การพัฒนาเทคนิคระดับโมเลกุลเพื่อประเมินและจัดการความเสี่ยงของ *Listeria monocytogenes* และ *L. innocua* ในโรงงานผลิตไก่ปรุงสุกแช่เยือกแข็ง

นายจิรพิพรรธ แพรไพศาล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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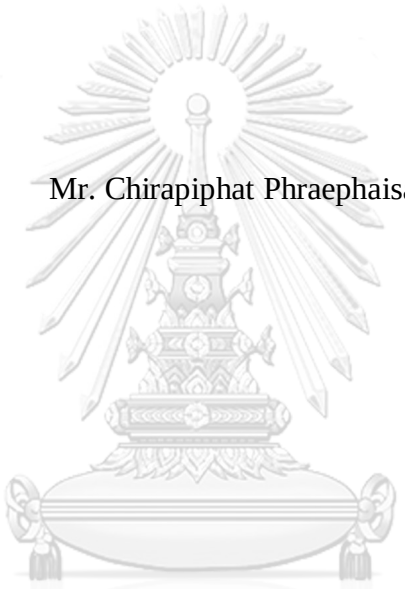
DEVELOPMENT OF MOLECULAR TECHNIQUE FOR RISK ASSESSMENT AND RISK MANAGEMENT OF *Listeria monocytogenes* AND *L. innocua* IN FROZEN COOKED CHICKEN PLANT

Mr. Chirapiphat Phraephaisarn



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By	Mr. Chirapiphat Phraephaisarn
Field of Study	Biotechnology
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จิรพิพรรธ แพรไพศาล : การพัฒนาเทคนิคระดับโมเลกุลเพื่อประเมินและจัดการความเสี่ยงของ *Listeria monocytogenes* และ *L. innocua* ในโรงงานผลิตไก่ปรุงสุกแช่เยือกแข็ง (DEVELOPMENT OF MOLECULAR TECHNIQUE FOR RISK ASSESSMENT AND RISK MANAGEMENT OF *Listeria monocytogenes* AND *L. innocua* IN FROZEN COOKED CHICKEN PLANT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.สุวิมล กีรติพิบูล, 144 หน้า.

ประเทศไทยเป็นประเทศที่ส่งออกเนื้อไก่ปรุงสุกเป็นอันดับต้นๆของโลก อย่างไรก็ตามผลิตภัณฑ์เหล่านี้สามารถตรวจพบการปนเปื้อนของ *L. monocytogenes* ซึ่งเป็นสาเหตุของโรค Listeriosis อัตราการเสียชีวิตจากโรคนี้มีมากถึง 30-40 เปอร์เซ็นต์ ดังนั้นในหลายประเทศรวมถึงญี่ปุ่นและกลุ่มประเทศสหภาพยุโรปจึงต้องการผลิตภัณฑ์ที่ปลอด *L. monocytogenes* โดยผู้นำเข้าได้กำหนดมาตรการที่เข้มงวดยิ่งขึ้น คือ ไม่ให้พบทุกสปีชีส์ของ *Listeria* เลย ในปีหนึ่งๆ พบว่ามีสินค้าที่ถูกปฏิเสธการนำเข้าเนื่องจากปนเปื้อน *Listeria* spp. เป็นมูลค่า 5-20 ล้านบาทต่อโรงงานผู้ส่งออก ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อเสนอเทคนิคระดับโมเลกุลที่ประหยัดต้นทุนสำหรับตรวจหา จำแนก และระบุสายพันธุ์ของ *L. monocytogenes* และ *L. innocua* เพื่อการตรวจติดตามแหล่งการปนเปื้อน ขั้นแรก ได้พัฒนาเครื่องหมายโมเลกุล BE-LisAll เพื่อตรวจหา *Listeria* โดยใช้โปรแกรม in silico พบว่ามีความจำเพาะต่อสปีชีส์ของ *Listeria* 100 เปอร์เซ็นต์ และสามารถแยก *Listeria* ออกจากแบคทีเรียชนิดอื่นได้ ขั้นที่สอง ได้พัฒนาเทคนิค high resolution melting analysis โดยใช้ *rar*A และ *ldh* เพื่อจำแนก *Listeria* จำนวน 9 สปีชีส์ พบว่ามีความสามารถเพียงพอที่จะจำแนกสปีชีส์ของ *Listeria* ที่แยกได้จากโรงงาน โดยมีอัตราความสำเร็จ 92.6 เปอร์เซ็นต์ ขั้นที่สาม ได้พัฒนาเทคนิค capillary electrophoresis-based MLVA โดยใช้ชุดไพรเมอร์ VNTR ของ *L. monocytogenes* ของ Chenal-Francisque และคณะ และชุดไพรเมอร์ VNTR ของ *L. innocua* จากการออกแบบใหม่โดยอาศัยข้อมูลรหัสจีโนม *L. innocua* CLIP 11262 เพื่อจำแนกความแตกต่างระหว่างสายพันธุ์ (subtyping) ของ *L. monocytogenes* และ *L. innocua* พบว่าเทคนิค CE-based MLVA มีศักยภาพในการจำแนกความแตกต่างระหว่างสายพันธุ์ของ *Listeria* ทั้งสองสปีชีส์ได้สูงกว่าเทคนิค random amplified polymorphic DNA (RAPD) ขั้นที่สี่ ได้พัฒนาเทคนิค next generation sequencing-based MLVA ด้วยเครื่อง Ion Torren Personal Genome Machine (PGM) โดยใช้ไพรเมอร์ VNTR ของ *L. monocytogenes* ที่ได้รับการทวนสอบแล้ว จำนวน 9 คู่ (JLR1 JLR2 JLR4 LisTR1317 LisTR881 LMTR4, LMV1 LMV6 และ LMV9) และไพรเมอร์ VNTR ของ *L. innocua* ที่ถูกออกแบบใหม่ จำนวน 6 คู่ (TR1 TR3 TR5 TR6 TR10 และ TR13) เพื่อใช้ในการจำแนกความแตกต่างระหว่างสายพันธุ์ของ *L. monocytogenes* และ *L. innocua* โดยพบว่าเทคนิค NGS-based MLVAมีศักยภาพในการจำแนกความแตกต่างระหว่างสายพันธุ์ของ *Listeria* ทั้งสองสปีชีส์ได้สูงกว่าเทคนิค CE-based MLVA และขั้นสุดท้าย ได้นำเทคนิค MLVA ที่พัฒนาขึ้นไปสำรวจแหล่งและเส้นทางการปนเปื้อนของ *L. monocytogenes* และ *L. innocua* ในโรงงานผลิตไก่ปรุงสุกแช่เยือกแข็ง พบว่าจากการประเมินความสัมพันธ์ระหว่างรูปแบบลายพิมพ์ดีเอ็นเอของ *L. monocytogenes* และ *L. innocua* ที่ปนเปื้อนในผลิตภัณฑ์กับสิ่งแวดล้อม แสดงให้เห็นว่า *Listeria* ทั้งสองสปีชีส์ที่พบในผลิตภัณฑ์มีโอกาสปนเปื้อนได้จากหลายแหล่ง เช่น เครื่องหั่น พื้น และล้อรถเข็น โรงงานจึงได้ปรับเปลี่ยนวิธีการล้างทำความสะอาดและฆ่าเชื้อให้เหมาะสมขึ้น พบว่าสามารถลดการปนเปื้อนของ *Listeria* ในผลิตภัณฑ์และสิ่งแวดล้อมได้อย่างมีนัยสำคัญ งานวิจัยนี้แสดงให้เห็นว่าเทคนิคที่ถูกพัฒนาขึ้นมีศักยภาพในการใช้เป็นเครื่องมือสำหรับตรวจสอบ ติดตามเส้นทางและหาแหล่งการปนเปื้อนของ *L. monocytogenes* และ *L. innocua* ในโรงงานผลิตไก่ปรุงสุกแช่เยือกแข็งได้อย่างมีประสิทธิภาพ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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CHIRAPIPHAT PHRAEPHAISARN: DEVELOPMENT OF MOLECULAR TECHNIQUE FOR RISK ASSESSMENT AND RISK MANAGEMENT OF *Listeria monocytogenes* AND *L. innocua* IN FROZEN COOKED CHICKEN PLANT. ADVISOR: PROF. SUWIMON KEERATIPIBUL, Ph.D., 144 pp.

Thailand is one of the largest cooked chicken meat exporting country in the world. Unfortunately, these products can be contaminated with *L. monocytogenes* causes the disease Listeriosis which carries a fatality rate of 30 to 40 percent. Therefore, the various importing countries including Japan and the European countries require a zero tolerance for *L. monocytogenes* in the cooked chicken products. Besides, all *Listeria* spp. occurrence, and not only *L. monocytogenes*, in these products is also unacceptable to the importers. Rejection of *Listeria* contaminated product can reach over 5 to 20 million baht per factory per year. Hence, the aim of this study was to introduce a cost-effective molecular approach to detect, identify, and subtype *L. monocytogenes* and *L. innocua* for tracking sources of contamination. Firstly, a new comprehensive BE-LisAll biomarker for *Listeria* detection using in silico scheme was developed. The results showed that biomarker has a 100% specificity for *Listeria* species detection and could differentiate *Listeria* species from a variety of non-*Listeria* bacteria. Secondly, HRMA of *rarA* and *ldh* method which identified 9 species belonging to the genus *Listeria* was established. The method can be considered sufficiently applicable as method for identifying the species of *Listeria* isolates from the food factory with a success rate of 92.6%. Thirdly, a recently validated PCR primer set targeting the VNTR of *L. monocytogenes* of Chenal-Francisque et al. and a novel PCR primer set targeting the VNTR designed based on completed genome sequence of *L. innocua* CLIP 11262 were validated and verified. The CE-based MLVA protocols provided higher discriminatory power for subtyping of *L. monocytogenes* and *L. innocua* than RAPD method. Fourthly, a cost-effective NGS-based MLVA by PGM using 9 adopted (JLR1, JLR2, JLR4, LisTR1317, LisTR881, LMTR4, LMV1, LMV6, and LMV9) and 6 novel (TR1, TR3, TR5, TR6, TR10, and TR13) VTNR loci was developed. This method provided higher discriminatory power for differentiating between *L. monocytogenes* and *L. innocua* strains than CE-based MLVA. Finally, the developed MLVA was applied to investigate sources and routes of *Listeria* contamination for reducing the risk of *L. monocytogenes* and *L. innocua* contamination in cooked frozen chicken meat process. The relationships of the *L. monocytogenes* and *L. innocua* in the final products and those in the environment were evaluated. The results showed that *L. monocytogenes* and *L. innocua* in finished products can be contaminated from various sources such as dicer, floor, and cart wheel. After revised the suitable procedures, the prevalence of *Listeria* spp. in the finished product and processing environment were decreased, significantly. These demonstrated that the developed techniques and approaches have the potential to provide an efficient tool for investigating and tracking the routes and sources of contamination of *L. monocytogenes* and *L. innocua* in frozen cooked chicken plant.

Field of Study: Biotechnology Student's Signature .....

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bp	base pair
^{O}C	Degree Celcius
cfu	Colony-forming unit
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
g	Gram
GI	GenInfo Identifier
h	hour
kb	Kilobase
KCl	Potassium Chloride
kV	Kilo Volt
$MgCl_2$	Magnesium Chloride
mM	Millimolar
mm	Millimeter
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic acid
rpm	Revolution per minute
s	Second
Tris-HCl	Tris-Hydrochloride
U	Unit
µL	Microliter
µM	Micromolar
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I
COMPREHENSIVE LITERATURE REVIEW

1.1 Introduction

Thailand is one of the world's biggest producers of food products. Thus, many people worldwide has dubbed Thailand as the "kitchen of the world". In 2016, Thailand exported approximately 30 billion U.S. dollar of food products to major countries such as Japan, the US, China, UK and ASEAN countries with the top 5 exports being rice, sugar, chicken, tuna, and shrimp. For chicken, Thailand is one of the largest cooked chicken meat exporting country in the world. The value of export for cooked chicken meat in Thailand was 2.14 billion U.S. dollars as of 2016. Unfortunately, these products can be contaminated with *Listeria* species, especially *L. monocytogenes*, the species significantly associated with listerosis. This microorganism often affects severe underlying condition such as encephalitis, abortion or blood poisoning in human. Therefore, it directly affects the frozen ready-to-eat food industries. Besides, other *Listeria* species, *Listeria* presence in food products is entirely unacceptable by the customers. Several research studies showed that *L. innocua* presence could indicate risk of *L. monocytogenes* contamination (Friedly et al., 2008; Keeratipibul & Techaruwichit, 2012). Therefore, the discovery of routes of transmission and emerging of these particular foodborne pathogens is important task to develop a better prevention and control in food industries.

1.2 Genus *Listeria*

Listeria, a genus named after the British pioneer of sterile surgery Joseph Lister, are gram positive, rod-shaped, and non-endospore-forming bacteria. They are facultative anaerobes, which prefer microaerophillic conditions (Wagner M & J, 2008). The genus *Listeria* currently comprises 17 recognized species. Phylogenetic analysis based on 16S rDNA sequences and amino acid sequences of 31 conserved loci revealed the existence of four well-supported monophyletic clades within the genus *Listeria*: (i) a first clade named *Listeria* sensu stricto consisting of *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*, (ii) a second clade consisting of

L. fleischmannii, *L. aquatica* sp. nov., and *L. floridensis* sp. nov., (iii) a third clade consisting of *L. rocourtiae*, *L. weihenstephanensis*, *L. cornellensis* sp. nov., *L. grandensis* sp. nov., *L. riparia* sp. nov., and two new species, *L. newyorkensis* and *L. booriae*, and (iv) a fourth clade containing *L. grayi* (den Bakker et al., 2014; Orsi & Wiedmann, 2016; Weller et al., 2015). Among these species, *L. monocytogenes* and *L. ivanovii*, are considered pathogens. The potential of these pathogens to cause disease correlates with their capacities to survive within macrophages, to invade non-phagocytic cells and replicate therein (Gaillard et al., 1991). They both invade host cells, replicate in the cytosol after phagosomal escape, and spread from cell to cell by polymerizing actin. However, *L. monocytogenes* infects humans and ruminants, whereas *L. ivanovii* is only thought to infect ruminants (Vázquez-Boland et al., 2001).

1.3 Subtyping technique

Several culture and biochemical methods have been routinely used to detect *Listeria* spp. from different food categories such as raw meats, seafood, and retail products. The common methods have been used such the FDA bacteriological and analytical method (BAM), the International Organization of Standards (ISO) 11290 method, and USDA and Association of Analytical Chemists (AOAC/IDF). However, there has been a limitation to find the real source of product contamination due to different strains of the same species. The conventional methods identify the organism found on those contaminated areas; only species level but not strain level. As a result, implementation of control and prevention strategies for *Listeria* elimination could not be handled, correctly.

The subtyping methods with higher degree of discrimination to differentiate *Listeria* strains are required in foodborne pathogen surveillance, outbreak detection, and source tracking throughout the food chain. These have assisted epidemiologists to understand virulent and ecological characteristics of the food borne pathogens. These subtyping methods are essential for timely tracking of sources of bacterial contamination therefore they can help to prevent further occurrences of *Listeria* contamination and allow for development of control strategies. In general, bacterial subtyping methods can be divided into phenotypic and genetic based methods (Wiedmann, 2002). The phenotypic subtyping methods are generally less sensitive and

do not consistently reproduce, whereas the genotypic approaches are more sensitive and reliable. Some of the conventional subtyping methods relying on phenotype, restriction-digestion based methods and PCR based methods are discussed below.

1.3.1 Phenotypic subtyping techniques

Serotyping scheme is based on somatic cell wall (O) and flagellar (H) antigens. O-antigens are distinctive structures on the bacterial cell wall, such as lipoteichoic acids and membrane proteins, and H-antigens are different structures of flagellas. For *Listeria*, serotyping was the first typing method used for serological detection with corresponding monoclonal and polyclonal antibodies. There are 15 *Listeria* somatic (O) antigen subtypes (I–XV), flagellar (H) antigens comprise four subtypes (A–D) (Table 1) (Seeliger & Jones, 1986). The serotypes of individual *Listeria* strains were determined by their unique combinations of O and H antigens. The examination of group-specific *Listeria* O and H antigens in slide agglutination, at least 12 serotypes have been recognized in *L. monocytogenes*, in *L. seeligeri*, one in *L. ivanovii*, and a few in *L. innocua*, *L. welshimeri* and *L. grayi* (Kathariou, 2002; Seeliger & Jones, 1986).

Multilocus enzyme electrophoresis (MLEE) is based on differences in electrophoretic migration patterns of the enzymes exhibiting differences in their amino acid profiles in bacteria (Sauders et al., 2004). The MEE provides a highly discriminatory method for detection of bacterial clones since many bacterial enzymes are polymorphic. MLEE and its application to the study of clonal diversity and genetic structures for many bacterial pathogens have been published. For *Listeria* species, seventy-three strains of the seven recognized *Listeria* species were examined 18 enzyme loci using MLEE. The mean number of alleles per locus was 9.5 and all of the loci were polymorphic. Fifty-six electrophoretic types were distinguished and revealed that there were six principal clusters at the species level. *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. ivanovii* each corresponded to one of these clusters with no overlap, while *L. grayi* and *L. murrayi* electrophoretic types formed a unique cluster (Boerlin & Piffaretti, 1991). This method allows clear differentiation of phenotypically similar species and evaluation of their genomic relatedness within a genus.

