al., 2012, Lefeber et al., 2011b, Endo et al., 2009, Endo et al., 2010). Metabolically, FLAB are obligately heterofermentative LAB that are grouped as either "obligately" or facultatively fructophilic, based on their ability to grow in the presence of glucose and their ability for ethanol production (Endo et al., 2009). *Fructobacillus* spp. and *Lb. kunkeei* have been classified as "obligately" FLAB (Endo et al., 2012, Endo et al., 2011a, Endo and Okada, 2008) while, *Lb. florum* and one strain of *Lb. brevis* as facultatively FLAB (Neveling et al., 2012, Endo et al., 2010).

2.2.2 Habitats of FLAB

Figure 2.1 gives an overview on the various habitats from which FLAB have been isolated. Recently, fructophilic *Lb. florum* was isolated from Valencia orange leaves (Kim et al., 2013) and this finding is unique as aerial surface of orange leaves is not generally regarded as a fructose-rich niche.

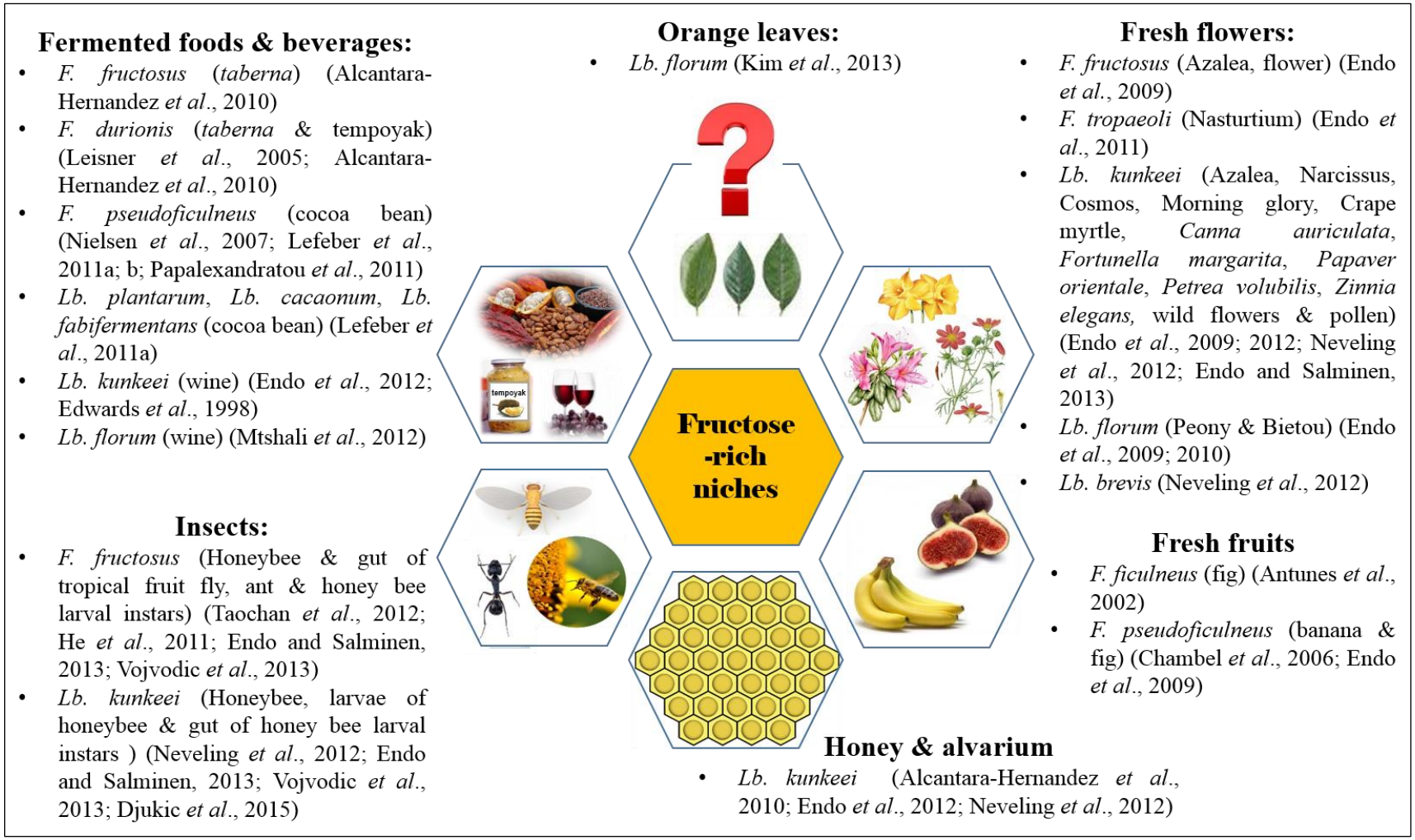

Figure 2.1 Ecological niches of FLAB

2.2.3 Unique characteristics of FLAB

FLAB grow well on fructose or on glucose in the presence of external electron acceptor (e.g. pyruvate, fructose or oxygen) but poorly on glucose in the absence of external electron acceptors. They prefer aerobic conditions rather than anaerobic conditions for growth, and generally only metabolise a limited number of carbohydrates (Endo et al., 2009). These characteristics are unusual in LAB, which are obligately or facultatively anaerobic and grow well on glucose, and thus these phenotypic characteristics are regarded as unique characteristics of FLAB.

2.2.3 (a) Poor carbohydrates fermentation abilities and the preference of fructose

Generally speaking, all FLAB utilise only a limited number of carbohydrates including both fructose and glucose. Fructose is utilised preferentially and metabolized at a faster rate compared to glucose (Endo et al., 2012). *Lb. florum* utilises only fructose and glucose while potassium gluconate is weakly utilised (Endo et al., 2010). *F. fructosus*, *F. pseudoficulneus* and *F. tropaeoli* utilises only