ISOLATION OF *Lactobacillus brevis* C23 FOR PRODUCTION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCE AGAINST FOOD BORNE PATHOGEN, *Listeria monocytogenes*

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LIST OF ABBREVIATIONS AND SYMBOLS

AU/ml	arbitrary unit per mililitre
BLIS	bacteriocin-like inhibitory substances
CFS	cell free supernatant
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
g	gram
g/L	gram per litre
GC	guanine-cytosine
GRAS	Generally Regarded As Safe
h	hour
L	litre
L/hr	litre per hour
M	molar
mg	milligram
mg/ml	milligram per millilitre
min	minute
ml	millilitre
ml/min	millilitre per minute
mM	millimolar
mm^2	millimeter square
MRS	deMan, Rogosa & Sharpe
nm	nanometer
OD	Optical density
PCR	Polymerase chain reaction
rpm	Rotation per minute
SDS	Sodium dodecyl sulfate
TEM	Transmission electron microscope
USM	Universiti Sains Malaysia
w/v	weight per volume

WHO	World Health Organization
v/v	volume per volume
α	alpha
ß	beta
µ	micro
>	more than
<	less than
±	plus minus
%	percent
°C	degree Celsius

LIST OF APPENDICES

PEMENCILAN *Lactobacillus brevis* C23 UNTUK PENGHASILAN BAHAN PERENCAT SEAKAN BAKTERIOSIN TERHADAP PATOGEN PEMBAWAAN MAKANAN, *Listeria monocytogenes*

ABSTRAK

Listeria monocytogenes ialah salah satu daripada patogen pembawaan makanan yang menjangkiti manusia dengan penyakit listeriosis dan berisiko membawa maut. Penularan penyakit listeriosis ini adalah melalui makanan yang tercemar. Beberapa kaedah telah dilaksanakan oleh sektor makanan untuk menentang *L. monocytogenes* dan salah satu kaedah adalah menggunakan bahan pengawet kimia. Walaubagaimanapun, pengguna sekarang juga sedar tentang risiko buruk pengawet kimia terhadap kesihatan. Oleh sebab itu, bakteriosin daripada LAB boleh menggantikan pengawet kimia kerana bakteriosin mampu menentang *L.monocytogenes* dan juga selamat untuk digunakan. Tujuan kajian ini adalah untuk memperolehi strain LAB berpotensi untuk menyekat *L. monocytogenes* melalui pengasingan daripada sumber makanan.untuk digunakan sebagai pengawet dan bungkusan makanan. Kaedah mikrodilusi telah digunakan untuk menyaring asingan yang paling berkesan untuk menentang *L. monocytogenes*. Aktiviti antimikrobial yang tertinggi telah dipilih dan dikenal pasti melalui sekuensi 16S rRNA. Pencirian strain dan bahan perencat seakan bakteriosin (BLIS) daripada strain tersebut telah diuji. Media kultur seperti sumber fosfat, karbon, nitrogen dan NaCl serta kondisi media, suhu dan pH telah dikaji menggunakan kaedah satu faktor pada satu masa (OFAT) untuk meningkatkan aktiviti BLIS. Fermentasi strain ini juga telah dilaksanakan dengan menggunakan teknik fermentasi sesuapan kelompok dalam 2L bioreaktor. Jumlah 68 strain daripada pengasingan makanan telah diuji dan asingan kubis, SCW

2 telah memperolehi aktiviti antimikrobial yang tertinggi, 73.94%. Asingan kubis tersebut telah dikenal pasti sebagai *Lactobacillus brevis* C23. Sekuensi strain itu telah dimasukkan ke dalam GenBank dengan nombor aksesi, MN880215. Aktiviti BLIS daripada *L. brevis* C23 stabil dalam pH kurang daripada pH 6 dan juga suhu 70°C. Ujian enzim telah membuktikan bahawa aktiviti antimikrobial CFS adalah protein. BLIS juga stabil apabila diuji dengan beberapa detergen dan tidak terlekat atas permukaan sel. *L. brevis* C23 juga mampu bertahan dalam garam hempedu (bile salts) yang berpekatan tinggi. Aktiviti antimikrobial terhadap *L. monocytogenes* telah dibuktikan apabila lisis sel dapat dilihat melalui mikroskop transmisi elektron. Aktivti antimikobial juga berjaya ditingkatkan apabila 1.5% (w/v) of KH_2PO_4, 1.5% (w/v) laktosa dan 1 % (w/v) ekstrak daging ditambah ke dalam media MRS dengan pH 5 dan suhu 37°C. Ujian sinergi juga membuktikan bahawa gabungan media MRS + KH_2PO_4 + Ekstrak Daging and MRS + Laktosa + KH_2PO_4 berjaya meningkatkan aktiviti BLIS. Kajian 2L fermentasi telah merekodkan aktiviti antimikrobial BLIS dan jisim sel yang lebih tinggi dengan media ekstrak daging.

ISOLATION OF *Lactobacillus brevis* C23 FOR PRODUCTION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCE AGAINST FOOD BORNE PATHOGEN, *Listeria monocytogenes*

ABSTRACT

Listeria monocytogenes is one of the food-borne pathogens transmitted to humans via contaminated food causes listeriosis, a potentially fatal food-borne disease. For decades, *L. monocytogenes* had plagued the food sector. Several measures were taken over the years to overcome contamination of *L monocytogenes* including chemical preservatives. However, chemical preservatives bear risk to our health and consumers nowadays are increasingly aware of it. Therefore, bacteriocins have drawn interest due to the ability to inhibit pathogen and safe. This study aims to isolate lactic acid bacteria (LAB) from various food sources to obtain a potent strain against *L. monocytogenes* for a potential use in food preservation and packaging. Broth microdilution method was utilized to measure the effectiveness of isolated strains against *L. monocytogenes*. Isolate that produced the highest antimicrobial activity against *L. monocytogenes* was selected for identification using 16S rRNA sequencing. The identified strain and the bacteriocin-like inhibitory substances (BLIS) produced was comprehensively characterized. To further improve the BLIS of strain, the culture media such as phosphate, carbon, nitrogen source and NaCl as well as culture conditions, pH and temperature were investigated by one-factor-at-a-time (OFAT) method. Finally, the strain was subjected to large scale production using fed-batch fermentation technique in 2L stirred tank bioreactor. A total of 68 strains were tested in this study with the isolate from cabbage, SCW 2 recorded the highest activity at 73.94%. The isolate was identified as *Lactobacillus brevis* C23 and the sequence was