Table 1: Compositions of somatic (O) and flagellar (H) antigens in *Listeria* serotypes.

Serotype	O antigen	H antigen
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VII, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C
5	(V), VI, (VIII), X	A, B, C
6a	V, (VI), (VII), (IX), XV	A, B, C
6b	(V), (VI), (VII), IX, X, XI	A, B, C
7	XII, XIII	A, B, C

1.3.2 Genotypic-based subtyping techniques

Ribotyping is a molecular technique that allows identification and typing of bacteria to the strain level by the analysis of band pattern differences obtained through cleavage ribosomal RNA or rDNA with an endonuclease (Regnault-Roger, 1997). Most importantly for the purpose of ribotyping, the DNA sequences encoding rRNA should be highly conserved even between different bacterial species, but the flanking and spacer sequences may vary (Bingen et al., 1994). For *Listeria* species, the potential of PCR ribotyping for discriminating between and within various species of *Listeria* were examined. A total of 388 *Listeria* isolates from 20 different dairy processing facilities were examined along with 44 silage, 14 raw milk bulk tank, and 29 dairy cattle isolates were examined using ribotyping method. These 475 isolates included 93 *L. monocytogenes*, 362 *L. innocua*, 11 *L. welshimeri*, 6 *L. seeligeri*, 2 *L. grayi*, and

1 *L. ivanovii* strains. Thirty-seven different *Listeria* ribotypes (RTs) comprising 16 *L. monocytogene*, 12 *L. innocua*, 5 *L. welshimeri*, 2 *L. seeligeri*, 1 *L. ivanovii*, and 1 *L. grayi* were identified (Arimi et al., 1997). This demonstrated that ribotyping scheme has potential value in epidemiological studies through subtyping of diverse bacteria.

Pulsed-field gel electrophoresis (PFGE) is a form of RFLP typing in which the bacterial genomes are digested with rare cutting enzymes, and are separated using specialized electrophoresis techniques. These restriction enzymes cut genomic DNA infrequently and thus generate a smaller number of DNA fragments with a wide range of sizes from 20 kb to 10,000 kb. The differences in the restriction profiles are used to carry out genetic comparisons among isolates (Herschleb et al., 2007). For *Listeria* species, a total of 131 *Listeria* isolated from a meat processing plant were detected by sampling different processing areas and meat products within a 2-year period. The isolates were differentiated by means of phenotypic characteristics. Furthermore, the genomic ApaI and SmaI fragment patterns of all isolates were examined by using PFGE. The PFGE provided 15 *L. monocytogenes*, 20 *L. innocua* and 6 *L. welshimeri* pulsotypes. The environmental *L. monocytogenes* isolates the predominating PFGE-type B was clearly associated with processing area A whereas PFGE-type E predominated in the meat products (Senczek et al., 2000). Although, PFGE is capable of discriminating between isolates of *Listeria* strains, the method is a laborious, time-consuming procedure that needs to be examined by a well-trained technician (Brolund et al., 2010).

Random amplified polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation with prior knowledge of genetic information. This method detects nucleotide sequence polymorphisms in a DNA amplification-based assay using a single arbitrary primer in a PCR reaction, resulting in the amplification of many diverse PCR amplicons. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as the presence or absence of a particular RAPD

band. The RAPD profiles allowed discrimination of strains. For *Listeria* species, a total of 415 *Listeria* contaminated samples were further subtyped using RAPD analysis to evaluate the relationship of the contaminants in the final product and those in the environment. The results showed that *L. innocua* type LI 1.1, *L. welshimeri* type LW 1.5 and *L. seeligeri* type LS 1 were the only isolates found in the finished product, whilst *L. innocua* type LI 1.1 was persistently found in the surfaces of the food processing plant throughout the sampling period (Keeratipibul & Techaruwichit, 2012). Although RAPD is an inexpensive and a rapid method, the lack of pattern reproducibility between laboratories has been observed (Bingen et al., 1993; Liu & Berry, 1995).

Multilocus sequence typing (MLST) is a sequence-based typing method that detects DNA sequence polymorphisms in a variable number of metabolic or housekeeping genes to identify isolates into clusters defined as sequence type (ST). The major advantages of the MLST method in bacterial typing are to allow rapid generation of clear results and easy comparisons between STs. For *Listeria* species, sixty-four strains of *L. monocytogenes*- and *L. innocua* obtained from ready-to-eat food and environment were classified using MLST based on the sequences of 9 unlinked genes gyrB, sigB, dapE, hisJ, ribC, purM, gap, tuf and betL. The method classified *Listeria* strains into 61 unique sequence types (ST) in the MLST scheme with a high discrimination index (DI = 0.99, 0.76 to 0.98 per gene). The sequence data of *L. innocua* revealed significantly greater polymorphism than *L. monocytogenes* (Chen et al., 2010). MLST, a DNA sequencing-based approach, is also one of the most accurate and simplest procedures for inter-laboratory comparison. However, due to the sequence conservation in housekeeping genes, MLST sometimes lacks the discriminatory power to distinguish bacterial strains, which limits its use in epidemiological investigations (Zhang et al., 2004).

Multilocus variable number of tandem repeat analysis (MLVA) is one of the most widely used molecular typing methods which is performed by utilizing the number of repeat units in the variable number tandem repeat (VNTR) loci varying depending on the strain. The numbers of repeats in multiple VNTR loci are used to compare and allow highly specific strain classification. VNTR loci are located both in coding and non-coding regions of genome. In recent decades, MLVA has been developed as a subtyping method coupled with a DNA sequence analysis. Generally, bacterial pathogens use high frequency of insertions and deletions of tandem repeat in genes to rapidly adapt to specific host niches or avert the host's immune response by promoting variations of the protein sequence and possibly lead to changed encoded proteins (Zhou et al., 2014). For *Listeria* species, MLVA has been use to subtype *L. monocytogenes* and *L. innocua*. The MLVA was developed to subtype 45 *L. monocytogenes* isolated from salmon and other sources. The results demonstrated that MLVA can be used to clearly differentiate the *L. monocytogenes* serotype 4b isolated from Salmon from other sources (Murphy et al., 2007). Besides, The MLVA technique was also used to subtype 60 *L. innocua* strains isolated from a food factory. The method classified 60 *L. innocua* strains into 11 patterns. Many of the strains were classified into ST-6, revealing that this MLVA strain type can contaminate each manufacturing process in the factory (Takahashi et al., 2014). These demonstrated that MLVA is powerful tool for subtyping of *Listeria* species for epidemiological investigation. The MLVA may also be a useful tool for tracking source of bacterial contamination.

Next generation sequencing typing (NGST) is a high-throughput methodology that enables rapid sequencing of the base pairs in DNA or RNA samples. The concept utilized in NGS is to sequence millions of small fragments of DNA prepared from an entire genome, transcriptome, or smaller targeted regions in a single run of the instrument. The NGS revolutionized sequence data generation in molecular subtyping method to track source of microbial contamination since several NGS platforms have been employed and developed to produce numerous high quality bases at low costs in single sequencing run. Subtyping methods with greater discriminatory power can be used to detect contaminated subtypes and therefore improve the accuracy of certain contribution studies. The NGS was established for subtyping of *Listeria* isolates (Hyden

et al., 2016; Takahashi et al., 2017; Yao et al., 2016). The microbial source contribution consequently provides crucial information in the risk assessment and risk management of *Listeria* contamination.

Bacterial subtyping methods provide tools to track sources of bacterial contamination throughout the food system. The subtyping methods provides an opportunity to better understand the epidemiology, and ecology of different foodborne pathogens (Wiedmann, 2002). Subtyping methods with greater discriminatory power can be used to detect contaminated subtypes and therefore improve the accuracy of certain contribution studies. The microbial source contribution consequently provides crucial information in the risk assessment and risk management of foodborne pathogen contamination. The developed molecular subtyping methods may also facilitate the development of a novel framework for tracking, preventing, and regulating other foodborne pathogens in the food processing plant.

1.4 Objectives

This study aimed to develop the molecular methods including polymerase chain reaction, multiplex polymerase chain reaction, high resolution melting analysis, and next generation sequencing technology for the detection, identification and subtyping of *L. monocytogenes* and *L. innocua*. The study conducted in several stages: (i) development of *Listeria* genus detection using PCR based technique; (ii) development of *Listeria* species identification using PCR based technique; (iii) development of molecular subtyping of *L. monocytogenes*; (iv) development of molecular subtyping of *L. innocua*; (v) development of high-throughput molecular subtyping of *L. monocytogenes* and *L. innocua*; (vi) tracking contamination sources, risk assessment and risk management of *L. monocytogenes* and *L. innocua* in the plant. Overall, molecular methods are rapid, sensitive, specific, and time-saving. These provided tools to track sources of *L. monocytogenes* and *L. innocua* contamination in Thai frozen cooked chicken plants.

CHAPTER II
DEVELOPMENT OF MOLECULAR METHOD FOR *Listeria* GENUS DETECTION

2.1 Introduction

Listeria spp., a Gram-positive rod shaped bacteria, comprises 17 species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, *L. marthii*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, including the recently classified *L. aquatica*, *L. cornellensis*, *L. floridensis*, *L. grandensis*, *L. riparia* (den Bakker et al., 2014), *L. booriae*, and *L. newyorkensis* (Weller et al., 2015). Among these species, *L. monocytogenes* is the species widely associated with listerosis in humans. Although the regulations for *L. monocytogenes* in RTE foods in Thailand and various countries, including USA, Australia, and Japan, require absence of the pathogen in 25 g of RTE food (zero tolerance) (DLD Thailand, 2010; FDA/FSIS, 2003; FSANZ, 2014), the occurrence of any *Listeria* species, not only *L. monocytogenes*, is definitely unacceptable to both exporters and importers (Keeratipibul & Techaruwichit, 2012). Many studies support that the presence of any *Listeria* species in a specific environment can indicate the presence of other species, including *L. monocytogenes* (Barros et al., 2007; Ryu et al., 2013). Thus, elimination and prevention of *Listeria* contamination in food products and processing environments is crucial to reduce economic losses in food industries.

Listeria detection by molecular methods, such as multiplex PCR (Chen & Knabel, 2007; Cocolin et al., 2002; Doumith et al., 2004; Liu et al., 2015), real-time PCR (Barbau-Piednoir et al., 2013; Liu et al., 2015), DNA microarrays (Hmaïed et al., 2014; Volokhov et al., 2002) and PCR ribotyping (Sontakke & Farber, 1995), provides a powerful tool to investigate the source of *Listeria* contamination. The major advantages of these molecular methods over conventional culture methods are their speed and accuracy of the results. However, most of these molecular methods mainly depend on specific biomarker genes. A biomarker is defined as biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a

therapeutic intervention (Naylor, 2003). The use of biomarkers derives from the premise that each species of pathogen carries a unique DNA or RNA signature that differentiates it from other organisms. Most commonly used biomarker genes for *Listeria* detection are *iap* (invasion-associated protein) and *hly* (hemolysin) (Rodríguez-Lázaro et al., 2004; Volokhov et al., 2002). The *iap* gene was successfully applied to PCR-based assay for rapid detection of each *Listeria* species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, and *L. welshimeri* within the genus, except *L. grayi* (Bubert et al., 1999). While the PCR assay targeting *hly* gene via Real-Time PCR could detect *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*, it could detect *L. innocua* and *L. welshimeri* (Leimeister-Wächter & Chakraborty, 1989). In addition, the recent emergences of 7 new *Listeria* species may negatively affect the detection efficiency of current molecular methods of *Listeria*. These new species may not be detected by the current used biomarker genes, leading to false negative results in samples contaminated with the new species. Therefore, a new comprehensive biomarker is needed for the effective detection of all *Listeria* species.

During the past few decades, the success of genomic mining has distributed an enormous flow of valuable sequence data that have served as powerful resources for biomarker exploration. However, finding potential biomarkers that can be used to specifically distinguish *Listeria* spp. from other food-borne pathogens in environmental mixed bacterial populations is still very challenging. A major obstacle for the development of genetic-based detection methods for specific pathogens is the identification of suitable target sequences. *Listeria* genomes of every species are needed to sequence and compare them with other microbial genomes to identify and verify the *Listeria*-specific gene sequences. By using in-silico scheme, a number of genomes of *Listeria* species (34 genomes) can be compared with over 2,700 microbial genomes in the public database in a short time. The in-silico scheme is useful to help manage the large quantities of data and facilitate the identification of the target sequences by computation-based analysis.

In this study, an innovative in-silico scheme was introduced to explore and characterize the alternative biomarkers through the massive bacterial genome databases and present a new biomarker as an alternative replicon for rapid *Listeria* detection in the food processing industries. Three frameworks for biomarker detection and utilization were conducted: (1) collection and verification of potential genetic biomarkers through literature-based data mining, (2) comparative genomic comparisons, and (3) validation of specific selected biomarkers for detection of *Listeria* spp. in food products.

2.2 Material and Methods

2.2.1 Bacterial strain, DNA Extraction, and PCR amplification

Some of *Listeria* spp. and other bacterial strains were obtained from American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), and the Collection of Institute Pasteur (CIP). *Listeria* spp. isolated from a food processing plant or the environment were also used. A total of 17 *Listeria* strains were investigated. These included 5 *L. monocytogenes*, two *L. fleischmannii*, two *L. innocua*, two *L. rocourtiae*, two *L. seeligeri*, one *L. grayi*, one *L. ivanovii*, one *L. marthii*, and one *L. welshimeri* (Table 2). In addition, 58 common gram-positive and negative bacterial strains were included in this study to verify the specificity of the developed biomarkers. All bacterial strains were individually grown in Trypticase Soy Broth (TSB) (Becton Dickinson, U.S.A.) overnight at 30 °C. Bacterial cells were harvested from 1 mL TSB medium by centrifugation at 8,000×*g* for 3 min and the supernatant was removed. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel, Germany) according to the manufacturer's protocol. The primer set for Putative hypothetical protein, BE-LisAll F: GAAMGAATGAAAGCGCTACGAGAYAARGT and BE-LisAll R: TCCCCACCW GCTAAATARTGRCTTTC, was novelly designed to amplify unique bands under standard PCR amplification conditions. The PCR amplification was performed in a final volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM BE-LisAll forward primer, 100 nM BE-LisAll reverse primer, 25 ng template DNA, and 0.5 U Takara Taq DNA polymerase (Takara Bio,

Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters were used for amplification: 95 ^{O}C for 5 min, 35 cycles of 95 ^{O}C for 30 s, 60 ^{O}C for 40 s, 72 ^{O}C for 1 min, and 72 ^{O}C for 4 min. PCR products were visualized and confirmed by electrophoresis in a 2% agarose gel.

Table 2: Bacterial strains used in this study and their corresponding PCR amplification results

Bacterial strains		Origin	BE-LisAll biomarker for *Listeria* detection
Family	Species		
Listeriaceae	*L. fleischmannii*	DSM24998	+
	L. fleischmannii	river water	+
	L. grayi	ATCC19120	+
	L. innnocua	ATCC33090	+
	L. innnocua	river water	+
	L. ivanovii	ATCC19119	+
	L. marthii	DSM23913	+
	L. monocytogenes	ATCC19114	+
	L. monocytogenes	ATCC19115	+
	L. monocytogenes	ATCC19116	+
	L. monocytogenes	CIP103575	+
	L. monocytogenes	CIP107776	+
	L. rocourtiae	DSM22097	+
	L. rocourtiae	River water	+
	L. seeligeri	ATCC35967	+
	L. seeligeri	River water	+
	L. welshimeri	ATCC35897	+
	Brochothrix thermosphacta	JCM12167	-

Table 2: Bacterial strains used in this study and their corresponding PCR amplification results (continued).

Bacterial strains		Origin	BE-LisAll biomarker for *Listeria* detection
Family	Species		
Aerococcaceae	*Aerococcus sanguinicola*	JCM11549	-
Aeromonadaceae	*Aeromonas hydrophila*	42523	-
	Aeromonas hydrophila	JCM1027	-
Alicagenaceae	*Alcaligenes faecalis*	JCM20522	-
Burkhoderiaceae	*Burkholderia caledonica*	JCM21561	-
Carnobacteriaceae	*Carnobacterium divergens*	JCM5816	-
	Carnobacterium maltaromaticum	NBRC15164	-
	Trichococcus massiliensis	13-A2	-
Chromatiaceae	*Rheinheimera* sp.	13-A4	-
Comamonadaceae	*Comamonas* sp.	42522	-
Dermabacteriaceae	*Brachybacterium* sp.	8-A1	-
Enterobacteriaceae	*Aerobacter asbuiae*	6-A2	-
	Enterobacter aerogenes	ATCC43175	-
	Enterobacter asburiae	4-A4	-
	Enterobacter cloacae	IAM12349	-
	Escherichia coli	ATCC11775	-

Table 2: Bacterial strains used in this study and their corresponding PCR amplification results (continued).