fructose, glucose and mannitol (Antunes et al., 2002, Chambel et al., 2006, Endo & Okada, 2008, Endo et al., 2009, 2011). However, De Bruyne et al. (2009) reported that *Lb. fabifermentans* isolated from Ghanian cocoa fermentations were able to utilise up to 18 different carbohydrates. Similarly, Neveling et al. (2012) reported that the fructophilic *Lb. brevis* and certain strains of *Lb. kunkeei* were able to utilise up to 14 and 15 carbohydrates, respectively. This suggests that there are FLAB species that may represent intermediaries on the evolutionary path during adaptation to fructose-rich niches.

2.2.3 (b) Require external electron acceptors (pyruvate, fructose or oxygen) for glucose metabolism

"Obligately" FLAB need an external electron acceptor for better metabolism or utilisation of glucose. On the other hand, facultatively FLAB grow on fructose or glucose in the presence or absence of pyruvate or O_2 as an electron acceptor but growth will be enhanced by the presence of electron acceptors (Endo et al., 2009). Absence of the bifuntional acetaldehyde/alcohol dehydrogenase gene (*adhE*) in the "obligately" FLAB prevents the conversion of acetyl-CoA, and subsequent conversion of acetyldehyde to ethanol, thus inhibiting reoxidation or recycling of NAD(P)H, resulting in the lack of NAD+ required for glucose metabolism (Endo et al., 2015, Maicas et al., 2002, Richter et al., 2003b, Richter et al., 2003a, Richter et al., 2001, Veiga-da-Cunha et al., 1993, Zaunmuller et al., 2006). The presence of external electron acceptors such as pyruvate (leading to formation of lactate), fructose (leading to formation of mannitol) and O_2 enhances glucose metabolism in "obligately" FLAB. The carbohydrate metabolism pathway of *F. durionis*, an "obligately" FLAB is presented in Figure 2.2. *F. durionis* also harbour the genes encoding for the formation of erythritol from erythrose-4-P and ultimately fructose-6-P. Thus, erythritol (formed from erythose-6-P and ultimately fructose-6-P may serve as an additional pathway in this genus for recycling NADP(H).