deposited in GenBank with accession number of MN880215. The BLIS of *L. brevis* C23 was stable in temperature and pH less than 70°C and pH 6. BLIS proteinaceous nature was confirmed when tested with proteolytic enzyme. The BLIS was stable at different types of detergent tested. *L. brevis* C23 strain was able to tolerate high bile salt concentration and the BLIS produced did not adhere on the surface of producer cells. The bactericidal effect of BLIS towards *L. monocytogenes* was observed under Transmission Electron Microscopy (TEM) In the culture medium and conditions study, BLIS was successfully induced higher with addition of 1.5% (w/v) KH_2PO_4, 1.5% (w/v) lactose, 1 % (w/v) meat extract into MRS media at pH 5 and temperature at 37°C. The combined media of MRS + KH_2PO_4 + Meat Extract and MRS + Lactose + KH_2PO_4 showed positive synergistic interaction. In large scale production, feeding media meat extract were successful to produce higher BLIS and cell biomass of *L. brevis* C23.

CHAPTER 1

INTRODUCTION

1.1 Background

Food borne diseases had really impacted world-wide in terms of civilian health and economy. The root of food-borne diseases is mainly through transmission of polluted food and drinking water (Newell et al., 2010). In United States itself, foodborne illnesses infected approximately 6-80 million populations which lead to 9000 deaths as well as 5 billion U.S. dollars loss (Altekruse, 1997). Some of the infamous bacterial species that dreaded the human populations are *Salmonella spp.*, *Campylobacter spp*. and *E. coli* while *L. monocytogenes* in recent years are emerging as among the burdening food borne diseases especially in food industry (Newell et al., 2010).

L. monocytogenes is an omnipresent, Gram positive food borne infectious microbes that cause listeriosis. Listeriosis is one of the food-borne disease which result in meningitis and sepsis. Individuals with impaired immune system such as pregnant woman and aged people are susceptible to listeriosis (Yin et al., 2019). Being among the top food borne pathogen to cause fatalities, *L. monocytogenes* also capable of infecting animals especially ruminants causing rhombencephalitis and abortions (Maury et al., 2019). Over the years, *L. monocytogenes* burdened the food industry with the pathogen capabilities to withstand most sterilization processes (Bahrami et al., 2020).

Hence, chemical preservatives are applied in food industry as a means to combat *L. monocytogenes*. Sodium and potassium salts such as sodium benzoate and potassium acetate are the chemical preservatives used to control *L. monocytogenes* (M.

R. Islam et al., 2012; Kin et al., 2011). However, chemical preservatives cause adverse effects to health (Choi & Chin, 2003). Physical methods such as radiation, high pressure handling and ultrasound are even utilized to combat *L. monocytogenes* but these methods are less effective compared to chemical preservatives (H Neetoo, 2008; Pietrysiak et al., 2019).

Biopreservations had become a popular alternative to chemical preservatives as it is environmental friendly and risk free (Li et al., 2019; Strack et al., 2020). Bacteriocins produced by lactic acid bacteria possess antimicrobial properties that have the potential to combat *L. monocytogenes* as well as improve the gastrointestinal tract (Jawan et al., 2019). Moreover, lactic acid bacteria (LAB) are certified as Generally Regarded As Safe (GRAS) by US Food and Drug Administrations (FDA). Bacteriocins are ribosomally produced by lactic acid bacteria (LAB) where they exert antimicrobial killing mechanism against large spectrum of pathogens by forming pore on the cell membrane onto the target cell. This is why bacteriocins are a good candidate for food industry applications as preservatives and packaging (Daba & Elkhateeb, 2020).

The properties and characteristics of the peptides is another reason why bacteriocins are good choice to use in food applications (Dussault et al., 2016). Bacteriocins are thermally-stable, able to withstand wide range of pH as well as strong resistance towards detergent (Saad et al., 2015). These properties of bacteriocins enable them to survive during pasteurization, in acidic food and surfactants added during food processing.

Nisin are widely known Class I bacteriocin synthesised by *Lactococcus lactis* are applied in food industries due to the antimicrobial properties against Gram-positive bacteria (Delves-Broughton et al., 1996; Field et al., 2016; E. Yang et al., 2012). Nisin, the pioneer of bacteriocins utilized in food industry at 1953, also certified as GRAS by FDA as well by WHO to be utilized as food additives (O'Sullivan et al., 2019). Despite many bacteriocins had been discovered over the years, Nisin remain the only bacteriocin that is legally authorized by WHO in only 50 countries as commercial food biopreservatives (Castilho et al., 2020; Figueiredo & Almeida, 2017).

However, the reliability of Nisin had become an uncertainty due to uncontrolled usage in the food industry as biopreservatives. The continuous usage of Nisin allowed pathogen strains to develop resistance towards its bactericidal effect. Several studies had demonstrated that *L. monocytogenes* had acquired immunity towards Nisin either by altering cellular metabolism or forming sophisticated biofilm (Ibarra-Sánchez et al., 2018; Stincone et al., 2020). As of 2015, 185 bacteriocins produced by LAB had been identified and documented. However, only 53% of these bacteriocins were comprehensively characterized to the molecular level (Woraprayote et al., 2016).

The characterized bacteriocins are presumably safer to consume and use in food applications. Therefore, this research study emphasizes the screening and identification of a LAB strain that produces BLIS capable to effectively inhibit *L. monocytogenes*. This study also aimed to well characterize the BLIS and the identified LAB for the potential food industrial application in near future.

1.2 Research Scope and Objectives

The main aim of this study is to isolate BLIS produced by lactic acid bacteria that is highly efficient against food borne pathogen *L. monocytogenes* for potential use as food preservatives and antimicrobial packaging.