Bacterial strains		Origin	BE-LisAll biomarker for *Listeria* detection
Family	Species		
	Escherichia sp.	42370	-
	Hafnia alvei	JCM1666	-
	Klebsiella planticola	ATCC43176	-
	Klebsiella pneumonias sprhinoscleromatis	JCM1664	-
	Morganella morganii	ATCC35200	-
	Morganella morganii	JCM1672	-
	Proteus mirabilis	JCM1669	-
	Proteus vulgaris	IAM12542	-
	Rahnella aquatilis	JCM1683	-
	Raoultella planticola	JCM7251	-
	Yersinia sp.	42414	-
Enterococcaceae	*Enterococcus faecalis*	JCM5803	-
Flavobacteriaceae	*Empedobacter* sp.	42708	-
	Flavobacterium sp.	9-A4	-
Intrasporagiaceae	*Janibacter limosus*	JCM10980	-
Leuconostocaceae	*Leuconostoc carnosum*	JCM9695	-
Micrococcaceae	*Kocuria varians*	JCM7238	-
	Microbacterium oxydans	JCM12414	-
	Micrococcus luteus	JCM1464	-
	Rothia dentocariosa	JCM3067	-
Morexellaceae	*Acinetobacter baumannii*	JCM6841	-

Table 2: Bacterial strains used in this study and their corresponding PCR amplification results (continued).

Bacterial strains		Origin	BE-LisAll biomarker for *Listeria* detection
Family	Species		
	Acinetobacter beijerinckii	42431	-
Pseudomonadaceae	*Pseudomonas aeruginosa*	JCM12689	-
	Pseudomonas argentinensis	42388	-
	Pseudomonas fluorescens	42676	-
	Pseudomonas fluorescens	IFO14160	-
	Pseudomonas migulae	42386	-
	Pseudomonas monteilii	42461	-
	Pseudomonas psychrophila	42476	-
	Pseudomonas putida	IFO14164	-
	Pseudomonas sp.	42401	-
Rhodospirillaceae	*Tistrella mobilis*	5-A1	-
Shewanellaceae	*Shewanella putrefaciens*	NBRC3908	-
Sphingomonadaceae	*Sphingomonas molluscorum*	JCM14122	-
Staphylococcaceae	*Staphylococcus* sp.	14-A2	-
Vibrionaceae	*Listonella anguillarum*	NCIMB2286	-
	Photobacteriumdamselae	ATCC33539	-
	Vibrio hollisae	JCM1283	-
Xanthomonadaceae	*Stenotrophomonas maltophilia*	6-A3	-
	Xanthomonas oryzae	JCM20241	-

2.2.2 In silico discovery of *Listeria* genus specific biomarker

A set of genus-specific genes of *Listeria* was identified by sequential blast analysis method using 34 complete genome sequences of *Listeria* species and other bacterial genomes available in the public database (Figure 1). *Listeria* genus-specific genes were considered as genes that (i) must have been found in all species and strains of *Listeria* and (ii) share no significant homology to other bacterial genes. Identification of these genes was performed at protein level (BlastP search). Protein sequences derived from all complete genomes of bacteria were downloaded from GenBank (ftp://ftp.ncbi.nlm.nih.gov) and formatted into two separate local databases as (i) *Listeria* protein database, which contains 34 complete genomes of *Listeria* and (ii) non-*Listeria* bacterial protein database, containing 2,711 genomes of other bacteria. All BlastP analysis was performed using default parameters, except the e-value threshold was set to 1e-40 and 1e-10 when blast against the *Listeria* and non-*Listeria* protein databases, respectively, while minimum alignment length of hit sequences was set to 80% of query sequence length. A custom perl script was written to parse all blast output files and extract desired hit sequences to match above-mentioned criteria.

A complete set of protein sequences derived from a single genome of *Listeria* (*Listeria monocytogenes* serotype 4b strain F2365; 2,821 protein-coding genes) was used as queries. First, these protein sequences were blasted against the *Listeria* protein database. Those that found significant matches to all genomes of *Listeria* in the database were considered as *Listeria* core genes. Second, these core genes were extracted and used as queries to blast against the non-*Listeria* protein database. Those core genes that found no match to this database were then potential *Listeria* genus-specific gene candidates. Finally, manual inspection was performed to identify *Listeria* specific genes that share highest homology among *Listeria* species, which is suitable for primer design.

2.2.3 Verification of biomarker for *Listeria* spp. detection

For application of specific primer for *Listeria* spp. detection in actual food processing plant, the verification was performed using *Listeria* spp. strains obtained from food processing plant. Sixty isolates from the plants, including *L. monocytogenes* CIP103575, were employed. The swab samples were suspended in preservative buffer. One millimeter of suspensions was transferred into Half-Fraser broth and then incubated at 30 ^{O}C overnight. After incubation, bacterial cultures were divided into 2 tubes (1 mL each). The first portion was kept at -20 ^{O}C for DNA extraction as Half-Fraser step, while the second portion was added into 9 mL Fraser broth and incubated at 30 ^{O}C overnight.

After incubation, the broth was separated into 2 tubes (1 mL each), one for spread-plating on PALCAM agar and incubated at 30 OC for 48 hours and the second one for DNA isolation. On PALCAM agar, *Listeria* colonies appear gray-green, approximately 1.5-2 millimeter in diameter, and black sunken in the centers. The *Listeria* colonies were picked up to perform colony PCR using Listeria genus specific primer to confirm the presence or absence of *Listeria* in the factory samples. Sequencing of 16S rDNA was also performed to identify bacterial species. A consensus region (contig) was resolved and compared by BLAST search of the DNA Data Bank of Japan (DDBJ), and a bacterial species assigned where homology was greater than 98%.

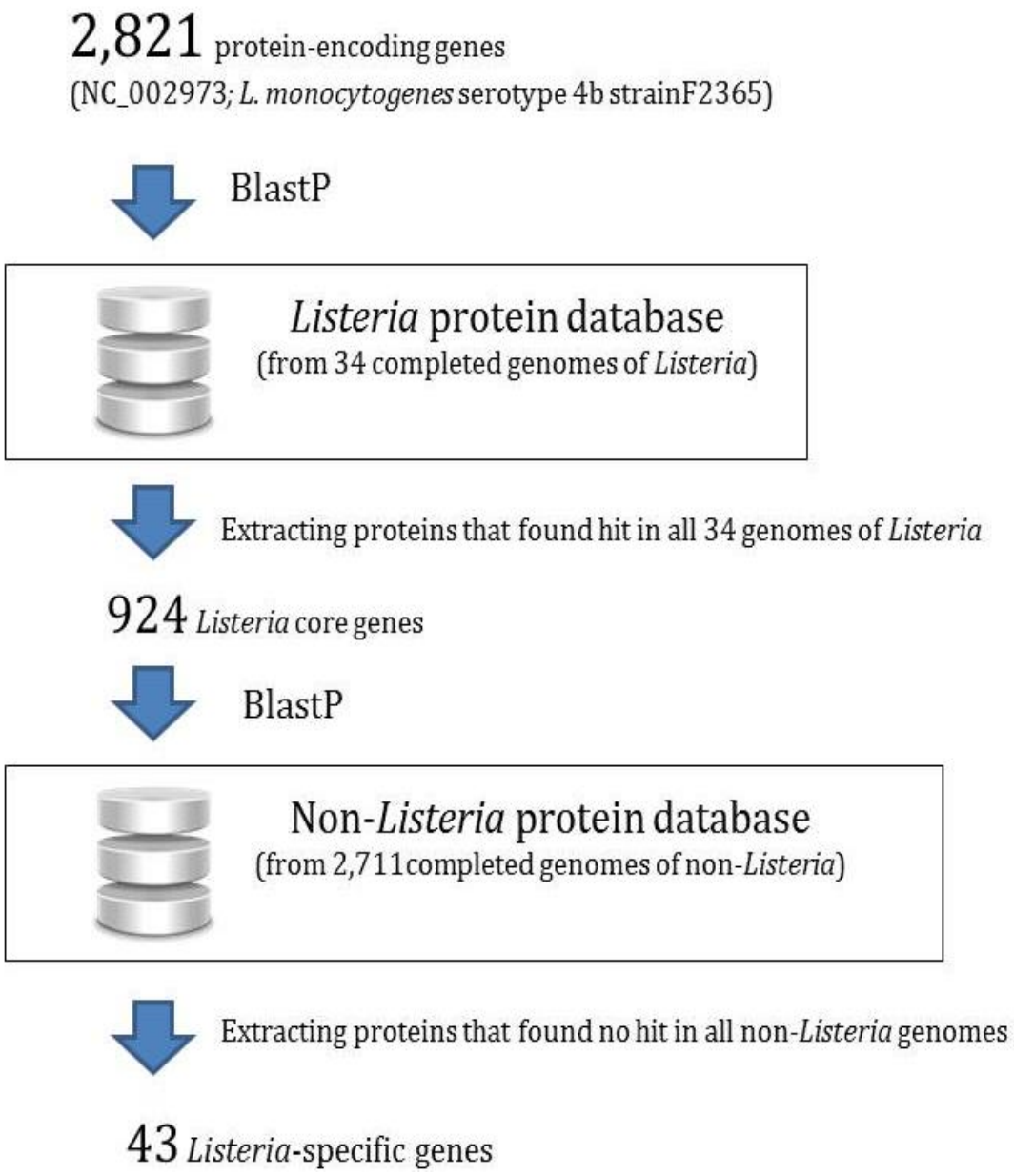


Figure 1: Identification of *Listeria* specific genes by sequential BlastP analysis against *Listeria* and non-*Listeria* protein databases.

2.3 Results and discussion

To search for potential biomarker genes, the in-silico approach was used to investigate all bacterial sequence information available in GenBank. Two thousand eight hundred and twenty-one protein-encoding genes of *L. monocytogenes* serotype 4b strain F2364 were used as reference sequences to perform sequential blast analysis. By comparing 34 genomes of *Listeria* in GenBank, a total of 924 genes were found in all *Listeria* species and were considered as the core genes of *Listeria*. After comparing 924 core genes with 2,711 genomes of non-*Listeria* bacteria, 43 out of 924 genes were detected to be particularly specific to *Listeria* and determined for sequence homology to design suitable primers. The highest percentage of sequence homology among *Listeria* species was found in 3 genes, encoding hypothetical proteins LMOf2365_0738, LMOf2365_0403 and LMOf2365_0404. However, gene LMOf2365_0738 could not be amplified in the DNA sample of *L. grayi* (data not shown). Since gene LMOf2365_0738 was found in flagellar operon, this might be due to the variation of phase flagella among *Listeria* species (Appendix 1). Besides *L. grayi*, phenotypic assay indicated the lack of mobility, and genome sequence analysis revealed that flagellar operon is also absent in all new five species of *Listeria*: *L. aquatica*, *L. cornellensis*, *L. floridensis*, *L. grandensis*, and *L. riparia* (den Bakker et al., 2014). This indicated that a *Listeria* specific biomarker targeting a gene in flagellar operon might not be applicable to those *Listeria* species. Without gene LMOf2365_0738, genes LMOf2365_0403 (Appendix 2) and LMOf2365_0404 (Appendix 3) were then developed for an effective PCR detection assay of all *Listeria* species. LMOf2365_0403gene was very specific to all 17 *Listeria* species; however, a part of LMOf2365_0404 gene was found in some *Bacillus* species. Meanwhile, the upstream gene of *Bacillus* homologues of LMOf2365_0404 gene shared no sequence homology to LMOf2365_0403. Therefore, a new primer for *Listeria* was developed by placing the forward primer on LMOf2365_0403 and reverse primer on LMOf2365_0404 gene, called BE-LisAll (Figure 2). The BE-LisAll is a potential biomarker that can overcome the phenotypic variation among *Listeria* species. Besides the lack of mobility of *L. aquatica*, *L. cornellensis*, *L. floridensis*, *L. grandensis*, and *L. riparia*, all new 7 recently classified species which the exception of *L. floridensis* are capable of reducing nitrate to nitrite, an ability not present among typical *Listeria* and

L. monocytogenes. Moreover, *L. floridensis* and *L. aquatica* are unable to grow at low temperature, one the major characteristics of the genus *Listeria* (Orsi & Wiedmann, 2016).

The BE-LisAll biomarker was evaluated for the specificity of primer with 17 different *Listeria* species and 58 non-*Listeria* bacteria isolates. The 436-bp PCR product of BE-LisAll biomarker was obtained in all *Listeria* samples, but it was not detected in any isolates of non-*Listeria* bacteria (Table 2). The BE-LisAll biomarker showed 100% specificity to *Listeria* and could differentiate *Listeria* species from non-*Listeria* bacteria. To determine the value of the BE-LisAll biomaker-based method for the detection of *Listeria* in samples from the food industries, biomarker-based PCR amplification and the conventional culture method (Half-Fraser broth, Fraser broth, and PALCAM agar plate) were compared using the results generated from 60 environmental swab samples collected from a food-processing factory. The positive results (changing to black color) of both Half-Fraser and Fraser Broths were observed in 9 swab samples, indicating the presence of bacterial growth. After continually culturing in PALCAM agar, 9 out of 60 samples were observed with typical colonies of *Listeria* species. Therefore, the DNA of a total of 129 samples (60, 60, and 9 samples from Half-Fraser Broth, Fraser Broth, and PALCAM agar, respectively) were extracted and served as the sources of DNA isolate for the biomarker-based PCR amplification. The BE-LisAll biomaker demonstrated results corresponding 100% to the results of the conventional culture method (Table 3). Fifty-one samples and 9 samples were negative and positive for *Listeria*, respectively.

Table 3: Comparison of PCR positive with different diagnostic procedures for *Listeria* spp. in food samples isolated from the food-processing plants.

Sample number	Half-Fraser broth	Fraser broth	PALCAM plate	PCR result	Sample number	Half-Fraser broth	Fraser broth	PALCAM plate	PCR result
1	+	+	+	+	31	-	-	-	-
2	-	-	-	-	32	-	-	-	-
3	-	-	-	-	33	-	-	-	-
4	-	-	-	-	34	-	-	-	-
5	-	-	-	-	35	+	+	+	+
6	-	-	-	-	36	-	-	-	-
7	-	-	-	-	37	-	-	-	-
8	-	-	-	-	38	-	-	-	-
9	-	-	-	-	39	-	-	-	-
10	+	+	+	+	40	-	-	-	-
11	-	-	-	-	41	-	-	-	-
12	-	-	-	-	42	-	-	-	-
13	-	-	-	-	43	-	-	-	-
14	-	-	-	-	44	-	-	-	-
15	-	-	-	-	45	-	-	-	-

Table 3: Comparison of PCR positive with different diagnostic procedures for *Listeria* spp. in food samples isolated from the food-processing plants (continue).

Sample number	Half-Fraser broth	Fraser broth	PALCAM plate	PCR result	Sample number	Half-Fraser broth	Fraser broth	PALCAM plate	PCR result
16	-	-	-	-	46	+	+	+	+
17	-	-	-	-	47	-	-	-	-
18	-	-	-	-	48	-	-	-	-
19	-	-	-	-	49	-	-	-	-
20	-	-	-	-	50	-	-	-	-
21	+	+	+	+	51	-	-	-	-
22	-	-	-	-	52	-	-	-	-
23	+	+	+	+	53	-	-	-	-
24	-	-	-	-	54	-	-	-	-
25	+	+	+	+	55	-	-	-	-
26	-	-	-	-	56	-	-	-	-
27	+	+	+	+	57	-	-	-	-
28	-	-	-	-	58	-	-	-	-
29	+	+	+	+	59	-	-	-	-
30	-	-	-	-	60	-	-	-	-

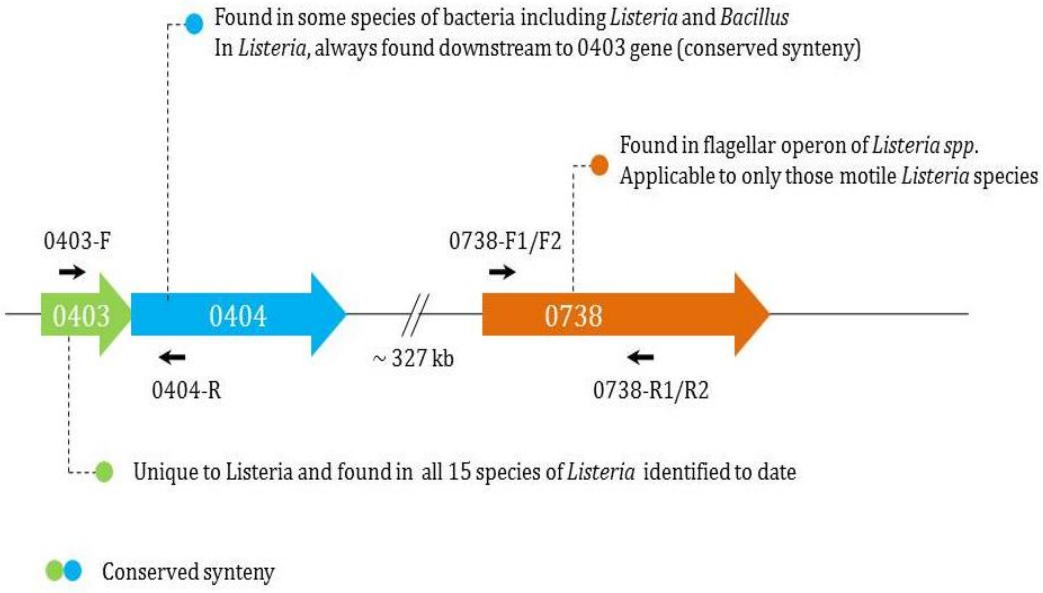


Figure 2: BE-LisAll primers for *Listeria* detection was developed by placing the forward primer on LMOf2365_0403 gene and the reverse primer on LMOf2365_0404 gene.