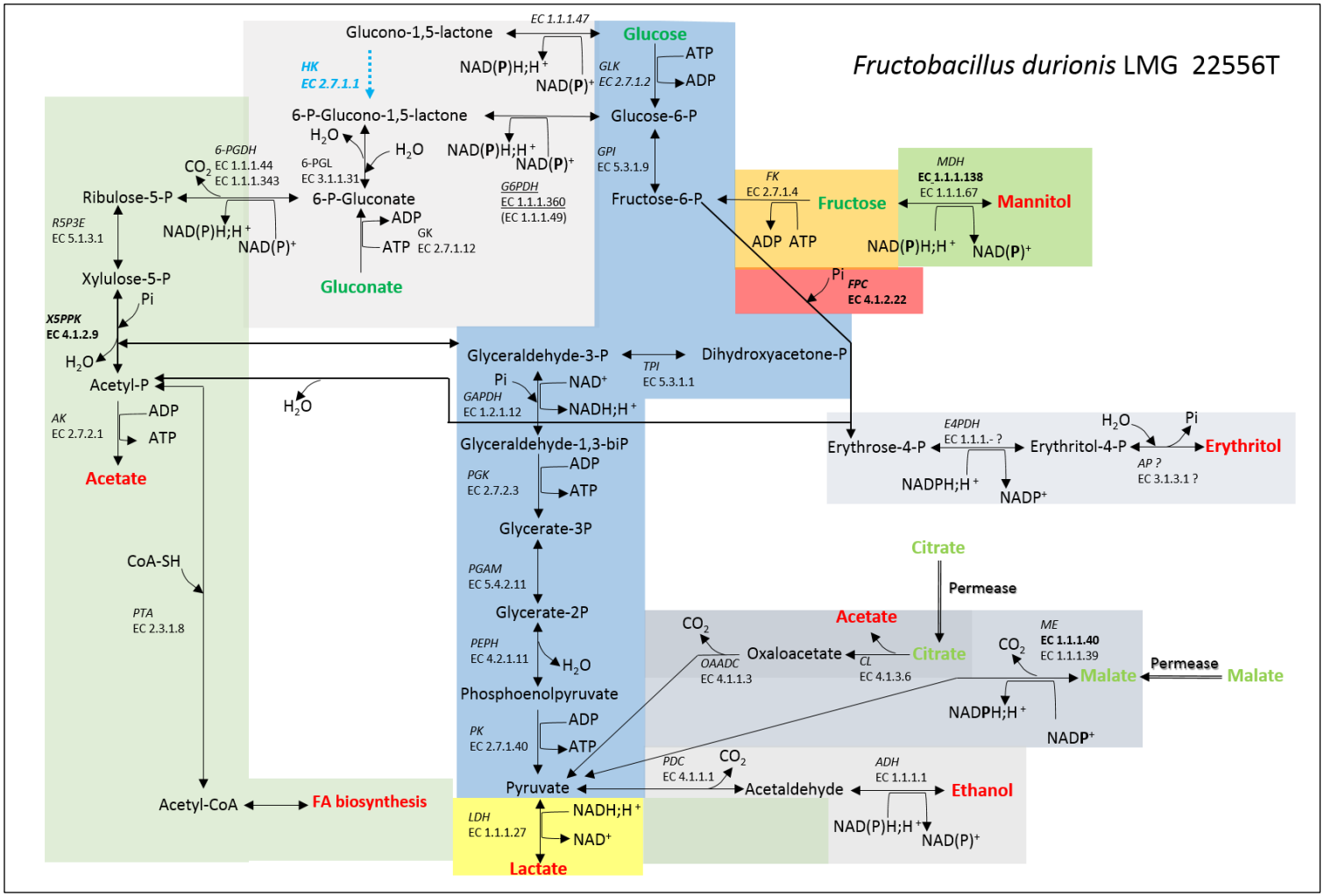

Figure 2.2 Carbohydrate metabolism pathway of *F. durionis* LMG 22556^{T}

2.2.3 (c) Types of fermentative metabolisms and end-products from glucose utilisation

The other distinguishing feature of FLAB is the production of ethanol from glucose. "Obligately" FLAB mainly produce lactic acid and acetic acid, with very little or no ethanol (Endo et al., 2009) but facultatively FLAB produce substantial amount of ethanol from glucose metabolism (Endo and Okada, 2008). Genomic analysis revealed the absence of genes encoding the subunits for pyruvate dehydrogenase complex in "obligately" FLAB (*Fructobacillus* spp. and *Lb. kunkeei*). This suggests that pyruvate generated during glucose metabolism is metabolised to lactate instead of being channelled into the TCA cycle. The absence of the bifuntional acetaldehyde/alcohol dehydrogenase gene (*adhE*) and acetaldehyde dehydrogenase activity in the fructophilic *Fructobacillus* spp. (Endo et al., 2015) are consistent with findings that no alcohol is produced by "obligately" FLAB, despite they being obligately heterofermentative (Endo et al., 2009). Facultative FLAB, however, produce predominantly lactic acid and small amounts of acetic acid, as well as a substantial quantity of ethanol, which is unique when compared to "obligately" FLAB (Neveling et al., 2012).

2.2.4 FLAB in food fermentation

In fruit fermentations, FLAB maybe one of the predominant bacterial groups at the onset or during initial stage of the fermentations. This has been shown during natural (spontaneous) fermentation of cocoa bean (Lefeber et al., 2011a, Nielsen et al., 2007, Papalexandratou et al., 2011a). Almost all of the reported FLAB-associated

fermentations are natural, except for *F. pseudoficulneus* which has been suggested as a starter culture in controlled cocoa bean fermentation, using cocoa pulp simulation medium (Lefeber et al., 2011b). *F. pseudoficulneus* is well-adapted to the cocoa pulp ecosystem which contains high levels of fructose, which could serve as an energy source as well as external electron acceptor. Indeed, *F. pseudoficulneus* has been previously isolated from naturally fermenting cocoa beans (Lefeber et al., 2011a, Nielsen et al., 2007, Papalexandratou et al., 2011a).