The objectives of this research were:

- To isolate LAB from local sources and screen for BLIS producer that are effective against *Listeria monocytogenes*
- To identify the selected strain and characterise its BLIS
- To investigate the influence of medium composition and culture conditions using OFAT for improvement of BLIS production by the selected strain in shake flask culture and stirred tank bioreactor

LITERATURE REVIEW

2.1 *Listeria monocytogenes*

Listeria monoyctogenes, an advantageous microbe is notable for the root of an infectious disease, Listeriosis. Listeriosis burst onto the scene during the 1920s when it was declared as an infection but only heeded until 1980s after arduous research and experiments to enlighten the prevalence, spreading as well as pathogenesis of *L. monoyctogenes* (Zhu et al., 2019). In May 1926, research was conducted after epidemic struck among rabbits but the pathogen was foreign with no preceding records. Hence, the pathogen was named as *Bacterium monocytogenes* (Murray et al., 1926). However, the genus name was switched to *Listerella* then finally fixed to *L. monoyctogenes* (Schlech & Acheson, 2000).

While Listeriosis disease able to spread to all human ages and populations, it is more susceptible to immune impaired people namely, geriatrics, conceived women, foetuses and newborns. Listeriosis infection result in 24% of death cases but 99.1% of the infected patients require hospitalization (Farber & Peterkin, 1991; Shamloo et al., 2019). Between 1998 to 2003, epidemic of listeriosis impacted various countries but USA saw a decline in listeriosis outbreak with the introduction of PulseNet at 1996. With the PulseNet, USA was efficient in curbing the spread of listeriosis disease (Camargo et al., 2017).

L. monoyctogenes being an infectious strain, is the only species in genus *Listeria* that are able to induce and transmit disease in humans as well as animals (Schuchat et al., 1991). Meanwhile infection among other species of genus *Listeria, Listeria ivanovii* only prevalent in ruminants (Orsi et al., 2011). *L. monoyctogenes*

comprised of three distinguish genetic lineage, lineage I, II and III. Among the serotype from the three lineage, serotype from lineage I and II (1/2c, 1/2a, 1/2b, and 4b) are linked to the epidemic (Abdollahzadeh et al., 2016). According to (Abdollahzadeh et al., 2016), the serotypes involved in the outbreak in different countries are not similar proving that the serotype assortment vary between location and time.

L. monoyctogenes is a tiny microbe, with the length measuring about 1-2 µm and width at 0.5 µm. *L. monoyctogenes* is a facultative anaerobe and aerobic which allow these microbes to thrive inside the cells of human and animal body. The morphology of *L. monocytogenes* are rod shaped and it develop short chains or placed aligned to each other (Low & Donachie, 1997). *L. monocytogenes* also better known as saprotroph allowing these pathogen to survive on raw meat and vegetables (Orsi et al., 2011). *L. monocytogenes is* omnipresent in vast array of geography including mud, soil, water and sewage. *L. monocytogenes* also present on food materials such as raw meat and vegetables, dairy products, fruits and ready to eat (RTE) foods (Beuchat, 1996). On top of the pathogens virulence, *L. monocytogenes* are sturdy in nature as it is able to survive with little nutrients (Jeyaletchumi et al., 2012). *L. monocytogenes* able to thrive on large margin of temperature from refrigeration temperature to 45°C. Moreover, *L. monocytogenes* possess incredible resistance towards range of pH (3-12) and high NaCl concentration at 40% (w/v). (Kathariou, 2002; Liu, 2006; Liu et al., 2005).

Due to the extreme endurance of *L. monocytogenes* at wide extent of temperatures, pH and osmolarity, food industries struggle to curb the pathogens growth. Preservation methods such as pasteurization, pickling and refrigeration are futile as *L. monocytogenes* able to withstand and proliferate. The dawn of antibiotic

resistance strains are due to the excessive use of antibiotics including *L. monocytogenes* is an unsettling issue for the general human health and even the food industry's perspective (White et al., 2002). The earliest antimicrobial resistant strain of *L. monocytogenes* was discovered in France at 1998 extracted from a patient. The *L. monocytogenes* strain was resistant to few antibiotics (streptomycin, chloramphenicol and tetracycline as well as erythromycin) (Olaimat et al., 2018).

According to (Poyart-Salmeron et al., 1992), antibiotic resistance gene of *L. monocytogenes* was discovered when both *tet* (M) gene and *tet* (L) were in the a 5-kb plasmid. The *tet* (M) gene encode for tetracycline resistant commonly present in streptococci and enterococci indicating transfer of genetic component to *L. monocytogenes*. As a result, food industries are shifting their attention towards bacteriocin produced by LAB as they exhibit antimicrobial activity against food-borne pathogen, *Listeria monocytogenes*.

2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are as a group of microaerophilic, Gram-positive, non-motile, non-spore forming, constitute of low fractions of G+C in their DNA (<55%). Lactic acid bacteria (LAB) are organisms capable of breaking down hexose sugars to produce lactic acid via homofermentative or heterofermentative pathway (Makarova et al., 2006). LAB are broadly utilized in food-based industry mainly as starter culture for manufacturing dairy products and other application in products like raw vegetables and meats (Bharti et al., 2015). By morphology, LAB comprise of two distinctive shape which is cocci and rod shape. The genera fall in LAB group are *Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc,*

Pediococcus, *Streptococcus Tetragenococcus, Vagococcus*, and *Weisella* (Table 2.1) (Henning et al., 2015; Mathur & Singh, 2005).

Lactic acid bacteria (LAB) are stringently fermentative and have several auxotrophies. They require meticulous sustenance to grow and are unable to thrive on simple growth media (Bringel, 1998; Hébert et al., 2004). Strains of LAB are popularly known for its production of diverse and useful compounds such as enzymes and antimicrobial compounds as they are able to undergo different metabolic activities. (Hayek & Ibrahim, 2013). Metabolic activities are vital for LAB growth and viability. All genera of LAB predominantly grouped according to their metabolic pathway which is either homofermenters or heterofermenters.

Heterofermenters breaks down hexose to pentose using enzyme phosphoketolase through an alternate pentose monophosphate pathway. Heterofermenters release lactic acid and several by products such as carbon dioxide, acetic acid, and ethanol when breaking down hexose. On the other hand, homofermenters produce lactic acid directly as their substantial end product through glycolysis by break down hexose. The examples of heterofermenters are *Leuconostoc*, *Oenococcus*, *Weissella*, *Carnobacterium*, *Lactosphaera*, and some lactobacilli. As for homofermenters, list of the genera including *Pediococcus*, *Streptococcus, Lactococcus*, *Vagococcus* and some of the lactobacilli. LAB from heterofermenters are frequently utilized in dairy industry due to the fragrance and taste boosting constituent such as acetylaldehyde and diacetyl produced during the process of pentose monophosphate pathway (Carr et al., 2002; Condon, 1987; Kandler, 1983; *Modern Food Microbiology*, 2005; Wood & Holzapfel, 1992; Zúñiga et al., 1993).