To confirm the accuracy of the comparison results, 16s DNA sequencing of 9 *Listeria*-positive samples was performed using the universal primer 27F. All the samples were identified as *Listeria* (*L. seeligeri* and *L. innocua*), as shown in Table 4. This result highlighted the specificity and capability of BE-LisAll biomarker for detection of *Listeria* species, even in the mixed population of bacteria. With Half-Fraser and Fraser Broths used for the selective enrichment of *Listeria*, neither broth could completely inhibit *Enterococcus faecalis* (Schalau, 2015). So, the development of black color in the broths after incubation may not be the result of *Listeria* alone. Moreover, *Listeria* was detected by the BE-LisAll biomarker-based PCR method since the Half-Fraser Broth enrichment step. Half-Fraser Broth was used in the first step of the conventional culture method for *Listeria* as the pre-enrichment broth (16-h incubation) to aid in the recovery of injured cells. These results indicated that BE-LisAll biomarker exhibited high sensitivity of the detection of *Listeria* species.

Table 4: 16S rRNA sequence analysis of bacterial isolated strain as compared to GenBank Database.

Sample number	GenBank accession number	BLAST result	% Homology
1	FN557490.1	*L. seeligeri*	546/546 (100%)
10	AL592102	*L. innocua*	536/536 (100%)
21	AL592102	*L. innocua*	536/536 (100%)
23	AL592102	*L. innocua*	550/550 (100%)
25	AL592102	*L. innocua*	568/568 (100%)
27	AL592102	*L. innocua*	469/469 (100%)
29	AL592102	*L. innocua*	474/474 (100%)
35	AL592102	*L. innocua*	569/569 (100%)
46	FN557490.1	*L. seeligeri*	409/409 (100%)

In conclusion, BE-LisAll biomarker-based PCR amplification can now be proposed for early detection of all *Listeria* species with a high degree of accuracy and sensitivity. It is hoped that this discovery can establish this new biomarker as a tool for controlling *Listeria* contamination in food factories. Consequently, commercial food processing businesses will be able to employ this finding to prevent or reduce economic losses and assure the safety of food products they produce.

CHAPTER III
DEVELOPMENT OF MOLECULAR METHOD FOR *Listeria* SPECIES IDENTIFICATION

3.1 Introduction

Listeria spp. are widely present the entire environments such as livestock, soil, plants, river water, silage, and seafood products (Farber, 2000; Miya et al., 2010; Vongkamjan et al., 2012). The *Listeria* genus consists of comprises 17 species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, *L. marthii*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. aquatica*, *L. cornellensis*, *L. floridensis*, *L. grandensis*, *L. riparia*, *L. booriae*, and *L. newyorkensis* (den Bakker et al., 2014; Weller et al., 2015). *L. monocytogenes* can be transmitted among humans and animals, and it is the cause of listeriosis. In the U.S.A., FDA standards have zero tolerance for L. monocytogenes contamination in processed foods, but with certain exceptions (Swaminathan et al., 2001). Likewise, the contamination level of L. monocytogenes in processed foods is strictly regulated in the EU at <100 cfu/g (Jadhav et al., 2012). Strict contamination management for *L. monocytogenes* is therefore necessary at food processing plants (Fox et al., 2011; Vogel et al., 2001). In the food industry, the FDA Bacteriological and Analytical Method (BAM) and the International Organization of Standards (ISO) 11290 method are established to detect *Listeria* spp. (Anon, 1997). In both methods, the *Listeria* are cultured in a liquid culture medium containing a selective agent and are then followed by the isolation of typical colonies and their culturing on selective media such as Oxford or PALCAM. The main drawback in these methods is that the process is time consuming and laborious. Alternatively, molecular methods such as 16S rDNA sequence (Hellberg et al., 2013), multiplex PCR (Huang et al., 2007), and multilocus sequence typing (MLST) (Salcedo et al., 2003), have all been used to identify *Listeria* spp. isolated by these methods. Methods that use sequence analysis, such as 16S rDNA sequencing and MLST, have high accuracy and reproducibility; however, they are complicated and expensive (Cai et al., 2013), making them unsuitable

for evaluating large quantities of samples. Rapid testing is critical for the food industry, and it is necessary that the methods be inexpensive and relatively easy to perform.

High-resolution melting analysis (HRMA) utilizes the different temperatures which the double-stranded DNA is dissociated. The time required for is approximately 1 hour at maximum, which results in relatively reduced time required for identification. This has gained attention for its usefulness for large-scale testing. To date, HRMA method has been developed for typing of *Cronobacter* spp. in milk (Cai et al., 2013), *Salmonella* (Zeinzinger et al., 2012), and *L. monocytogenes* (Pietzka et al., 2011). This technique has received a lot of attention in fields other than food microbiology; by using HRMA for examining specific genes, methods have been developed to identify the other ingredient oil which is mixed with olive oil (Ganopoulos et al., 2013), as well as hookworm infection in humans (Ngui et al., 2012). Therefore, this study aimed to develop an HRMA method for identifying all the 9 *Listeria* species by using the novel gene targets, and evaluated the new method by using it for analyzing bacterial isolates from the food industry.

3.2 Material and Methods

3.2.1 Bacteria strains and DNA extraction

Strains used in this study are listed in Table 5. Thirteen strains of 9 *Listeria* spp. were obtained from American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Collection of Institute Pasteur (CIP) and 6 strains of 3 *Listeria* spp. were isolated from a food processing plant or the environment. One strain of *L. seeligeri* used in this study was isolated from the river water located in Hokkaido, Japan (140.15596, 42.29360). The sampling site is located in open access area and no specific permissions are required to collect samples. Additionally, endangered or protected species were not collected. Strains were grown in Trypticase Soy Broth (TSB) (Becton Dickinson, U.S.A.) at 37 ^{O}C overnight. Bacterial cells were harvested from 1 mL TSB by centrifugation at 12,000 rpm for 3 min and the supernatant was removed. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey Nagel, Germany) according to the manufacturer's protocol.

Table 5: Bacterial strains used in this study.

Species	Strain no.
L. monocytogenes	ATCC19114
	ATCC19115
	ATCC19116
	CIP103575(SottA)
	CIP107776(EGDe)
L. innocua	ATC33090
	1-2
	8-1
	1-25
	26-1
L. seeligeri	ATCC35967
	12.9.11.2-1
L. rocouriae	DSM22097
L. ivanovii	ATCC19119
L. grayi	ATCC19120
L. welshimeri	ATCC35897
	019-3w
L. marthii	DSM23913
L. fleischmannii	DSM24998

3.2.2 Primer design

The *rar*A, which encodes a recombination factor protein, and the *ldh*, which encodes L-lactate dehydrogenase, were chosen as target genes. Sequence date for the rarA of *L. monocytogenes* strain FSL S4-465 (GenBank accession number: GU475922.1), *L. innocua* strain FSL R6-556 (GU475917.1), *L. seeligeri* FSL S4-009 (GU475926.1), *L. rocourtiae* strain CIP 109804 (JQ287768.1), *L. ivanovii* subsp. ivanovii FSL F6-600 (GU475932.1), *L. grayi* DSM 20601 (CCR02000005.1), *L. welshimeri* FSL S4-182 (GU475919.1), *L. fleischmannii* LU2006-1 c28 (ALWW01000009.1), *L. marthii* FSL S4-120(GU475909.1), and sequence date for

the *ldh* of *L. monocytogenes* EGDe (AL591824.1), *L. innocua* Clip11262 (NC_003212.1), *L. seeligeri* FSL S4-171 (GU475600.1), *L. ivanovii* subsp. ivanovii PAM 55 (NC_016011.1), *L. grayi* DSM 20601 (ACCR02000005.1), *L. welshimeri* serovar 6b SLCC5334 (NC_008555.1), *L. fleischmannii* LU2006-1 c9 (NZ_ALWW01000007.1), and *L. marthii* FSL S4-120 (GU475572.1) were obtained from GenBank. Multiple sequence alignment was performed using the Genetyx-Win program (Software Development Co., Japan). Unique and specific primer pairs for *rar*A and *ldh* were developed using the above information (Table 6).

Table 6: Sequence of primers used in this study.

Forward primer	Sequence (5′→3′)	Reverse primer	Sequence (5′→3′)
For *rarA* amplification			
*rar*A-f universal	GGYGCVACRACDA GTAATCC	*rar*A-r universal	CCRTTRCTSGCHGTT GC
*rar*A-f *L.grayi*	CGCTACCACCAGTA ATCC	*rar*A-r *L.rocourtiae*/ *seeligeri*	CCATTACTCGCMGTC GC
*rar*A-f *L.innocua*	GCAACGACGAGTA ACCC	*rar*A-r *L.innocua*/ *ivanovii*	CCRTTACTYGCYGTG GCAA
*rar*A-f *L.fleischmannii*	GCCACAACGAGCA ATCC	rarA-r *L.fleischmannii*	CCACCACTTGCTGTC GC
For *ldh* amplification			
ldh-f	GGYAAAATCGCAT TTTCGTTA	*ldh*-r	CCAGCWTGGAGCCA YACAAC

3.2.3 PCR Amplification of *rarA* Gene and *ldh* Gene

Partial *rarA* and *ldh* gene fragments were amplified for the 19 bacterial strains listed in Table 5. PCR was performed in a final volume of 50 µL. The PCR reaction mix for *rarA* contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM rarA-f universal primer, 167 nM *rar*A-f *L. grayi* primer, 167 nM *rar*A-f *L. innocua* primer, 167 nM *rar*A-f *L. fleischmannii* primer, 375 nM *rar*A-r universal primer, 250 nM *rar*A-r *L. rocourtiae*/*seeligeri* primer, 250 nM *rar*A-r *L. innocua*/*ivanovii* primer, 125 nM *rar*A-r *L. fleischmannii* primer, 25 ng template DNA, and 0.5 U Takara *Taq* DNA polymerase (Takara Bio, Japan). The PCR reaction mix of *ldh* contained 1 µM *ldh*-f and *ldh*-r primers instead of *rarA* primers. Primer sequences used in this study are shown in Table 6. Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters ware used for amplifying rarA: 95 ^{O}C for 5 min, 35 cycles of 95 ^{O}C for 10 s, 56 ^{O}C for 30 s, 72 ^{O}C for 30 s, and 72 ^{O}C for 1 min. For amplification of ldh, the following conditions were used: 95 OC for 5 min, 35 cycles of 95 ^{O}C for 10 s, 60 ^{O}C for 30 s, and 72 ^{O}C for 1 min. PCR products were confirmed by electrophoresis in a 2% agarose gel.

3.2.4 HRMA

Following confirmation of target gene amplification, 1 µL of 20x Resolight Dye (Roche, Germany) was added to 19 µL of the PCR reaction, and HRMA was carried out with a LightCycler 480 at 75 acquisitions/°C with the following steps: 95 ^{O}C for 1 min and 40 ^{O}C for 1 min, followed by increasing the temperature from 60 to 99 ^{O}C at 0.01 ^{O}C/s. For *rarA* analysis, Light Cycler 480 gene scanning program (Roche) was used. The straight-line parts of the DNA dissociation curve at the time of dissociation (73.06–79.05 ^{O}C and 90.09–91.77° ^{O}C) were selected for normalization, and differences in the shape of the normalized and temperature-shifted plots according to each *Listeria* spp. were determined. The melting peak of *L. monocytogenes* CIP103575 was used as a baseline control. For *ldh* analysis of *L. monocytogenes* and *L. welshimeri*, Tm calling was performed using the Light Cycler 480 software and the

Tm value of the amplification products determined. All samples of *ldh* were examined in triplicate and obtained the standard deviation (SD) for the Tm value.

3.3 Results and discussions

High resolution DNA melting analysis (HRMA) is a low-cost, rapid, and high throughput closed-tube method for analyzing genetic variation in PCR amplicons (Dhakal et al., 2013). In this present study, a method was developed for identifying *Listeria* spp. via HRMA of the polymorphic regions in *rar*A and *ldh*. It was considered that the polymorphism of these sequences which were different from species to species showed diverse HRM peak patterns and Tm values. In an evaluation of 9 species – *L. monocytogenes, L. innocua, L. seeligeri, L. rocourtiae, L. ivanovii, L. grayi, L. welshimeri, L. marthii,* and *L. fleischmannii* – by using the newly developed HRMA method. The HRMA was performed on the *rar*A of 19 strains from 9 species of *Listeria* spp. (Figure 3), resulting in the classification of 5 strains of *L. monocytogenes* into 2 patterns. The 5 *L. innocua* strains could be grouped together. *L. seeligeri* isolated from the environment showed a specific pattern; however, typestrain of *L. seeligeri* was grouped with *L. monocytogenes* strains. *L. fleischmannii, L. grayi, L. ivanovii, L. rocourtiae,* and *L. marthii* each showed specific patterns. However, the type strain and the isolated strain from food industry of *L. welshimeri* could not be distinguished from *L. monocytogenes*.

There were 2 problems that arose with the initial attempt to identify *Listeria* spp. by using *rar*A alone. First, the HRM peak pattern differed between the type strain of *L. seeligeri* and the environmental isolate of *L. seeligeri*. The environmental isolate of *L. seeligeri* showed a completely different specific peak compared to other species, while the type strain showed the same peak as that of *L. monocytogenes*. To determine the reason for this difference, the sequences of *rar*A regions of the 2 *L. seeligeri* strains and *L. monocytogenes* CIP103575 were compared. The sequences of the 2 strains of *L. seeligeri* differed by 3 out of 202 bases; therefore, even within the same species, the sequence is not identical. Furthermore, 34 differences in the sequence were found between the type strain of *L. seeligeri* and *L. monocytogenes* CIP103575, but because the respective GC content were 41.1% and 41.6%, both species produced similar Tm

values. HRMA uses the differences in GC content, the composition of bases, and sequence lengths (Ngui, Lim, & Chua, 2012).

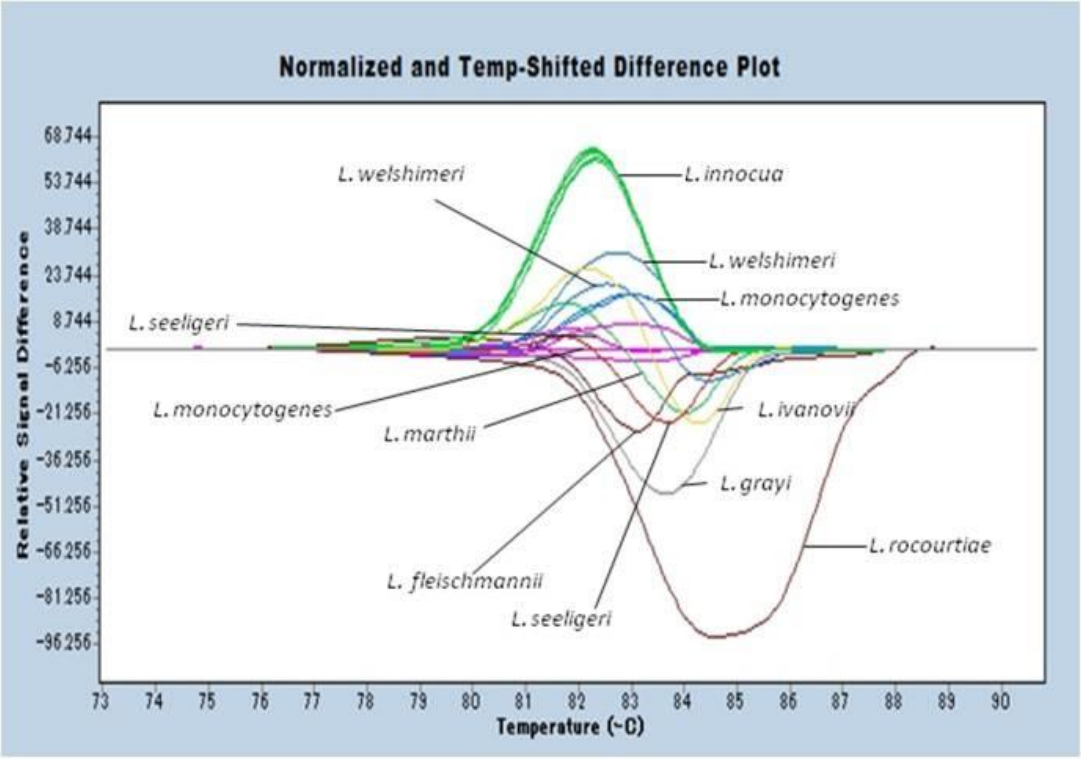


Figure 3: The result of high resolution melting analysis of *rar*A for 19 strains of 9 *Listeria* spp.

Representative profiles of the high resolution melting curves (normalized and temperature shifted difference plot) of rarA amplicons for *L. innocua* (upper green line), *L. welshimeri*^{T} (upper blue line), *L. welshimeri* 019-3w (blue line in the middle), *L. monocytogenes* ATCC19114, ATCC19116 (lower blue lines), *L. monocytogenes* CIP107776, CIP103575 (base line), ATCC19115 (pink lines), *L. seeligeri*^{T} (pink line), *L. fleischmannii*^{T} (upper brown line), *L. seeligeri* 2–1 (red line), *L. marthii*^{T} (lower green line), *L. ivanovii*^{T} (yellow line), *L. grayi*^{T} (gray line) and *L. rocourtiae*^{T} (lower brown line). T: type strain.