In addition, FLAB have been isolated from fermented beverages (Figure 2.1). Mtshali et al. (2012) reported that *Lb. florum* strains isolated from grapes and wines harbours the genes responsible for the encoding of malolactic enzyme, peptidases, phenolic acid decarboxylase and citrate lyase, which associated with proteolysis and peptidolysis in wine-making (Juillard et al., 1995) and organoleptic properties in wine-making (Björkroth and Holzapfel, 2006). Also, a strain of *F. fructosus* has been examined for its ability to produce mannitol from fructose during fermentation of wheat bran (Prückler et al., 2015). This species was not, however, among those recommended for utilisation in industrial wheat bran fermentation.

2.2.5 FLAB as probiotics in honeybees

Recent studies reported on symbiosis between LAB and honeybees, and the presence of LAB, including *Fructobacillus* and other FLAB, in fresh honey (Olofsson et al., 2014, Olofsson and Vasquez, 2008, Vásquez et al., 2012, Mattila et al., 2012). Various researchers have reported on the probiotic effects of *Lb. kunkeei* in honeybees, as observed by their ability to inhibit the bee pathogen *Melissococcus plutonius* and *Paenibacillus larvae* responsible for the European Foulbrood disease and American

Foulbrood (AFB), respectively (Forsgren et al., 2010, Vásquez et al., 2012). Analysis of the draft genome of *Lb. kunkeei* EFB6, isolated from a honeybee larva infected with European foulbrood, together with other *Lb. kunkeei* strains isolated from wine and honey, indicated the presence of *Lb. kunkeei*-specific genes encoding for cell surface or secreted proteins which are involve in biofilm formation and cell adhesion (Djukic et al., 2015). In addition, genes encoding for lysozyme (Butler et al., 2013) and lysozyme-like enzymes (Djukic et al., 2015), which confer antimicrobial properties, were also detected in *Lb. kunkeei* strains isolated from honeybees. These findings suggest a symbiotic relationship between *Lb. kunkeei* and honeybees, whereby *Lb. kunkeei* colonise the bees protecting their niche and also inhibit honeybee pathogens and microorganisms present in pollen and nectar. In another study, Olofsson and Vasquez (2008) suggested that putative FLAB may also play a role in the fermentation of honey. Hence, application of *Lb. kunkeei* as probiotic additive to improve honey bee health might be plausible.

Furthermore, researchers have also proposed that paratransgenesis of commensal FLAB, *F. fructosus* and *Lb. kunkeei*, as a feasible approach to promote honeybee health (Rangberg et al., 2012, Maddaloni et al., 2014, Rangberg et al., 2015). Their studies showed that both fructophilic *F. fructosus* and *Lb. kunkeei* are transformable and are able to survive well in the honeybee gut upon reintroduction without causing any adverse effect on honeybee health and survival. Transgenesis of FLAB isolated from honeybee serve as a molecular toolbox by which genetic modification of FLAB to produce desired effector molecules (e.g. bioactive metabolites with inhibitory effects against bee pathogens) is achievable.

2.3 Metabolites produced by LAB

LAB metabolites are intermediate and final products of metabolism in LAB. Fermentation reduces the amount of available carbohydrates and results in a range of small molecular mass organic molecules known as metabolites. LAB produce organic acids, acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Özcelik et al., 2016; Papagianni, 2012; Zacharof and Lovitt, 2012), some of which may act as antimicrobials. The metabolites produced by LAB are in high demand in the food industry due to the GRAS status.

2.3.1 Oxygen Metabolites and Catabolism End-products

Oxygen metabolites (hydrogen peroxide) and end-products of carbohydrate catabolism such as organic acids, diacetyl, acetaldehyde and D-isomers of amino acids are produced by LAB (Piard and Desmazeaud, 1991). Organic acids are major metabolites produced by LAB, where lactic acid is the major end-product of carbohydrate catabolism. More than 50% of lactic acid is produced from pyruvate by lactate dehydrogenase in order to regenerate pyrimidine nucleotides which are necessary for sugar break down. Thus, the general name "*Lactic Acid Bacteria*" has been given to this group of bacteria due to the predominance of lactic acid in the conversion of the carbohydrate (Orla-Jensen, 1919).

The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, as these acids disrupts intracellular pH homeostasis (Kuipers et al., 2000; Suskovic et al., 2010). The cell membrane is impermeable to ionized hydrophilic acids, while the non-ionized hydrophobic acids diffuse passively through the membrane,