Table 2.1 Classifications of Lactic Acid Bacteria

Genera	Species	References
Lactobacillus	*L. acidophilus, L. helveticus, L. fermentum, L. curvatus, L. rhamnosus, L. crispatus, L. gasseri, L. reuteri, L. salivarius, L.paracasei, L. brevis, L. casei, L.helveticus, L. paraplantarum, L. jensenii, L. johnsonii, L. plantarum, L. sakei*	(Bernardeau et al., 2008)
Lactococcus	*L. lactis, L. lactis subsp. cremoris*	(Ramalho et al., 2019)
Weisella	*W. confuse, W. cibaria, W. viridescens*	(Björkroth et al., 2002)
Streptococcus	*S. thermophiles, S. salivarius*	(Wescombe et al., 2009)
Enterococcus	*E. faecium, E. durans, E. faecalis*	(Hanchi et al., 2018)
Pediococcus	*P. acidilacti, P. pentosaceus*	(Cai et al., 1999)
Bifidobacterium	*B. infantis, B. breve, B. longum, B. adolescentis, B. thermophilum*	(Cheikhyoussef et al., 2008)
Leuconostoc	*L. pseudomesenteroides, L. mesenteroides subsp. mesenteroides*	(Diana et al., 2015; Yaping Wang et al., 2018)

2.3 *Lactobacillus*

Lactobacilli are non-spore-forming, gram-positive rod-shaped bacteria. The genus of *Lactobacillus* encompass of substantial and myriad genus of lactic acid bacteria (LAB) (Kleerebezem et al., 2003). In fact, *Lactobacillus* consist of over 200 species and subspecies that can be found in plants, environment, fermented dairy products and meat as well as human and animal digestives tract (Zhihong Sun et al., 2015). There was a spike in the number of *Lactobacillus* species discovery over the

last few years, particularly in the span between 2004 to 2010. 74 new species of new *Lactobacillus* species were discovered in that six years. The advancement of molecular and identification technique had elevated the number of *Lactobacillus* species identification (Liu et al., 2014). As of March 2020, 261 species of the *Lactobacillus* genus were identified (Zheng et al., 2020). Being the genera comprising of the highest number of species, the *Lactobacillus* is the most researched among all genera of LAB (Giraffa et al., 2010). *Lactobacillus* also comes in various sizes spanning from 1.23 Mb *Lactobacillus sanfranciscensis* to 4.91 Mb. *Lactobacillus parakefiri*, which is quadruple in size. (Zhihong Sun et al., 2015). *Lactobacillus* are similar to other LAB which seem non motile with a morphology of thin rods in different length while some appear as coryneform with irregular shape and chain formation (Slover & Danziger, 2008).

Lactobacillus predominantly are facultative anaerobes though they are capable to thrive in either aerobic and anaerobic environment. However, approximately 20% of isolated *Lactobacillus* species are obligate anaerobes (Slover & Danziger, 2008). Similar to LAB, *Lactobacillus* produce lactic acid at the end of glucose fermentation and also low GC (Guanine Cytosine) content. The GC content of *Lactobacillus* generally range between 31.93% to 57.02% (Slover & Danziger, 2008; Zhihong Sun et al., 2015; Versalovic et al., 2011). Most of *Lactobacillus* species are popularly known as beneficial microbe as well as attaining the GRAS (Generally Recognized as Safe) status, some *Lactobacillus* species may result in infection especially on immunosuppressive patients. (Brook, 1996; De Angelis & Gobbetti, 2004; S Salminen, 1998; Slover & Danziger, 2008). Some of the more common infection caused by *Lactobacillus* are bacteraemia and endocarditis along with dental caries (Alvarez-Olmos & Oberhelman, 2001; Cannon et al., 2005). Dental caries particularly

more prominently caused by *Lactobacillus* species (Hahn et al., 1991). Among the *Lactobacillus* species, *Lactobacillus acidophilus* is the predominant species that result in dental carries (Byun et al., 2004).

Regardless of negative impact of *Lactobacillus* species, *Lactobacillus* are widely applied as starter cultures in food industry due to their ability to ferment food. Dairy products and beverages such as beer, wine and fruit juices are the most renowned food products where *Lactobacillus* strains are utilized for fermentation (Aguirre & Collins, 1993). Human and animal gastrointestinal tract are the source of most of isolated *Lactobacillus* species. Fermented foods and vegetables account the second highest source of *Lactobacillus* species right after the human and animal gastrointestinal tract (Mokoena, 2017). This is a testament to the astounding survivability of *Lactobacillus* in diverse environment conditions. The extensive adaptability of *Lactobacillus* is the result of genomic evolution of niche-specific adaptation (Makarova & Koonin, 2007).

One of the evident way *Lactobacillus* genetically evolved is through Horizontal Genetic Transfer (HGT). Horizontal Genetic Transfer (HGT) involve transfer of gene via transformation, transduction or conjugative pathways (Lerner et al., 2017). In order to keep up with the continuous environment change, *Lactobacillus* need to conserve the functional genes either by attaining or dropping DNA (Lawrence, 1999). One of the evident horizontal genome transfer (HGT) was observed between (*Lactobacillus acidophilus, Lactotobacillus johnsonii*) found in gastrointestinal and *Lactobacillus delbrueckii ssp.bulgaricus* found in yoghurt. There was a substantial genetic transfer occur between the *L. acidophilus* and *L.johnsonii* while only minimal gene transfer happen between *L. delbrueckii ssp.bulgaricus* and *L.johnsonii*, indicating that environment factor was instrumental in gene transfer (Nicolas et al., 2007). The

potential of *Lactobacillus* to populate wide array of environment had attracted the application in food preservation.

2.4 Source of Lactic Acid Bacteria

Lactic acid bacteria are generally known for its omnipresent nature. They are able to be found diversely in ever-changing habitat when carbohydrates are present including fruits, vegetables, raw meat products, fermented foods products, dairy products, gastrointestinal tract, water source, soil and many more (Table 2.2) (Lonvaud-Funel, 2001; Merrifield et al., 2014; O'Sullivan et al., 2002). The first isolated LAB was from milk (Carr et al., 2002). Despite that, different genera of LAB usually isolated from food sources due to easy availability.