In this case, although the sequence differed, the GC content was very similar across the species, thereby producing a common HRMA peak pattern. The second issue was that *L. monocytogenes* and *L. welshimeri* could not be distinguished by using the HRMA peak pattern of *rar*A alone. Comparison of the sequences of the *rar*A region used in HRMA of *L. monocytogenes* and *L. welshimeri* showed that 35 out of 200 base pairs were different. However, as with *L. seeligeri*, discrimination was difficult due to similar GC levels (41.6 and 41.4%, respectively). Previous report demonstrated the relationship between mutations, single nucleotide polymorphisms (SNPs), and HRMA curves, and demonstrated that even if the sequence was different, the same melting curve results if the Tm values were similar (Pietzka et al., 2011). In this study, strains that could not be distinguished were those with similar Tm values. Identification using *ldh* was attempted for the 2 strains that could not be distinguished by *rar*A HRMA. The Tm value was then investigated for the *ldh* of *L. monocytogenes* and *L. welshimeri*, which could not be determined by HRMA of rarA. Tm values of the 5 strains of *L. monocytogenes* ranged from 82.57±0.30–83.25±0.32°C, and Tm of the 2 *L. welshimeri* strains were 83.88±0.15 and 84.08±0.12°C, respectively. The results of HRMA using *ldh* of 5 *L. monocytogenes* strains, as well as the 2 strains of *L. welshimeri*, are shown in Table 7.

Table 7: Tm value of *5 L. monoytogenes* strains and 2 *L. welshimeri* strains for *ldh* gene

species	strain	Tm (°C)
L. monocytogenes	ATCC19115	82.91
	ATCC19114	83.22
	CIP103575	83.36
	CIP107776	83.36
	ATCC19116	83.62
L. welshimeri	ATCC35897	84.02
	019-3w	84.20

Since the Tm values of the 2 species differed by at least 0.5°C, the Tm values can be used to differentiate between the 2 species. Besides, the Tm values of the 2 species were very different, and thus criteria could be established for their identification. To evaluate the newly established method of HRMA, identification of 81 strains isolated from the food-processing plant was performed. HRM peak patterns of *rar*A were classified broadly into 3 groups. Twenty-one were *L. innocua*, and 26 were *L. seeligeri*, while 33 strains were classified into the *L. monocytogenes*/*L. welshimeri* group (Figure 4).

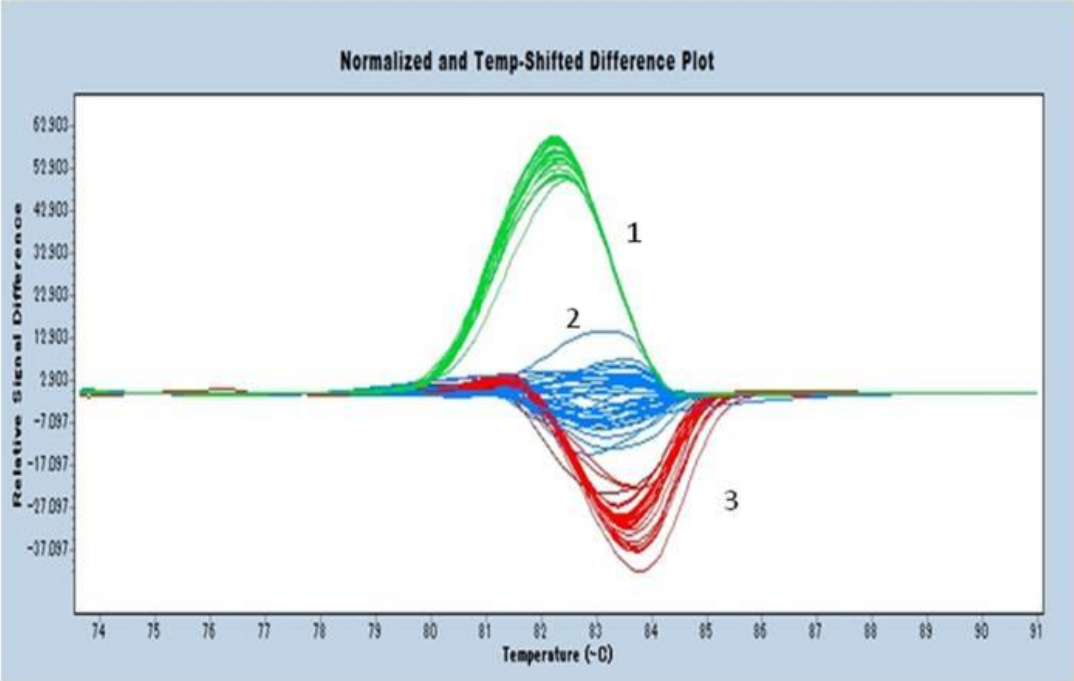


Figure 4: The result of high resolution melting analysis of *rar*A for 81 strains isolated from food processing plant.

The peak pattern of 1 strain did not fit into any group. The 33 strains classified into the *L. monocytogenes*/*L. welshimeri* group underwent species identification using ldh. Using the previously described method, strains with a Tm value of 83.31°C or below were designated *L. monocytogenes*, and those with a Tm higher than 83.82°C designated *L. welshimeri*, resulting in 18 identified as *L. welshimeri* and 14 as *L. monocytogenes*, with 1 strain remaining unidentified (Table 8).

Table 8: Tm value of 32 strains which could not identify by *rar*A gene HRMA.

Strain no.	Tm (°C)	*Listeria* species	Strain no.	Tm (°C)	*Listeria* species
31	83.06	*monocytogenes*	16	84.12	*welshimeri*
21	83.1	*monocytogenes*	2	84.14	*welshimeri*
76	83.12	*monocytogenes*	61	84.2	*welshimeri*
35	83.12	*monocytogenes*	4	84.2	*welshimeri*
23	83.12	*monocytogenes*	7	84.2	*welshimeri*
52	83.13	*monocytogenes*	14	84.22	*welshimeri*
12	83.13	*monocytogenes*	10	84.24	*welshimeri*
20	83.17	*monocytogenes*	9	84.25	*welshimeri*
11	83.18	*monocytogenes*	1	84.26	*welshimeri*
28	83.19	*monocytogenes*	38	84.27	*welshimeri*
22	83.2	*monocytogenes*	5	84.3	*welshimeri*
18	83.21	*monocytogenes*	37	84.32	*welshimeri*
25	83.21	*monocytogenes*	17	84.35	*welshimeri*
19	83.25	*monocytogenes*	15	84.42	*welshimeri*
46	83.64	*monocytogenes*	3	84.8	*welshimeri*
13	84.04	*welshimeri*	45	85.56	*welshimeri*

The results of HRMA identification were validated using 16S rDNA sequencing. The 21 strains identified as *L. innocua* by the HRMA of *rar*A gave the same results in 16S rDNA sequencing. Additionally, the 14 strains of *L. monocytogenes* and 18 strains of *L. welshimeri* that were identified by a combination of *rar*A and *ldh* data also produced the same results in 16S rDNA sequencing. Among the 26 strains identified as *L. seeligeri* from the HRMA of rarA, 22 strains were confirmed as *L. seeligeri* by 16S rDNA sequencing. Four strains showed results that were different for 16S rDNA sequencing from those of HRMA; 2 were identified as *L. monocytogenes* from 16S rDNA sequencing, while the other 2 were identified as *L. welshimeri*. Additionally, the strain not belonging to any group from the HRMA of *rar*A was determined to be *L. welshimeri*. The strain for which identification was

unfeasible due to the *ldh* Tm value of 83.64°C was also *L. monocytogenes*. The success rate of species identification by HRMA was 100% for *L. monocytogenes*, *L. innocua*, and *L. welshimeri*, and 84.6% for *L. seeligeri* (Table 9). The overall success rate for all 81 strains was 92.6%. In the present study, a method was established using HRMA of *rar*A and *ldh*, which identified 9 species belonging to the genus *Listeria*. The food industry uses FDA BAM and ISO methods for testing food products for *Listeria*, and if typical colonies are confirmed on a selective culture medium, species identification of the strain is necessary. Since strain identification can take several additional days, the present method, which needs only hours, can contribute significantly to increasing the rapidity of testing. The present study assumes the HRMA is carried out on pure, isolated colonies, and would be easy and appropriate to adopt for daily testing carried out by food companies. The method can be considered sufficiently applicable, as evaluation on actual isolates from the food factory identified *Listeria* spp. with a success rate of 92.6%.

Table 9: Comparison of results of identification by HRM and 16S rDNA sequencing *Listeria* spp. isolated from the food industry.

Listeria spp.	A: Number of strains identifiedby HRMA	B: Number of strains in agreement between the HRMAand 16S rDNA sequencing identificaation results	Success rate of the HRMA identification method (B/A)
L. monocytogenes	14	14	100%
L. innocua	21	21	100%
L. seeligeri	26	22	84.6%
L. welshimeri	18	18	100%
could not identify	2	-	-
total	81	75	92.6%

In addition to the 9 species used in the present study, *L. weihenstephanensis* has been identified recently as a member of the *Listeria* genus. Based on the present study, the likelihood of isolating this species in the food industry is low; however, it is necessary to have methods to identify such strains of *Listeria* spp. distributed in the environment. The simplicity and rapidity of HRMA method surpasses that of identification by sequence analysis, and its concurrence with the 16S rDNA sequence analysis was also high. Our newly developed method for identifying *Listeria* spp. is highly valuable; its use is not limited to the food industry, and it can be extended to identifying strains isolated from the natural environment.

CHAPTER IV
DEVELOPMENT OF MOLECULAR SUBTYPING METHOD FOR *Listeria monocytogenes*

4.1 Introduction

Listeria monocytogenes is the causative agent of listeriosis in humans and animals (Ferreira et al., 2014; Schuchat et al., 1991). In pregnant women, listeriosis may lead to miscarriage, fetal death, or babies being born with listeriosis (Cartwright et al., 2013). The fatality rate for listeriosis is 20–30%, which is much higher than that of diseases caused by other food poisoning bacteria. Most cases of outbreaks were related to contaminated foods. This bacterium has ability to survive and grow at refrigerated temperature. Moreover, it has ability to form biofilm in machine or on wet surface. For this reason, *L. monocytogenes* is widely distributed in the environment. It is also often detected in RTE foods such as processed meat (Gandhi & Chikindas, 2007; Montero et al., 2015), and salads (Cartwright et al., 2013). Several cases of listeriosis occurred in the EU, caused by cheese, fruit, vegetable, and meat products. Contamination of food by pathogens, including *L. monocytogenes*, not only increases the risk of disease outbreaks, but also results in tremendous economic losses due to product rejection (Lomonaco et al., 2015). Therefore, food companies utilize the intense care in minimizing contamination of products by such bacteria. To completely prevent contamination at food production facilities, it is important to identify and eliminate sources and routes used by bacteria to contaminate the factory and products. Therefore, product and environment sampling is conducted regularly at food production facilities to isolate bacteria.

Molecular methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and multiple locus variable-number tandem repeat analysis (MLVA), have been widely used to discriminate bacterial isolates (Bertrand et al., 2016; Chenal-Francisque et al., 2013; Kimura et al., 2008; Lüdeke et al., 2015; Malachowa et al., 2005; Moura et al., 2016). Several reports utilizing these methods to investigate contamination sources at food production facilities have been reported (Otero et al., 2013; Prendergast et al., 2011; Takahashi et al., 2014). Although bacteria

with diverse genotypes have been isolated from food production facilities, often such bacteria are genetically related to each other. This relatedness depends on the source location or the time of isolation (Barancelli et al., 2014). Therefore, a typing method with greater ability to differentiate isolates is needed.

Multiple-locus Variable number of tandem repeat Analysis (MLVA) has been used for subtyping the pathogenic bacteria such as *Salmonella* (Liu et al., 2003) *Staphylococcus aureus* (Sabat et al., 2003), *Escherichia coli* O157 (Lindstedt et al., 2004b), and *L. monocytogenes* (Murphy et al., 2007). Traditionally, variation of tandem repeat number can be analyzed by using capillary electrophoresis (CE). CE offers the advantages of high speed, great efficiency, as well as the requirement of minimum amounts of sample and buffer for the analysis of DNA. The MLVA was developed to subtype 45 *L. monocytogenes* isolated from salmon and other sources by using 6 loci (LM-TR1, LM-TR2, LM-TR3, LM-TR4, LM-TR5 and LM-TR6). The results demonstrated that MLVA can be used to clearly differentiate the *L. monocytogenes* serotype 4b isolated from Salmon from other sources (Murphy et al., 2007). Moreover, MLVA was developed to subtype *L. monocytoges* serotype 1/2a, 1/2b, and 4b by using 8 loci (Lm-2, Lm-3, Lm-8, Lm-10, Lm-11, Lm-15, Lm-23, and Lm-32). The results demonstrated that MLVA provides provided 7, 7, and 9 patterns from *L. monocytoges* serotype 1/2a, 1/2b and 4b (32, 31, and 60, respectively) (Sperry et al., 2008). Thus, the assay has a great potential as a high-resolution molecular typing tool for the study of *L. monocytogenes*. Hence, this study was conducted to develop MLVA for subtyping *L. monocytogenes* strain. The method in turn can help food industry in managing the risk of *L. monocytogenes* contamination in food industry by tracking their sources of contamination.

4.2 Materials and methods

4.2.1 Bacterial strain and DNA extraction

Thirty-two isolates of *L. monocytogenes* obtained from our laboratory collections were used in this study. Cell stocks of *L. monocytogenes* were activated by culturing on Tryptone Soy Agar (TSA) supplement with 0.6% yeast extract (YE) at 37 ^{O}C for 24 h. A single colony was inoculated into Tryptone Soy Broth (TSB)

supplement with 0.6% YE incubated at 37o C for 24h. Cell pellets were recovered from 3 mL TSB culture by centrifugation at 12,000 rpm for 10 minutes. Supernatant was discarded and cell pellets were stored at -20o C. Genomic DNA was extracted from cell pellets using Genomic DNA extraction kit (RBC bioscience, Taiwan) according to the manufacturer's protocol for Gram-positive bacteria. The purified DNA was stored at -20o C until use.

4.2.2 Subtyping of *L.monocytogenes* using MLVA

Previously validated PCR primer set targeting the variable number of tandem repeat loci of *L. monocytogenes* based on a literature reviews (Chenal-Francisque et al, 2013) were used in this chapter. Evaluation of adopted MLVA primers were conducted by investigating their reproducibility, typeability, and discriminatory power. The primer information are listed in Table 10.

4.2.3 MLVA primer validation

PCR reactions were performed using 9 pairs of primers specific for 9 VNTR loci (Table 10). The PCR amplification was performed in a final volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara Taq DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters were used for amplification: 95 ℃ for 5 min, 35 cycles of 95 °C for 30 s, 60 °C for 40 s, 72 °C for 1 min, and 72 ℃ for 4 min. PCR products were visualized and confirmed by electrophoresis in a 2% agarose gel. To analyze the variants further, the observed amplicons were subjected to capillary electrophoresis (CE; QIAxcel Advanced; Qiagen, Tokyo, Japan) for fragment analysis. DNA sequencing was used to determine the number of TRs in any novel-sized fragments (Appendix 5).

Table 10: Sequence of primers used in this study (Chenal-Francisque et al., 2013).

TR locus	Primer name	Sequence (5'→3')	Repeat sequence	Repeat size (bp)
JLR1	JLR1-F JLR1-R	GCGCTATAACCTGAGGAAAGC GTCTTAATCCATGCAGATGGAAC	CAGCAT	6
JLR2	JLR2-F JLR2-R	CCTTCCAGAGAAAGACAAAACAG RCTAATCCACCAGCAAATAGC	CAAAAGATACAC	12
TR1317	LIs-TR1317-F Lis-TR1317-R	TGATTTACAAAAAGCTTTGCC ACTTGGCACTTCTGGTTTA	AACACCAACACCAGA CCCAACACC	24
TR1869	Lis-TR1869-F Lis-TR1869-R	CCGCGCTATAACCTGAGGAAAGC CTGAAATCATTGCAATCAGATGCACC	TCAGCATCAGCG	12
TR881	Lis-TR881-F LIs-TR881-R	TGTAAATAAAGCTGGTACGTAC GTATGTTGCTTGTTATCAACTAC	AAAACCAATAAAACC ATC	18
JLR4	JLR4-F JLR4-R	AGAAATTCCAGTCCGCCAG GGARCAACAGAAGCTGATCCA	CTTCTGGAGCTTCTGG TA	18
LMTR4	LM-TR4-F LM-TR4-R	TCCGAAAAAGACGAAGAAGTAGCA TGGAACGACGGACGAAATAATAAT	GAAGAACCAAAA	12
LMV1	LMV1-F LMV1-R	CGTATTGTGCGCCAGAAGTA MAMCAACRCAACAACAAACAG	TTGTAT	6
LMV9	LMV9-F LMV9-R	AACGGTKRCKGATTTACTTC CTTGGYGTCGAGGCATTTA	AGAAAAACC	9

4.2.4 Tandem repeat analysis using capillary electrophoresis

The PCR products of 32 strains of *L. monocytogenes* were directly subjected into the QIAxcel Advanced system using the QIAxcel DNA High-Resolution Kit (QIAGEN). A custom alignment marker of 15–600 bp was run simultaneously with the samples, and the QX DNA size marker of 25–500 bp was used for size estimation. The reaction products were analyzed using the OM500 method at 5 kV and a 500 s separation time. The alignment marker was injected at 4 kV for 20 s and samples at 5 kV for 10 s. The following formula was used in MLVA to calculate the actual size of the repeat:

$$\textbf{Number of repeat unit } (\textbf{RU}) = \frac{\textbf{amplicon size} - \textbf{flanking size}}{\textbf{repeat unit size}}$$

where RU = number of repeat unit of interesting TR
amplicon size = size of PCR product
flanking size = size of PCR product without TR
repeat unit size = nucleotide number of TR

4.2.5 Subtyping of *L. monocytogenes* using Random Amplification of Polymorphic DNA (RAPD)

DNA of *L. monocytogenes* were used to analyze by RAPD described by Keeratipibul & Techaruwichi (2012). PCR reactions were performed using 4 pairs of primers (Table 11). The PCR amplification was performed in a final volume of 50 µL, containing 10 mMTris-HCl (pH 8.3), 50 mMKCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara Taq DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). Under the conditions listed in Table 11. PCR products were visualized and confirmed by capillary electrophoresis (QIAxcel, Germany).