One of the most prevalent sources of LAB are dairy products such as milk, yoghurt, buttermilk, kefir, yoghurt and cheese. Raw milk in particular is immensely nutritious which served as native habitat for primeval LAB (Wouters et al., 2002). LAB gradually acidy milk, producing sour flavour by breaking down lactose to lactic acid (Aslim et al., 2005; Bigret & Mäyrä-Mäkinen, 2004). The LAB in milk are the pioneer microbes used as starter culture for other dairy and fermented products such as cheese, yoghurt, buttermilk, wine and fermented vegetables as well as meat products (Khalil & Anwar, 2016).

Besides dairy products, plant products such as vegetables and fruits are also an excellent source to isolate LAB. Vegetables and fruits are filled with nutrients with steeper amount of carbohydrate while lesser protein content. and a much acidic pH compared to dairy products (Garcia et al., 2016). Hence, LAB isolated from vegetables and fruits able to survive the acidic pH in our gastrointestinal tract which may serve

as excellent probiotics (Naeem et al., 2012). The exotic microflora present in vegetables and plants are contributed by pollinators such as plants and insects, moving from plant to plant (Ruiz Rodríguez et al., 2019). Lately, LAB from plant products are sought after due to their extensive metabolic profile and considered riskless compared to raw meat , dairy products and seafood (Naeem et al., 2012; Teneva-Angelova & Beshkova, 2016).

Table 2.2 Source of isolated lactic bacteria strains

Source		Lactic acid bacteria strains	References
Dairy Product	Raw Cow Milk	*S. salivarius subsp.thermophilus, Lb. plantarum, Lb. delbrueckii subsp. Lactis, Lb. brevis, Lb. delbrueckiisubsp.delbrueckii, Lb. delbrueckiisubsp, Lb. helveticus, E. faecium, E. durans*	(Bennani et al., 2017; Bin Masalam et al., 2018; Zoumpopoulou et al., 2018)
	Yoghurt	*Lb. acidophilus, Lb. bulgaricus, Lb. delbrueckii, Lb. casei, Lb. fermentum*	(Hor & Liong, 2014; Marhamatizadeh & Sayyadi, 2019)
	Cheese	*Lc. lactis subsp. Cremoris, Leuc. mesenteroides subsp. Cremoris, Lb. plantarum, Lb. casei subsp. casei, Lb. helveticus, Lb. bulgaricus, Lb. delbrueckiisubsp*	(Ali, 2011)
Vegetables and Fruits	Radish	*W. confusa, E. durans, B. coagulans*	(Xu et al., 2018)
	Cabbage	*Lb. cellobiosus, W. cibaria, Lb. fermentum*	(Bamidele, 2011; Hor & Liong, 2014)
	Tomato	*Lb. paracasei, Lb. fermentum, Lb. plantarum*	(Hor & Liong, 2014)
	Ginger	*E. hirae*	(Xu et al., 2018)

Table 2.2 (cont)

Source		Lactic acid bacteria strains	References
	Broccoli	*Leuc. mesenteroides, E. gallinarum,*	(Xu et al., 2018)
	Paddy rice	*Lb. plantarum, Lb. brevis, Leuc. pseudomesenteroides, P. acidilactici, E. faecalis, Lac. Lactis, W. kimchii*	(Ennahar et al., 2003)
	Guava	*Lb. casei*	(Hor & Liong, 2014)
	Cherry	*E. gallinarum, P. pentosaceus*	(Xu et al., 2018)
	Mulberries	*W. cibaria, Leuc. pseudomesenteroides, Lb. plantarum,*	(Y.-S. Chen et al., 2010)
Fermented products	Dry sausages	*Lb. sakei, Lb. curvatus, Lb. plantarum, Lb. pentosus, E. faecalis, P. acidilactici,*	(Fontana et al., 2005)
	Fermented fish	*Lb. plantarum, Lb. pentosus*	(Muryany et al., 2017)
	Jeotgal	*Leuc. mesenteroides, S. salivarius*	(Cho & Do, 2006)
	Cucumber pickles	*E. casseliflavus, E. durans, E. mundtii, Lc. lactis lactis, Leuc. citreum, Leuc. mesenteroides, Lb. curvatus, Lb. plantarum, P. pentosaceus, W. confusa*	(Reina et al., 2005)
	Fermented oil palm sap	*Lb. pentosus, Lb. plantarum, Lb. brevis*	(Bertrand et al., 2015)
	Greek Wheat Sourdough	*Lb. sanfranciscensis, Lb. paralimentarius, Lb. brevis, W. cibaria*	(Luc De Vuyst et al., 2002)
Raw product	Raw meat	*Lac lactis subsp. lactis, Lb. paracasei, Lb rhamnosus*	(Lengkey et al., 2009)
Fermented beverages	Wine	*Lb. brevis, Lb. collinoides, Lb. hilgardii, Lb. paracasei, Lb. pentosus, Lb. plantarum, Lb. mali*	(Rodas et al., 2005)
	Beer	*Lb. paracasei subsp. paracasei, Lb. plantarum, Lb. pentosus, Lac. lactis subsp. lactis*	(Todorov & Dicks, 2004)

2.5 Bacteriocins

Bacteriocins are clusters of heterogenous peptides secreted by LAB with the purpose to eliminate its own species or different species organism to attain upper-hand in obtaining nutrient in the challenging environment. Bacteriocins also produced by LAB which serve as protection in their population against foreign microbes (Margaret Riley & Wertz, 2002). Lactic acid bacteria including the major genera such *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus Leuconostoc*, *Streptococcus* ribosomally-produced bacteriocin which are small (< 6 kDa) , low toxicity and heat stable (Hegarty et al., 2016). In fact, (Klaenhammer, 1988) stated that 99% of species minimally produce one bacteriocin.