4.2.6 Comparison of discriminatory power for subtyping *L. monocytogenes* between MLVA and RAPD

In order to compare the discriminatory power of the two different subtyping methods used in this study, diversity indices were calculated. The diversity indices are commonly used to measure the discriminatory power by defining the ability of method to distinguish between unrelated isolates. The higher the value of a diversity index defines the higher the discriminatory power of the subtyping method (Hunter et al, 1998). The discriminatory index (D-value) described by Hunter and Gaston of individual and combined TR loci was also evaluated by this equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

When N = number of isolates tested

S =number of different genotypes

nj = number of isolates belonging to genotype j.

Table 11: PCR amplification and primers used for RAPD in *L. monocytogenes*.

No.	Primer sequence	PCR reaction	PCR condition	
1	UFS	1X PCR Buffer	94°C 3 min	
	(5’	1.5 mM $MgCl_2$	94°C 45 sec	
	TTATGTAAAA	200 µM dNTP	26°C 2 min	4X
	CGACGGCCAG	0.5 µM UFSPrimer	72°C 2 min	
	T 3’)	0.1 U *Taq* DNA	94°C 45 sec	
		Polymerase	36°C 1 min	30X
			72°C 2 min	
			72°C 5 min	

Table 11: PCR amplification and primers used for RAPD in *L. monocytogenes* (continued).

No.	Primer sequence	PCR reaction	PCR condition	
2	HLWL74	1X PCR Buffer	94°C 4 min	
	(5' ACGTATCTGC 3')	1.2 mM $MgCl_2$	39°C 45 sec	
3	HLWL85	100 µM dNTP	72°C 1 min	
	(5' ACAACTGCTC 3')	0.3µ M DNA Primer	94°C 15 sec	
4	OMP-01	0.1 U *Taq* DNA	39°C 45 sec	43 x
	(5' GTTGGTGGCT 3')	Polymerase	72°C 1 min	
			94°C 15 sec	
			39°C 45 sec	
			72°C 10min	

4.3 Results and discussion

Listeria monocytogenes is the species of pathogenic bacteria that causes the listeriosis in human. This microorganism is most commonly found in fresh foods, ready to eat foods, plant facilities and plant environment. However, the *L. monocytogenes* which found on those environments might not be the bacteria that contaminated in the products since the conventional identification method, such as BAM (Hitchins & Whiting, 2001), ISO11290 method (Anon, 1997), and AOAC/IDF method 993.09 (AOAC, 2000), cannot be used to differentiate different strains of the same *Listeria* species. Recently, the molecular methods, such as RAPD, PFGE, MLST and MLVA, have ability to differentiate amongst different strains of bacteria, making it possible to determine the real source of contamination. Thus, development of molecular subtyping method is necessary for monitoring and tracking the sources of contaminated bacteria for food safety improvement. In this study, the MLVA method was applied to subtype the *L. monocytogenes*. This method has been successfully applied to subtype many other bacterial species by detecting variation of tandem repeat element (Grundmann et al., 2001; Pourcel et al., 2009). The relevance of this tool for the subtyping of *L. monocytogenes* was investigated by testing this method on nine VNTR loci in 30 strains previously characterized by RAPD method. The nine selected TR loci and primers were used to amplify 30 isolates of *L. monocytogenes*.

The PCR products were visualized and size determined by capillary electrophoresis. All 9 TR loci could be successfully amplified without non-specific amplicons (Figure 5). Representative amplicons were chosen from each TR loci and sequenced for examining the flanking region and repeat sequence. The sequences were then used to investigate the allele number of each TR loci. Overall length of PCR amplicons and DNA sequences were measured and analyzed to determine the size range and repeat sequences of TR loci (Appendix 4). The polymorphisms of all 9 TR loci were investigated. The 30 *L. monocytogenes* isolates produced 20 MLVA patterns. The JLR1 locus was divided into 5, JLR2 locus was divided into 4 types, Lis-TR1317 locus was divided into 2 types, Lis-TR1869 locus was divided into 4 types, Lis-TR881 locus was divided into 5 types, JLR4 locus was divided into 4 types, LM-TR4 locus was divided into 3 types, LMV1 locus was divided into 5 types, and LMV9 locus was divided into 4 types. The VNTR information are shown in Table 12. For RAPD analysis, the RAPD method described by Keeratipibul & Techaruwichi (2012) was used to investigate the diversity type. Thirty strains *of L. monocytogenes* were screened using 4 random primers (UFS, HLWL74, HLWL85, OMP-01), generated 15 RAPD profiles (Figure 6).

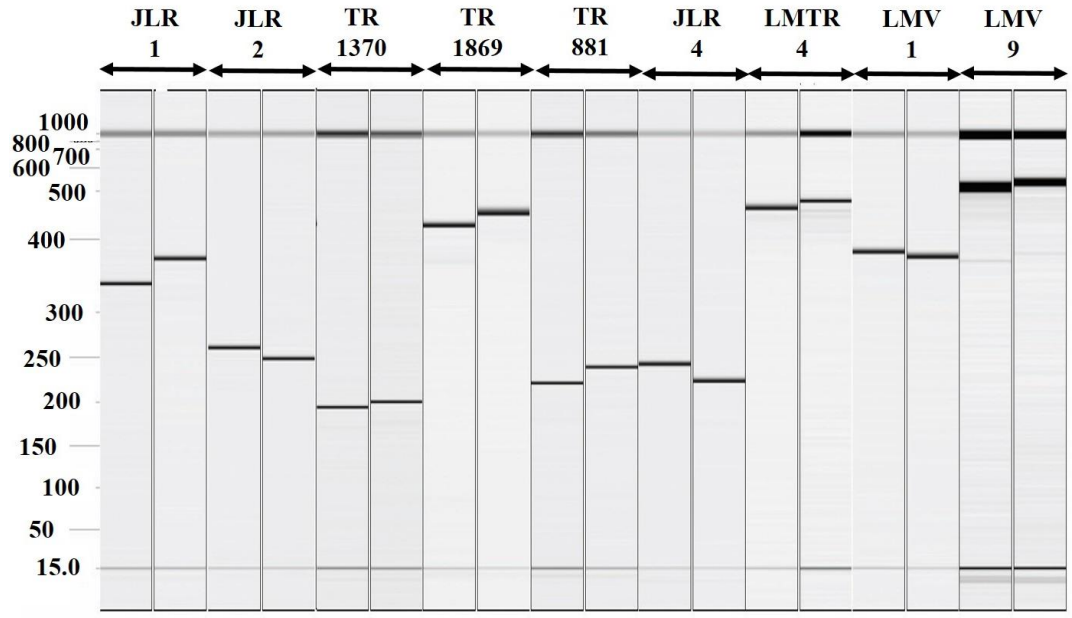


Figure 5: Visualization and size determination of VNTR in *L. monocytogenes* by capillary electrophoresis.

Table 12: The selected 9VNTR information.

Name	Repeat sequence	Repeat size (bp)	Size range (bp)	No. of alleles *	Allele range (copy no.)
JLR1	CDSCRT	6	343.1 – 380.7	5	11 – 17
JLR2	CAAAARATACAC	12	236.6 – 280.3	4	2 – 5
Lis-TR1317	AACACCGGACCCAACACC	18	168.7 – 201.1	2	0 – 1
Lis-TR1869	TCCGCATCGGCA	12	414.3 – 465.9	4	4 – 8
Lis-TR881	AAAACCTAT	9	217.2 – 289.7	5	1 – 9
JLR4	CTTCTGGAGCTTCTGGTA	18	171.3 – 313.8	4	0 – 5
LM-TR4	GAAGAACCAAAA	12	460.8 – 484.9	3	1 – 3
LMV1	TTGTAT	6	370.1 – 394.3	5	2 – 6
LMV9	AACCGGATA	9	488.9 – 539.1	5	NA – 4

* With null allele

Comparison of the two methodologies was undertaken with Simpson's index of diversity. The higher the value of a diversity index defines the higher the discriminatory power of the subtyping method (Hunter & Gaston, 1988). MLVA analysis with the nine selected loci discriminated the 30 *L. monocytogenes* isolates into 20 different patterns with a Simpson's index of diversity of 0.96, while RAPD resolved them into 15 patterns with an index of 0.92. The largest groups of identical RAPD profiles consisted of 7 isolate, most of which belonged to R6. All 7 of the R6 strains in this work clustered together but were resolved into 6 different MLVA patterns within this cluster; 2 of them had identical profiles (Table 13). Besides, RAPD is much more subjective in data interpretation and requires a trained specialist for RAPD pattern designation, leading to more variability in inter-laboratory results (Takahashi et al., 2014).

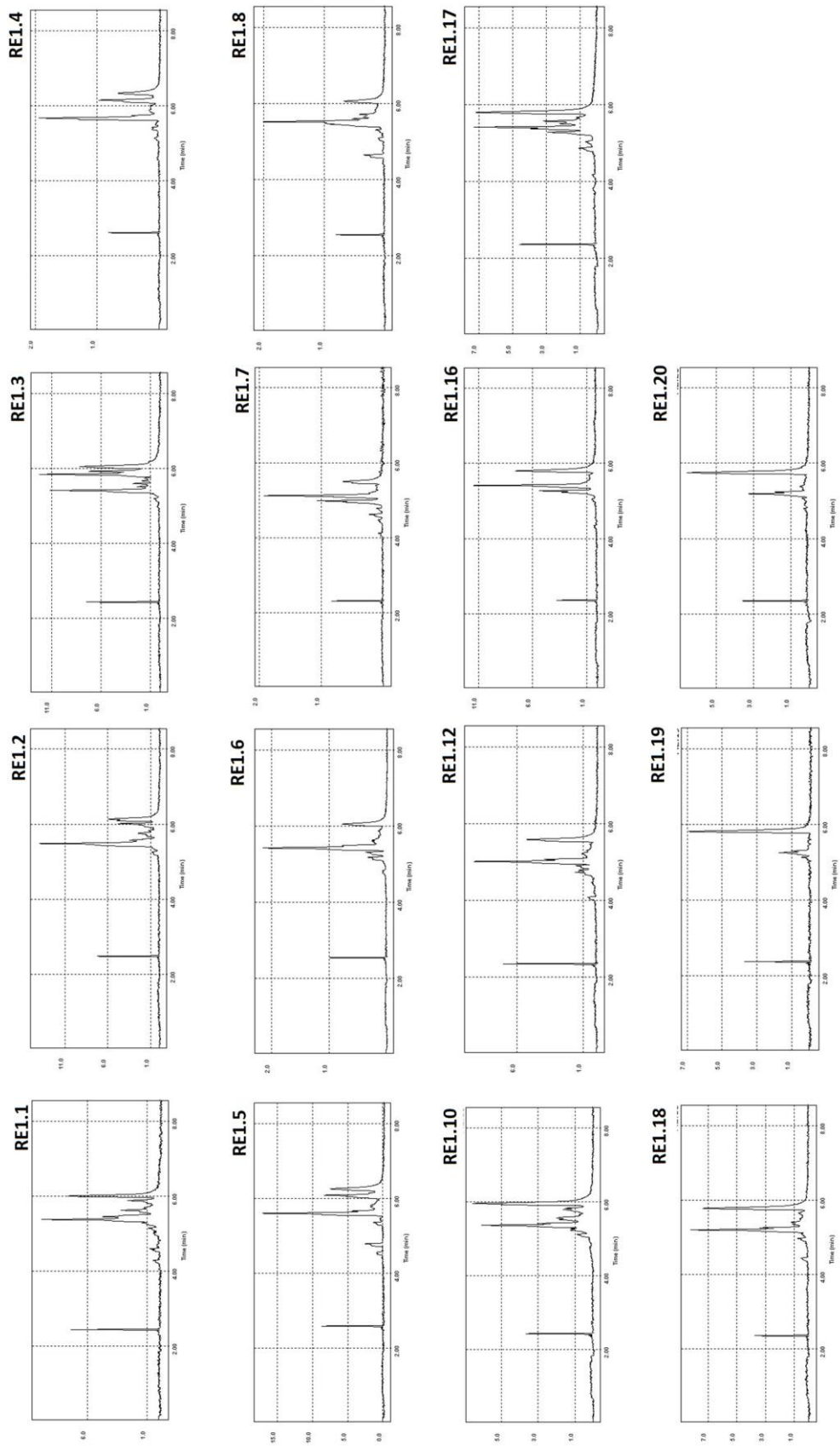


Figure 6: Fifteen RAPD profiles of *L. monocytogenes*.

Table 13: MLVA and RAPD type of 30 *L. monocytogenes* isolates.

Isolate	MLVA pattern	MLVA type	RAPD pattern	RAPD type
1	17-4-8-3-4-3-3-2	M1	RE1.1	1
2	16-4-8-3-4-3-3-2	M2	RE1.2	2
3	13-4-6-7-3-3-4-3	M3	RE1.3	3
4	17-4-8-3-5-3-3-NA	M4	RE1.4	4
5	16-3-8-0-4-2-2-1	M5	RE1.4	4
6	12-5-6-0-3-2-2-3	M6	RE1.5	5
7	17-4-8-3-4-3-3-1	M7	RE1.6	6
8	17-3-8-0-4-2-3-3	M8	RE1.6	6
9	17-4-8-3-4-3-3-1	M7	RE1.10	9
10	17-4-8-3-4-3-3-2	M1	RE1.10	9
11	17-4-8-3-4-3-3-1	M7	RE1.12	10
12	12-5-6-0-3-1-3-4	M9	RE1.8	8
13	12-5-6-0-3-1-3-4	M9	RE1.10	9
14	11-2-4-9-0-2-6-1	M10	RE1.7	7
15	11-2-5-9-0-2-6-1	M11	RE1.13	11
16	11-2-4-9-0-2-5-1	M12	RE1.7	7
17	13-3-8-0-4-2-2-3	M13	RE1.2	2
18	17-4-8-3-4-3-3-2	M1	RE1.1	1
19	16-3-8-0-4-2-2-3	M14	RE1.6	6
20	17-3-8-0-4-2-2-3	M15	RE1.6	6
21	16-3-8-0-4-2-2-3	M14	RE1.19	14
22	16-3-8-0-4-2-3-3	M16	RE1.6	6
23	16-3-8-0-4-2-3-3	M16	RE1.6	6
24	16-3-8-0-4-2-3-3	M16	RE1.10	9
25	17-4-8-3-4-3-3-2	M1	RE1.20	15
26	16-3-8-0-4-2-2-2	M17	RE1.6	6
27	11-2-6-6-0-2-6-1	M18	RE1.16	12
28	16-3-8-0-4-2-3-3	M16	RE1.19	14
29	12-5-6-0-3-2-3-3	M19	RE1.18	13
30	16-4-8-0-4-3-3-1	M20	RE1.19	14

In conclusion, the MLVA typing scheme which is simple and robust reproducible was developed in this study. The nine VNTR loci chosen for this assay showed a high level of diversity between 30 *L. monocytogenes* strains. These results demonstrated that the MLVA will help in effective monitoring approaches to identify environmental contamination by pathogenic strains of *L. monocytogenes* and investigation of outbreaks.

CHAPTER V
DEVELOPMENT OF MOLECULAR SUBTYPING METHOD FOR *Listeria innocua*

5.1 Introduction

L. monocytogenes is the species associated with listeriosis in human. Therefore, regulatory commissions of Department of Livestock Development of Thailand and various countries such as The Food and Drug Administration (FDA) of United States require zero-tolerance (negative in 25 g samples) for this microorganism. However, many food importers require that all species of Listeria, not only *L. monocytogenes*, are not to be found in food products. It has been reported that *L. innocua* is the most frequently found species in finished products and plant facilities such as equipment and plant environments. *L. innocua* in particular is considered to be similarly distributed and behave similarly to *L. monocytogenes* and is the most frequently isolated species in food manufacturing sites (Keeratipibul & Techaruwichit, 2012; Oliveira et al., 2011). For this reason, establishment of a monitoring method and clarification of contamination sources and routes are required. In order to meet customer requirements, controlling and preventing *L. innocua* contamination in food processing plants are necessary. Therefore, tracking possible sources of *L. innocua* contamination in food processing plants is mandatory for achieving this goal. Molecular subtyping of bacteria is a beneficial method for tracking sources of contamination because it has ability to discriminate among different strains of bacteria and also infers an intimate relationship of bacterial strains between products and environments. Pulsed-field gel electrophoresis (PFGE) is widely used to assess the distribution of bacteria in foods and food manufacturing sites. PFGE, considered the gold standard technique due to its high resolution, has obvious disadvantages, including a complicated protocol, skill requirement, and time-consuming analysis. In addition, multilocus sequence typing (MLST) and multilocus variable number of tandem repeat analysis (MLVA) have been developed as typing methods that use DNA sequence analysis. MLVA is a technique that types bacterial strains by utilizing the fact that the number of repeat units in the variable number tandem repeat (VNTR) region varies depending on the strain.

Comparison of the numbers of repeats in multiple VNTR regions allows highly specific strain classification. Analytical methods using VNTR regions have been developed for various food microorganisms, such as *L. monocytogenes*, *Escherichia coli*, *Bacillus anthracis*, *Salmonella* enterica, and *Vibrio parahaemolyticus* (Kimura et al., 2008; Lindstedt et al., 2004a), showing the efficacy of MLVA as a strain identification method. Variant detection of TR of bacteria is a powerful molecular subtyping method for tracking sources of bacterial contamination because TR is highly specific element for each strain and can be used as markers for subtyping. So identification of TR loci by bioinformatics is a potential method for subtyping bacterial strains. Hence, this study was conducted to develop the novel TR loci for *L. innocua* strain differentiation. The method in turn can help food industry in managing the risk of *L. innocua* contamination in food industry by tracking *L. innocua* sources of contamination.

5.2 Material and Methods

5.2.1 Bacterial strains and DNA extraction

Ninety-four isolates of *L. innocua* obtained from our *L. innocua* collections were used in this study. Cell stocks of *L. innocua* were activated by culturing on Tryptone Soy Agar (TSA) supplement with 0.6% yeast extract (YE) at 37 ^{O}C for 24 h. A single colony was inoculated into Tryptone Soy Broth (TSB) supplement with 0.6% YE incubated at 37 ^{O}C for 24 hours. Cell pellets were recovered from 3 mL TSB culture by centrifugation at 12,000 rpm for 10 minutes. Supernatant was discarded and cell pellets were stored at -20 ^{O}C. Genomic DNA was extracted from cell pellets using Genomic DNA extraction kit (RBC bioscience, Taiwan) according to the manufacturer's protocol for Gram-positive bacteria. The purified DNA was stored at -20 ^{O}C until use.

5.2.2 VNTR selection and primer design

Whole genome sequence of *Listeria* innocua (NC_003212.1) along with its annotation information were downloaded from GenBank. Repeats identified by TRF were mapped on to coding sequences. Protein sequences of these coding genes were then extracted and used as query to blast against local protein database of *Listeria*.

Protein sequences that found orthologues in other *Listeria* species with different number of internal repeats were subjected to primer design using Primer 3 Plus. The primer sequences are given in Table 14.

Table 14: Primer sequence and VNTR information.

Locus	Repeat unit	Location	Sequence (5'→3')
TR1	ACAAAT	118054	F:TCGGTCGATTAAAGTCGAAA
			R:CCACCTTCATTTGCGTTACC
TR2	AAAAT	678153	F:CGATAAACAGCAGGTCCATT
			R:GGCACAAAGCATGCTAGAAA
TR3	CGGTAGACC	1124290	F:GACAAAAGTAAGTCATGCGGG TATTT
			R:TAGCTACAATCGGATTAACGG
TR4	GCTGTAGAATGGTT TAATACGAACATTT GGGAACCGATTAAA TTAGCAGTTTCAACT GTGGCTTATGCAAT ATCAAGCTACTTTTC AACCTCATGGGAAA TGATAAAATTAGTTT GGTTCCTT	1285178	F:TGAAATCGTAGGCTCTGCA R:ACTGCATCGTGAGCAGTTTG
TR5	TGCTTT	1695412	F:CGATGTTTGTGATTTTGTCCTCT
			R:GAAGGAGGAAACGTGGATGA
TR6	CATCGG	1924244	F:GTACCTCCATTTGCTGTTCC
			R:ATGTTATCCACCTTCAAGTAACTG
TR7	TCGGCA	1924542	F:TGGATTGACAGTTGGTTTTTCA
			R:CTGATGCTGACGCCGATG
TR8	TCGGCA	1924709	F:GGATTGACAGTTGGTTTTTCAACT AGGA
			R:TGACGCCGACGCTGATGCTGACG
TR9	GCATCC	1924762	F:ATCAGCGTCAGCATCGGCATCA
			R:TGACGCTGATGCCGACGC

Table 14. Primer sequence and VNTR information (continued).

Locus	Repeat unit	Location	Sequence (5'→3')
TR10	CAATCG	2172229	F:CACTGATGATTCGAAAAGCAAC
			R:ACAAATCCTAAAACGTGGTCA
TR11	TAAAGA	2444015	F:CGCCGCCCGCCGCCCGCCCCCCGT
			GCCTAGACTAACTTCTAAGGCAAT
			R:TCCTTTTAAATCTTCATAAGAAAC
			AAG
TR12	TTTTA	2471516	F:AAAAACTGTCACCATTCGATGT
			R:ACTTAATCAATCAGTTCCAAATGC
TR13	TTTACTGGG	2741368	F:ATGTGCTGGATCTGCTGGT
			R:AAAATCCAGTAGTTCCGGTAGAC

5.2.3 MLVA primer validation

PCR reactions were performed using the designed specific primers for VNTR loci. The PCR amplification was performed in a final volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mMKCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara *Taq* DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters were used for amplification: 95 ^{O}C for 5 min, 35 cycles of 95 ^{O}C for 30 s, 60 ^{O}C for 40 s, 72 ^{O}C for 1 min, and 72 ^{O}C for 4 min. PCR products were visualized and confirmed by electrophoresis in a 2% agarose gel. To analyze the variants further, the observed amplicons were subjected to capillary electrophoresis (CE; QIAxcel Advanced; Qiagen, Tokyo, Japan) for fragment analysis. DNA sequencing was used to determine the number of TRs in any novel-sized fragments (Appendix 7). Tandem repeat analysis using capillary electrophoresis were analyzed. The PCR products of 93 strains of L.innocua were directly subjected into the QIAxcel Advanced system using the QIAxcel DNA High-Resolution Kit (QIAGEN). A custom alignment marker of 15–600 bp was run simultaneously with the samples, and the QX DNA size marker of 25–500 bp was used for size estimation. The reaction products were analyzed using the OM500 method at 5 kV and a 500 s separation time. The alignment marker was injected

at 4 kV for 20 s and samples at 5 kV for 10 s. The following formula was used in MLVA to calculate the actual size of the repeat:

$$\textbf{Number of repeat unit } (\textbf{RU}) = \frac{\textbf{amplicon size} - \textbf{flanking size}}{\textbf{repeat unit size}}$$

where RU = number of repeat unit of interesting TR
amplicon size = size of PCR product
flanking size = size of PCR product without TR
repeat unit size = nucleotide number of TR

5.2.4 Subtyping of *L. innocua* using Random Amplification of Polymorphic DNA (RAPD)

DNA of *L. innocua* were used to analyze by RAPD described by Keeratipibul & Techaruwichi (2012). PCR reactions were performed using selected 4 specific primers for VNTR loci (TR1, TR3, TR6, and TR9). The PCR amplification was performed in a final volume of 50 µL, containing 10 mMTris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara Taq DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). Under the conditions listed in Table 15. PCR products were visualized and confirmed by capillary electrophoresis (QIAxcel, Germany)

5.2.5 Comparison of discriminatory power for subtyping *L.monocytogenes* between MLVA and RAPD

In order to compare the discriminatory power of the two different subtyping methods used in this study, diversity indices were calculated. The diversity indices are commonly used to measure the discriminatory power by defining the ability of method to distinguish between unrelated isolates. The higher the value of a diversity index defines the higher the discriminatory power of the subtyping method (Hunter et al,

1998). The discriminatory index (D-value) described by Hunter and Gaston of individual and combined TR loci was also evaluated by this equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

When N = number of isolates tested
S =number of different genotypes
nj = number of isolates belonging to genotype j.

Table 15: PCR amplification and primers used for RAPD in *L. innocua.*

No.	Primer sequence	PCR reaction	PCR condition	
1	UFS	1X PCR Buffer	94°C 3 min	
	(5’	1.5 mM $MgCl_2$	94°C 45 sec	
	TTATGTAAAACGACGG	200 µM dNTP	26°C 2 min	4X
	CCAGT 3’)	0.5 µM UFSPrimer	72°C 2 min	
		0.1 U *Taq* DNA	94°C 45 sec	
		Polymerase	36°C 1 min	30X
			72°C 2 min	
			72°C 5 min	
2	HLWL74	1X PCR Buffer	94°C 4 min	
	(5’ ACGTATCTGC 3’)	1.2 mM $MgCl_2$	39°C 45 sec	
3	HLWL85	100 µM dNTP	72°C 1 min	
	(5’ ACAACTGCTC 3’)	0.3µ M DNA Primer	94°C 15 sec	
4	OMP-01	0.1 U *Taq* DNA	39°C 45 sec	43 x
	(5’ GTTGGTGGCT 3’)	Polymerase	72°C 1 min	
			94°C 15 sec	
			39°C 45 sec	
			72°C 10min	

5.3 Results and discussion

Several research studies showed that *L. innocua* was found to be the most widespread species which infer the presence of *L. monocytogenes*. Therefore, the discovery of routes of transmission and emerging of these particular foodborne pathogens is important task for development of better prevention and control in food industries. DNA typing methods that are widely used for epidemiological surveys and tracking contamination sources include PFGE. However, the disadvantages of PFGE range from a complicated protocols, time consumption, to difficulty in data comparison due the results being a band pattern (Chenal-Francisque et al., 2013). One DNA typing method reported for *L. innocua* is random amplified polymorphic DNA (RAPD) (Keeratipibul & Techaruwichit, 2012). RAPD has low reproducibility, and the analysis is cumbersome to share because the electrophoresis results have to be converted into comparable data. On the other hand, in MLVA, the protocol is easier, and the analysis is shorter than that of PFGE and RAPD. In addition, because the data obtained are not a band pattern, it is easy to share and compare the data (Jadhav et al., 2012). Because of those properties, MLVA is very convenient for food companies and other industries with multiple factories. In the MLVA protocol established in this experiment, 13 candidate VNTR regions were obtained as a result of the whole genome database search of the *L. innocua* CLIP11262 strain using Unipro UGENE program. All VNTR regions with the sequence length suitable for MLVA using CE, were chosen, and their corresponding primer sets were designed. Characteristics of the VNTR regions used in the MLVA were shown in Table 16. To determine the VNTR regions, eleven *L. innocua* strains stored in our laboratory derived from different food factories and the environment were each subjected to MLVA. Among them, eight VNTR regions (TR1, TR2, TR3, TR5, TR6, TR10, TR12, and TR13) were successfully amplified. The numbers of repeat units were then calculated by using the model size designed first after determining the length of the PCR product using CE.

Table 16: CE profiles of 13 VNTR loci of *L. innocua*.

Isolate	TR1 Size (bp)	TR2 Size (bp)	TR3 Size (bp)	TR4 Size (bp)	TR5 Size (bp)	TR6 Size (bp)	TR7 Size (bp)	TR8 Size (bp)	TR9 Size (bp)	TR10 Size (bp)	TR11 Size (bp)	TR12 Size (bp)	TR13 Size (bp)
05	250	140	458	-	125	244	-	-	-	99	-	124	149
18	250	140	494	-	125	244	-	-	-	99	-	124	167
24	250	140	485	-	125	244	-	-	-	99	-	124	149
28	250	140	485	-	125	244	-	-	-	99	-	124	167
52	250	140	485	-	125	244	-	-	-	99	-	124	167
55	250	140	485	-	125	244	-	-	-	99	-	124	77
58	250	140	485	-	125	244	-	-	-	99	-	124	149
59	256	140	395	-	125	226	-	-	-	99	-	124	104
60	250	140	431	-	125	256	-	-	-	99	-	124	167
67	250	140	449	-	125	244	-	-	-	99	-	124	149
91	250	140	485	-	125	244	-	-	-	99	-	124	140

As a result, the numbers of repeats in the VNTR regions were 250-256, 140, 395-494, 125, 226-256, 99, 124, and 77-167 in TR1, TR2, TR3, TR5, TR6, TR10, TR12 and TR13, respectively. The TR1 locus was divided into 2 patterns, TR3 locus was divided into 6 patterns, TR6 locus was divided into 3 types, TR13 locus was divided into 5 types, and other loci (TR2, TR5, TR10, and TR12) were divided into 1 pattern. The VNTR information are shown in Table. The length and number of tandem repeats was determined by analyzing the CE confirmed by sequencing the differing amplicons of the all MLVA patterns. Sequencing was done by Sanger sequencing performed by 1st BASE (Malaysia) (Appendix 6). The resulting sequences confirmed that the primers targeted their respective tandem repeat in each MLVA pattern and that no off-targets were amplified. The developed method was then evaluated by typing 93 *L. innocua* strains isolated from a food processing plant using 4 potential VNTR loci (TR1, TR3, TR6, and TR13). Overall length of PCR amplicons and DNA sequences were measured and analyzed to determine the size range and repeat sequences of TR loci. The polymorphisms of all 4 TR loci were investigated. The 93 *L. monocytogenes* isolates produced 10 MLVA patterns. The TR 1 locus was divided into 2, TR3 locus was divided into 6 types, TR6 locus was divided into 3 types, and TR13 locus was divided into 4 types The VNTR information are shown in Table 17.

Table 17: Characterization of 4 selected VNTR loci of *L. innocua*.

Name	Repeat sequence	Repeat size (bp)	Size range (bp)	No. of alleles*	Allele range (copy no.)
TR1	ACAAAT	6	250 – 256	2	3 – 4
TR3	CGGTAGACC	9	395 – 494	6	4 – 15
TR6	CATCGG	6	226 – 256	3	7 – 10
TR13	TTTACTGGG	9	77 – 167	4	5 – 12

* With null allele

RAPD method described by Keeratipibul & Techaruwichi (2012) was also used to investigate the diversity type. Ninety-three strains of *L. innocua* were screened using 4 random primers (UFS, HLWL74, HLWL85, OMP-01), generated 6 RAPD profiles (Figure 7).

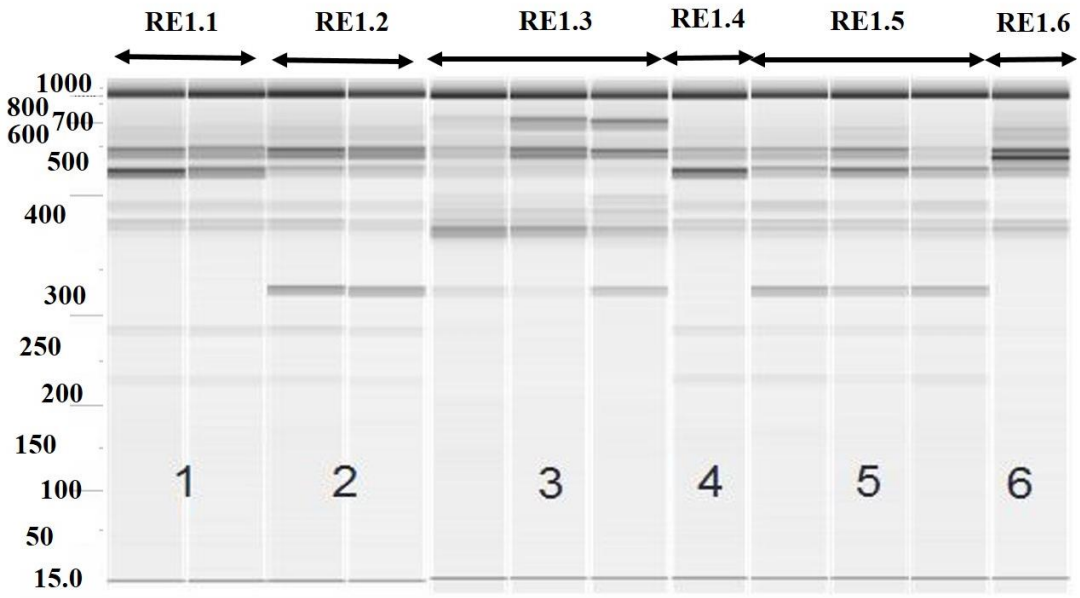


Figure 7: Six RAPD profiles of *L. innocua*

Comparison of the two methodologies was undertaken with Simpson's index of diversity. The higher the value of a diversity index defines the higher the discriminatory power of the subtyping method (Hunter et al, 1998). Ninety-three *L. innocua* strains were obtained from laboratory collection which covers the environmental monitoring isolates from various processes, including steam, chilling, cutting, freezing, and packing. These environmental swab samples were intentionally taken from the areas that are difficult to access for cleaning in order to monitoring the persistent of *L. innocua*. Many of the 93 strains isolated from the factory were classified as 3-14-10-10. MLVA analysis with the four selected loci discriminated the 93 *L. innocua* isolates into 10 different patterns with a Simpson's index of diversity of 0.45, while RAPD resolved them into 15 patterns with an index of 0.42. The largest groups of identical RAPD profiles consisted of 69 isolate, most of which belonged to R1. All 6 of the R6 strains in this work clustered together but were resolved into 4 different MLVA patterns within this cluster (Table 18).