Bacteriocins showcase narrow spectrum of antimicrobial activity against species that are closely related or extensive wider spectrum of antimicrobial activity against various genera meanwhile producer strains are resistant toward their own synthesised bacteriocin (Mills et al., 2011). Furthermore, bacteriocin produced by LAB are cationic, have amphipathic properties and also have low molecular weight (Rodríguez, 2003; Zacharof & Lovitt, 2012). Earliest records of bacteriocin was evident at 1925 by Gratia when the bacteriocins were formerly known as colicins which were synthesised by *Escherichia coli* (Balciunas et al., 2013). Being the earliest bacteriocin discovered, the colicins were thoroughly researched setting a standard for other bacteriocins study thereafter.

The properties bacteriocins vary with different strain and species including the size and molecular weight of peptides, antimicrobial targets and spectrum, mechanism of action as well as resistance (Mokoena, 2017; Naidu et al., 1999). Bacteriocins are synthesised by both Gram-positive and Gram-negative LAB although the bacteriocin

produced by Gram-positive bacteria are more distinctive and plenteous (Jack et al., 1995). The abundant properties of bacteriocins had attracted the usage in various sectors in food processing.

2.6 Classifications of bacteriocins

Gram-positive bacteria produced extensive array of bacteriocin with variable size and molecular weight, antimicrobial properties and spectrum as well as different properties. Due to the vast variety of bacteriocin, the bacteriocin are categorized based on the post-translational modifications. (Klaenhammer, 1993), grouped bacteriocins into 4 separate classes namely, Class I, Class II and Class III and Class IV. The class II were branched out to three distinguish category. However, (Nes et al., 1996), revamped the class II bacteriocin and removed the class IV bacteriocin. Class IV bacteriocin was eliminated and changed to bacteriolysins (Kumariya et al., 2019). Meanwhile, class IIc incorporated bacteriocins producing *sec*-dependent signal peptide (Franz et al., 2007). The substantial amount bacteriocin in class II prompted improper division of bacteriocin into the subclass of bacteriocin. This had negatively impacted bacteriocin in food processing, regarding production number of bacteriocin and regulation as well as reliability.

2.6.1 Class I: Lantibiotics

The class I bacteriocin, lanthionine-containing antibiotics which commonly abbreviate as lantibiotics comprise of tiny peptide undertook post translational modifications (PTM) which involve peculiar amino acids thioether lanthionine (Lan) and ß-methyllanthionine (meLan) forming the amphiphilic helical structure (Chen &

Hoover, 2003; J. Zhang et al., 2018). The lantibiotics are peptide with low molecular weight (<5kDa) and with peptide length range from 19-38 amino acids (Cotter et al., 2005). The organization of lantibiotics molecule also promote the heat stability of the peptide (Güllüce et al., 2013). The bacteriocins of this class eliminate target cells by transforming the cell membrane. This is possible due to the amphiphilic helical "ring" structure of the peptide granting easily permissibility into the target cell membrane which subsequently boring pores and causing the collapse of target cell membrane (Balciunas et al., 2013).

The lantibiotics begin with the enzymatic dehydration of the gene that encode serine and threonine occur resulting in dehydroalanine (Dha) and dehydrobutyrine (Dhb). Subsequently, lanthionine (Lan) or methyllanthionine (MeLan) bridge formed with the inclusion of Cys residue from the thiol group onto dehydroalanine (Dha) and dehydrobutyrine (Dhb) called as stereospecific intramolecular(Guder et al., 2000; Willey & van der Donk, 2007; Zacharof & Lovitt, 2012).

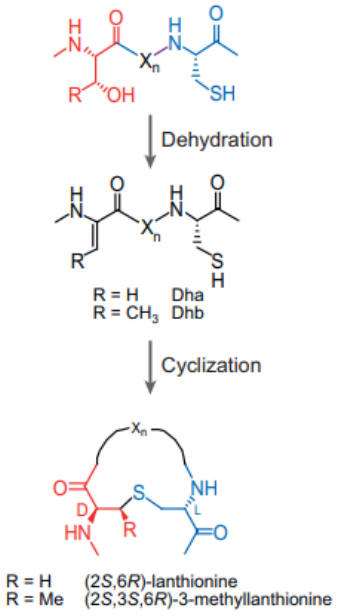

Figure 2.1 Synthesis of lanthionine and methyllanthionine into lantibiotics (Willey & van der Donk, 2007)

Gram-positive bacteria predominantly produce lantibiotics with over 50 different lantibiotics recorded synthesised by Gram-positive bacteria (Drider et al., 2006; Willey & van der Donk, 2007). There are 2 subdivision of Class I bacteriocin which are Class Ia and Class Ib. The distinctive difference between Class Ia and Class Ib are the structure of the molecules as well as the inhibitory activity of the lantibiotics. Class Ia lantibiotics take the form of a linear and malleable structure molecule while Class Ib lantibiotics has a spherical shape structure. The lantibiotics of Class Ia produce pore onto the target cell membrane as its inhibitory action. The peptide of Class Ia also possess positive charge and molecular weight between 2 to 4 kDa. Contrastingly, molecular weight of class Ib lantibiotics are in between 2 to 3 kDa and the peptide structure contain negative or no net charge. Lantibiotics of class Ib rely on particular enzyme release for antimicrobial action (Chen & Hoover, 2003; Deegan et al., 2006; Drider et al., 2006; Rea et al., 2011).

Examples of prominent lantibiotics fall on the class Ia and class Ib are Nisin and Mersacidin. Nisin is more popular product of LAB applied in food processing as food biopreservatives. Nisin is synthesised by *Lactococcus lactis* and has a large range of inhibitory action against an abundance of pathogenic bacteria namely *Listeria monocytogenes* and *Staphylococcus aureus* as well as multi-drug resistant strain resistant strain (Severina et al., 1998). On the other hand, Mersacidin is an antimicrobial compound synthesised by genus *Bacillus*. Despite its antimicrobial potential especially against multi-drug resistant pathogen and Gram-positive bacteria,

Mersacidin are not implemented in food industry because of not certified as GRAS (Ibrahim, 2019; Kruszewska et al., 2004).

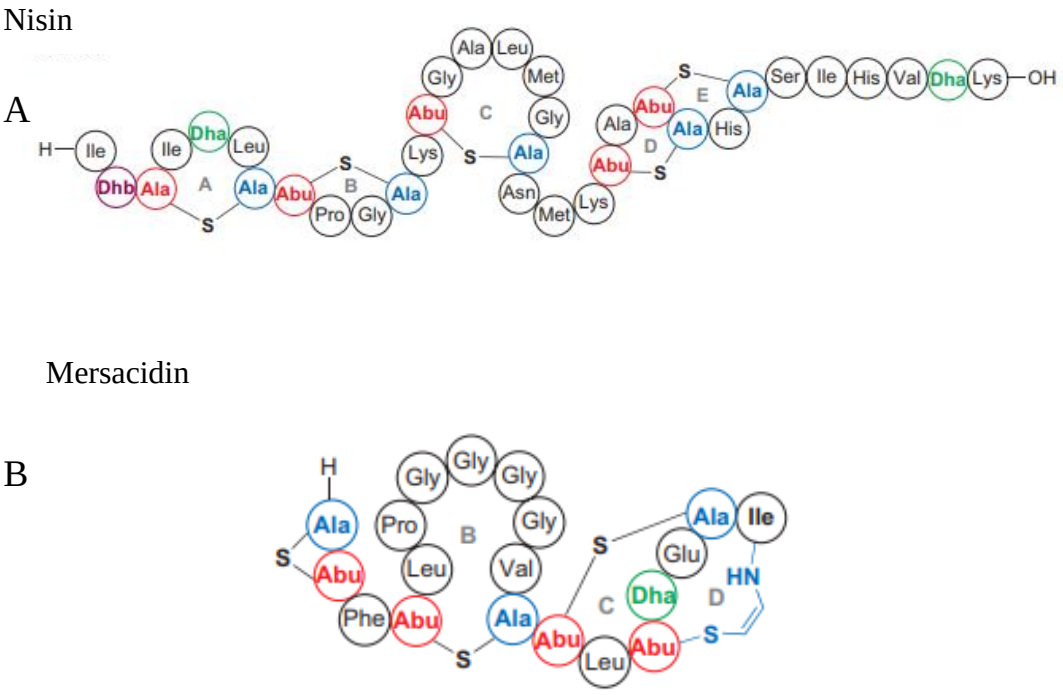

Figure 2.2 Molecular structure of A: Nisin from Class Ia and B: Mersacidin from Class Ib (Willey & van der Donk, 2007)

2.6.2 Class II: Non-lantibiotics

Bacteriocins fall in this group are also known as non- lantibiotics and opposingly to the complex structure of class I lantibiotics, the bacteriocins molecular structure are uncomplicated. Lack of post-translational modification in class II bacteriocin resulted in an unsophisticated molecular structure of class II bacteriocin. Similar to class I lantibiotics, class II bacteriocins are small heterogenous peptides with molecular weight (< 10kDa) (Güllüce et al., 2013; Sacchini et al., 2017). Class II bacteriocins also share other similar characteristics as class I bacteriocins including

thermostable and having an indistinguishable amphiphilic helical molecular structure (Cotter et al., 2005). Class II bacteriocin being a cationic peptide exhibit inhibitory action by penetrate into the target cell membrane resulting in the depolarization of the membrane (Moll et al., 1999). Class II bacteriocins can be further subdivided into three subgroup which are class IIa, class IIb and class IIc.

2.6.2(a) Class IIa Pediocin-Like Bacteriocins

Class IIa are known as pediocin-like bacteriocin and is one of the thoroughly studied class of bacteriocin. Many species of LAB notably *Lactobacillus*, *Pediococcus* and *Enterococcus* are known to produce pediocin-like bacteriocin (Eijsink et al., 2002). Being synthesised by "food grade" organism, pediocin-like bacteriocin are very well recognized in food industrial applications (Nissen-Meyer et al., 2009). Bacteriocins in this particular class consist of amino acids with varying length ranging from 37 to 58 and molecular size lesser than 10 kDa (Belguesmia et al., 2011). Furthermore, bacteriocins in class IIa also can be distinguished by the N-terminal sequence Tyr–Gly–Asn–Gly–Val with a pleated arrangement as well as two cysteines establishing a S-S bridge (Cleveland et al., 2001). Class IIa bacteriocins are characterized as having fine spectrum of antimicrobial activity but potent antimicrobial action against *Listeria* species. They exert bactericidal activity by severing the proton shifting force resulting in the exhaustion of ATP This result in the collapse of the target cell membrane (Fimland et al., 2005; Nissen-Meyer et al., 2009).

2.6.2(b) Class IIb: Two-Peptide Unmodified Bacteriocins

Class IIb bacteriocins are famed as two-peptide bacteriocins because of the corresponding action between two distinctive peptides to exhibit antimicrobial action against pathogenic bacteria (Masuda et al., 2012). With one operating peptide, the bacteriocin still able to exhibit but low antimicrobial action (Oppegård et al., 2007). Similarly to other class of bacteriocin, the two-peptide bacteriocin are cationic in nature and the homology of the two-peptide bacteriocin consist of hydrophobic region (Garneau et al., 2002). The two peptides exert antimicrobial action by depolarizing the cell membrane upon contact resulting in cell death (Nissen-Meyer et al., 2009).

2.6.2(c) Class IIc: Circular Bacteriocins

Circular bacteriocin as the name suggest consisted of a circular homology. The circular structure are the result of the covalent bond connection between N- and C-terminal amino acids (Kawai et al., 2004). The circular bacteriocin has a large span of antimicrobial activity. The amino acid sequences ranging from 58 to 70 with molecular mass of the peptide between 5.6 to 7.2 kDa (Gabrielsen et al., 2014). Notable examples of circular bacteriocins are gassericin A and butyrivibriocin AR10 (Cotter et al., 2005).

2.6.3 Class III: Bacteriolysins

Bacteriolysins, previously known as class III bacteriocins are heal labile as well as large in size, recording approximately 30 kDa (Gontijo et al., 2020; Hickey et al., 2003). Till date, only four bacteriolysins had been discovered with colicins one of the four bacteriolysins were well documented (Riley, 1993). Bacteriolysins consist of

three domain that responsible for translocation, binding of receptor and lethal activity (Zhilan Sun et al., 2018). Bacteriolysins has a unique bactericidal activity against target cell in comparison to other class of bacteriocins. This class of bacteriocins particularly aims and depolarize the cell wall where the N-terminal region are correspondent to the endopeptidase of target cells. Endopeptidase of the target cells involve in the building of cell wall. Meanwhile, the C-terminal of bacteriolysins accountable to the target cell identification (Lai et al., 2002)

2.7 Biosynthesis of Bacteriocins

Class I bacteriocin, lantibiotics production involve two genetics system which start off as pre-peptides. The pre-peptides do not have antimicrobial properties due to the existence of NH_2-extension (N-terminal), keeping the peptides dormant to safeguard the producer strain. NH_2-extension, a leader peptide consist of 57 amino acids linked to the C-terminal pro-peptide containing two glycine residues (Guder et al., 2000; Havarstein et al., 1995). The pre-peptides are synthesised by *lanA*, a structure gene among the gene cluster. During the maturation of lantibiotics, the 23 amino acid residues of N-terminal will detach from the 34 amino acid residue C-terminal pro-peptide and the C-terminal will undergo posttranslational modification (Sablon et al., 2000).

Within the region of pro-peptide consist of serine, threonine and cysteine residues. These residues undergo two-step procedure with aid of LanB and LanC enzymes to become lanthionine (Lan) or methyl-lanthionine (MeLan) (McAuliffe et al., 2001). In the two-step procedure of Lan/MeLan, the first-step involve dehydration process where hydroxyl amino acids Serine and Threonine are converted to α,ß-

unsaturated amino acids 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) by LanB. On the other hand, LanC are responsible for the catalyzation of thioether bond where intramolecular Michael addition including thiol groups in adjacent Cysteine residues and the double bond of Dha and Dhb producing Lan and MeLan (Guder et al., 2000; Twomey et al., 2002).

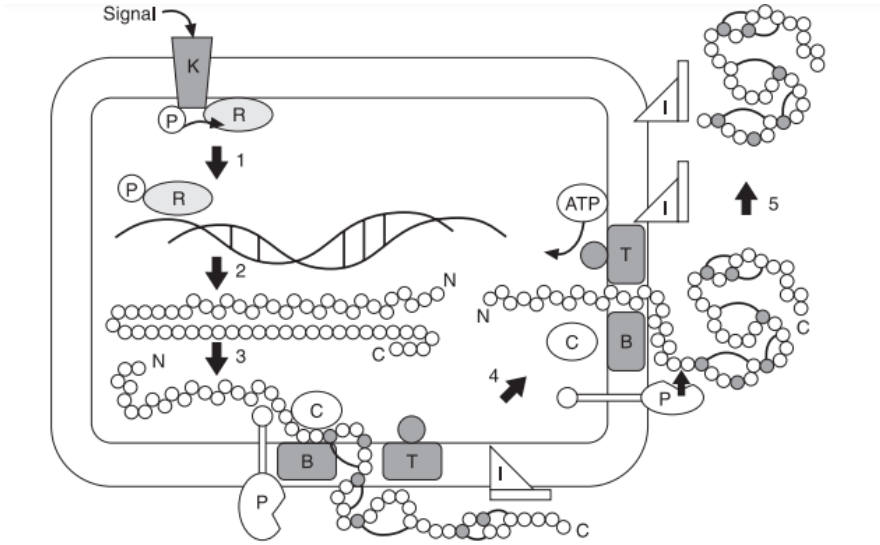

Figure 2.3 Biosynthesis pathway and transport process of class I lantibiotics (Sablon et al., 2000)

In contrast for type II non-lantibiotics, the dehydration process is conducted by LanM instead of LanB enzyme. The LanM possess the features of both the LanB and LanC enzyme, having a similar structure to Lan C (Asaduzzaman & Sonomoto, 2009). Proteolytic mechanism occurred where the N-terminal, leader peptide detached from the LanA pre-peptide. The leader peptide will be abolished by serine protease LanP while the LanA pre-peptide is transported out of the membrane through ATP binding cassette (ABC) transporter LanT (David et al., 2016; Fath & Kolter, 1993). Once the peptides are translocated out of the membrane, the gene LanI which is a lipoprotein

contribute self-resistance to the producer strain against the peptides. The gene LanI can be found on the membrane of producer strain, attach itself to the bacteriocin before reaching the cell membrane (Heidrich et al., 1998; Martínez et al., 2016).

The biosynthesis of bacteriocin often participate in quorum sensing through a two-step procedure in which histidine kinase (LanK) and LanR, a transcriptional response regulator is involved. The purpose of histidine kinase (LanK) and LanR are to coordinate equilibrium between synthesis and immunity during bacteriocin biosynthesis (Kareb & Aïder, 2020). On the other hand, the class II biosynthesis of bacteriocin also follow a similar path as the class I lantibiotics but they do not undertake posttranslational modification. The existence of double glycine leader on the C-terminal in class II bacteriocin function as signal peptides for synthesis and excretion of peptides through a allocated transport system (sec-independent transporter system) consisting of the ABC transporter and an accessory protein (Ennahar et al., 2003).

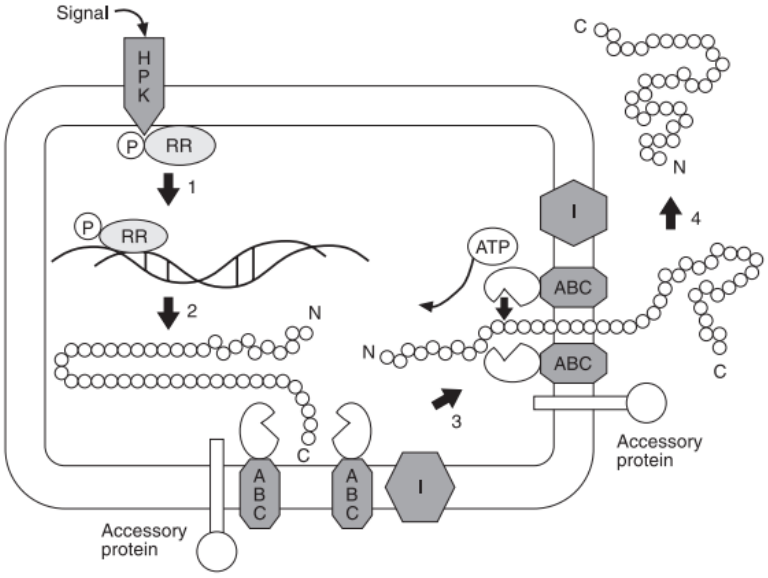

Figure 2.4 Biosynthesis pathway and transport process of class II non-lantibiotics (Sablon et al., 2000)