Table 18: MLVA and RAPD types of 93 *L. innocua* isolates.

Isolate	MLVA pattern	MLVA type	RAPD pattern	RAPD type
1	3-14-10-10	M3	RE1.1	R1
2	3-14-10-10	M3	RE1.1	R1
3	3-14-10-10	M3	RE1.1	R1
4	3-14-10-10	M3	RE1.1	R1
5	3-11-10-10	M2	RE1.1	R1
6	3-11-10-10	M2	RE1.1	R1
7	3-14-10-10	M3	RE1.1	R1
8	3-14-10-10	M3	RE1.1	R1
9	3-14-10-10	M3	RE1.1	R1
10	3-14-10-10	M3	RE1.1	R1
11	3-14-10-10	M3	RE1.1	R1
12	3-14-10-10	M3	RE1.1	R1
13	3-14-10-12	M4	RE1.2	R2
14	3-14-10-10	M3	RE1.1	R1
15	3-14-10-10	M3	RE1.1	R1
16	3-14-10-12	M4	RE1.2	R2
17	3-14-10-10	M3	RE1.1	R1
18	3-15-10-12	M7	RE1.2	R2
19	3-14-10-10	M3	RE1.1	R1
20	3-14-10-10	M3	RE1.1	R1
21	3-14-10-10	M3	RE1.1	R1
22	3-14-10-10	M3	RE1.1	R1
23	3-14-10-10	M3	RE1.1	R1
24	3-14-10-10	M3	RE1.1	R1
25	3-14-10-10	M3	RE1.1	R1
26	3-14-10-10	M3	RE1.1	R1
27	3-14-10-10	M3	RE1.1	R1
28	3-14-10-12	M4	RE1.2	R2
29	3-14-10-10	M3	RE1.1	R1

Table 18: MLVA and RAPD types of 93 *L. innocua* isolates (continued).

Isolate	MLVA pattern	MLVA type	RAPD pattern	RAPD type
30	3-14-10-10	M3	RE1.1	R1
31	3-14-10-10	M3	RE1.1	R1
32	3-14-10-10	M3	RE1.1	R1
33	3-14-10-10	M3	RE1.1	R1
34	3-14-10-10	M3	RE1.1	R1
35	3-14-10-10	M3	RE1.1	R1
36	3-14-10-10	M3	RE1.1	R1
37	3-14-10-10	M3	RE1.1	R1
38	3-14-10-10	M3	RE1.1	R1
39	3-14-10-10	M3	RE1.1	R1
40	3-14-10-10	M3	RE1.1	R1
41	3-14-10-10	M3	RE1.1	R1
42	3-14-10-10	M3	RE1.1	R1
43	3-14-10-10	M3	RE1.1	R1
44	3-14-10-10	M3	RE1.1	R1
45	3-14-10-10	M3	RE1.1	R1
46	4-4-7-5	M10	RE1.3	R3
47	3-14-10-10	M3	RE1.1	R1
48	3-14-10-10	M3	RE1.1	R1
49	3-14-10-10	M3	RE1.1	R1
50	3-14-10-12	M4	RE1.2	R2
51	3-14-10-10	M3	RE1.1	R1
52	3-14-10-12	M4	RE1.2	R2
53	3-14-10-10	M3	RE1.1	R1
54	3-14-10-12	M4	RE1.2	R2
55	3-14-10-2	M5	RE1.1	R1
56	3-14-10-10	M3	RE1.1	R1
57	3-14-10-12	M4	RE1.2	R2
58	3-14-10-10	M3	RE1.1	R1
59	4-4-7-5	M10	RE1.3	R3
60	3-8-12-12	M8	RE1.2	R2
61	3-14-10-10	M3	RE1.1	R1
62	3-14-10-10	M3	RE1.1	R1

Table 18: MLVA and RAPD types of 93 *L. innocua* isolates (continued).

Isolate	MLVA pattern	MLVA type	RAPD pattern	RAPD type
63	3-14-10-10	M3	RE1.1	R1
64	3-14-10-10	M3	RE1.1	R1
65	3-14-10-12	M4	RE1.2	R2
66	3-14-10-10	M3	RE1.2	R2
67	3-10-10-10	M1	RE1.1	R1
68	3-14-10-12	M4	RE1.2	R2
69	3-14-10-10	M3	RE1.1	R1
70	3-14-10-10	M3	RE1.1	R1
71	3-14-10-10	M3	RE1.1	R1
72	3-14-10-10	M3	RE1.1	R1
73	3-14-10-10	M3	RE1.1	R1
74	3-14-10-10	M3	RE1.1	R1
75	3-14-10-10	M3	RE1.1	R1
76	3-15-10-12	M7	RE1.2	R2
77	4-4-10-5	M9	RE1.3	R3
78	3-14-10-10	M3	RE1.1	R1
79	3-14-10-10	M3	RE1.4	R4
80	3-14-10-10	M3	RE1.4	R4
81	3-14-10-12	M4	RE1.5	R5
82	3-14-10-12	M4	RE1.5	R5
83	3-14-10-12	M4	RE1.5	R5
84	3-14-10-12	M4	RE1.2	R2
85	3-14-10-10	M3	RE1.1	R1
86	3-14-10-10	M3	RE1.1	R1
87	3-14-10-10	M3	RE1.1	R1
88	3-14-10-12	M4	RE1.2	R2
89	3-14-10-10	M3	RE1.1	R1
90	3-14-10-10	M3	RE1.1	R1
91	3-14-10-9	M6	RE1.6	R6
92	3-14-10-10	M3	RE1.1	R1
93	3-14-10-10	M3	RE1.1	R1

In this study, we established an MLVA protocol for *L. innocua* by using CE that allows a simpler work process, shorter analysis duration, and cost reduction. In the evaluation of the established technique using strains isolated from a food factory, this MLVA protocol allowed us to obtain information on the actual status of contamination and bacterial is residence in the factory, demonstrating the high usefulness for hygiene control in factories. These data indicate that the CE-based MLV Aprotocol is very effective as a highly identifiable and simple typing method.

CHAPTER VI
DEVELOPMENT OF MOLECULAR SUBTYPING METHOD FOR *Listeria monocytogenes* AND *L. innocua* USING NEXT GENERATION SEQUENCING

6.1 Introduction

Listeria monocytogenes is the species significantly associated with listerosis which often affects severe underlying condition such as encephalitis, abortion or blood poisoning in human. The overall mortality rate associated with the disease is 30-40% in susceptible groups of people (Siegman-Igra et al., 2002). Due to high mortality rate of listeriosis, it is necessary to establish the strict legislations for controlling *L. monocytogenes* in foods. The regulatory standards of the Department of Livestock Development of Thailand and various countries, including the EU and Japan for RTE meat and poultry products require a zero tolerance (negative in 25 g sample) for *L. monocytogenes* (Commission regulation, 2005; DLD Thailand, 2010). Thailand is one of the largest cooked chicken meats exporting country in the world. The value of export for cooked chicken meat in Thailand was 2.14 billion U.S. dollars as of year 2016 (FAS/USDA, 2017). Despite no regulatory standards for other *Listeria* species, *Listeria* presence in food products is entirely unacceptable by the customers. Several research studies showed that *L. innocua* presence could indicate risk of *L. monocytogenes* contamination (Friedly et al., 2008; Keeratipibul & Techaruwichit, 2012; Zhang et al., 2007). Therefore, the discovery of routes of transmission and emerging of *Listeria* species is important task for development of better prevention and control in food industries. However, microbiological testing methods used in food factory such as BAM (Hitchins & Whiting, 2001), ISO11290 method (Anon, 1997), and AOAC/IDF method 993.09 (AOAC, 2000) identify only the species level which restrict ability to identify the real contamination source and the root cause of product contamination.

As a consequence, higher degree of discrimination requires a molecular typing method to provide additional strain level. MLVA is one of the most widely used

molecular strain-typing methods (Chenal-Francisque et al., 2013; Kimura et al., 2008; Lindstedt et al., 2004a; Sabat et al., 2003). It is performed by utilizing the number of repeat units in the variable number tandem repeat (VNTR) loci varying depending on the strain. The numbers of repeats in multiple VNTR used to compare and allow highly specific strain classification. These VNTR loci are located both in coding and non-coding regions of genome. In recent decades, MLVA has been coupled with a capillary electrophoresis (CE) system to determine the fragment size and coupled with Sanger sequencing to determine the fragment sequence. However, it is laborious, time-consuming and expensive to employ MLVA with CE and DNA sequencing with a large number of isolates usually collected from a food factory. Moreover, discriminatory power and multiplex PCR capability are also limited by the use of MLVA with CE analysis due to the determination of length of amplicons.

To achieve cost and time efficiencies and increase the discriminatory power, NGS was introduced to support MLVA for the first time in this study. NGS is a high-throughput sequencing technique producing enormous DNA information to assists in several fields of study such as genetic variations, disease mechanism, and antibiotic resistance in microorganisms (Barba et al., 2014). NGS revolutionized sequence data generation in molecular subtyping method, since several NGS platforms have been employed and developed to produce numerous high quality bases at low costs in single sequencing run when compared with Sanger method (Boers et al., 2012; Singh & Prakash, 2008). This has created NGS as the selective method for large-scale complex genetic analyses. Recently, combination between multiplex PCR and multiplexed samples using barcodes with NGS library has been described as bacterial subtyping. For the reason that PCRs and samples combined libraries use different barcodes, sequences from the libraries can be differentiated during data analysis. Sequencing of entire PCR amplicons also allows the subtyping of additional SNPs, indels, and unique multiple base changes in the fragments (Bernardo et al., 2015).

The integration of MLVA with NGS approach has potential to generate MLVA sequence data which could be determined not only length polymorphism but also sequence polymorphism. Hence, this study aims to propose MLVA with NGS platform using the Ion Torrent Personal Genome™ Machine (PGM™) to subtype a collection of *L. monocytogenes* and *L. innocua*.

6.2 Material and Methods

6.2.1 Bacterial strain and DNA Extraction

A total of 48 *L. monocytogenes* and *L. innocua* strains were investigated. Six of *L. monocytogenes* and *L. innocua* strains were obtained from American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), and the Collection of Institute Pasteur (CIP). Forty-two of *L. monocytogenes* and *L. innocua* isolated from a food processing plant in Thailand and the environment in Japan were also used. All bacterial strains were individually grown in Trypticase Soy Broth (TSB) (Becton Dickinson, U.S.A.) overnight at 30 °C. Bacterial cells were harvested from 1 mL TSB medium by centrifugation at 8,000xg for 3 min. The supernatant was removed. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel, Germany) according to the manufacturer's protocol.

6.2.2 MLVA target gene amplification

Previously validated PCR primer set targeting the variable number of tandem repeat loci of *L. monocytogenes* based on a literature reviews (Chenal-Francisque et al., 2013) were blasted against protein database using BLASTN to identify the regions of genomic DNA that encodes genes. All usable validated PCR primers were re-designed to develop the MLVA using NGS approach to generate the sequence data set of *L. monocytogenes*. For *L. innocua*, we developed a pipeline using perl to filter and select these loci from TRF output. Whole genome sequence of *L. innocua* (NC_003212.1) along with its annotation information were downloaded from GenBank. Repeats identified by TRF were mapped on to coding sequences. Protein sequences of these coding genes were then extracted and used as query to blast against local protein database of *Listeria*. Protein sequences that found orthologues in other *Listeria* species with different number of internal repeats were subjected to primer design. The primer sequences are given in Table 19. The modified two-step PCR strategy described by Boers et al., 2012 for MLVA were used to amplify the barcode incorporate PCR amplicons using the selected primer sets (Appendix 8).

Table 19: Information of the selected VNTR loci.

Locus		Primer sequence (5'→ 3')	Function
JLR1	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGTTCCTCCATTGGTAGAAGGATT	Lmo1799, peptidoglycan binding protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTGAAAGCTGGAGATGTTATTCA	
JLR2	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTTCCAGAGAAAGACAAAACAG	Lmo0627, peptidoglycan binding protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGRCTAATCCACCAGCAAATAGC	
Lis-TR1317	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGATTTGCAAAAAGCTGCACC	Lmo1290, hypothetical bound protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTGGMACTTCTGGTTTA	
Lis-TR881	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTAAATAAAGCTGGTACGTAC	Lmo0842, peptidoglycan binding protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTATGTTGCTTGTTATCAACTAC	

Table 19: Information of the selected VNTR loci (continued).

Locus		Primer sequence (5'→ 3')	Function
JLR4	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAAA TTCCAGTCCGCCAG	Lmo0652, hypothetical protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAR CAACAGAAGCTGATCCA	
LM-TR4	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAAG ACGAAGAACCAGTAGGTAAA	FtsH, hypothetical protein, ATP-dependent metalloprotease
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGC CTCTTTGATTTTATTCGTCT	
LMV1	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGTTAC CACCCCATGAATAAG	Iap, probable endopeptidase p60
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAC AAACAGCACCTAAARCAC	
LMV6	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATATG AACTYGATACGACSCCAGT	Lmo0159, peptidoglycan binding protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGYTCGC TGTTTTCTGWTTTCTKAGG	

Table 19: Information of the selected VNTR loci (continued).

Locus		Primer sequence (5'→ 3')	Function
LMV9	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCACA GAGGCACTAAACGTAACCG	Lmo0160, peptidoglycan binding protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTAA KCCRCTCGCTGAAAT	
TR01	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAAC AACAAAAGATGAAACGAAA	Lin0132, hypothetical protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTC ATTTGCGTTACCCTTAGTA	
TR03	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGTTA ACAACAACGTATTCTGGT	Lin1101, cell wall surface anchor protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCAGT TGAATTTACTTGATTTGACG	
TR05	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTTCTC GATGTTTGTGATTTTGTC	citB, aconitate hydratase 1
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAG AGACCAGTCGTTTCAAGAAT	

Table 19: Information of the selected VNTR loci (continued).

Locus		Primer sequence (5'→ 3')	Function
TR06	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAGC CTGAGGAAAGCATTGTT	peptidoglycan-binding protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGTTA TCCACCTTCAAGTAACTGC	
TR10	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTGTTG TTCGAGAATTTTTGTTTC	FtsL, cell division protein
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATCA AATCTCGAGCCAAATAGAGT	
TR13	F:	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGTC ATCCATTAGATCGTCTTG	Internalin
	R:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAAC AAAATGCCTGCTAATGATTT	

The PCR amplification was performed in a final volume of 50 µL, containing 10 mMTris- HCl (pH 8.3), 50 mMKCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara Taq DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters were used for amplification: 95 ℃ for 5 min, 25 cycles of 95 ℃ for 30 s, 60 ℃ for 40 s, 72 ℃ for 1 min, and 72 ℃ for 4 min. After the PCRs. The reactions were purified using AMPure XP beads (Agencourt, Woerden, Netherlands) to eliminate excess dNTPs, primers, primer dimers and salts.

6.2.3 NGS using Ion PGM™ sequencing

All barcode incorporate amplicons were pooled in equimolar amounts of 50 pM to ensure equal representation of each fused amplicon in the sequencing step. The pooled amplicons were then subjected to emulsion PCR on Ion OneTouch™ 2 instrument (Thermo Fisher) with the Ion PGM™ Hi-Q Chef Kit (Thermo Fisher), and followed by template positive Ion Sphere Particles (ISPs) enrichment on the Ion OneTouch™ ES instrument (Thermo Fisher) according to manufacturer's protocol. NGS was accomplished on PGM™ platform (Thermo Fisher) using Ion 316v2™ Chip, with Ion PGM™ Hi-Q Sequencing kit and using 850-flow runs, on Ion PGM platform.

6.2.4 PGM data analysis

NGS raw data were generated, collected, and processed on the Torrent Suite software (v5.0.2). The reads were sorted by Ion Xpress barcodes and MLVA primer sequences. An in-house pipeline were employed to detect MLVA loci in FASTQ data. Raw sequence data in FASTQ format were demultiplexed into each sample using barcode sequences information. Low quality sequences were removed and trimmed using Trimmomatics (Bolger et al., 2014). BWA and SAMtools were used to map reads to reference sequences (Li, 2011; Li & Durbin, 2009). Assembled sequences were inspected using Tablet (Milne et al., 2009) and consensus sequences were extracted using tabix and bcftools in SAMtools package (Li, 2011). Consensus sequence of each amplicon from different samples was aligned using MUSCLE (Edgar, 2004).

6.3 Results and discussions

6.3.1 NGS data analysis

Due to a large number of samples and need of fast and accurate results, ability of NGS platform enabling multiplex analysis is a major advantage of the method in molecular subtyping in industrial scale. The PGM is one of multiplex NGS platforms providing multi-million read level output. In this study, we performed multiplex PCR amplification to discriminate *Listeria* strains by targeting VNTR loci in specific coding regions from several genes. Generally, bacterial pathogens use high frequency of insertions and deletions of tandem repeat in genes to rapidly adapt to specific environment by promoting variations of the protein sequence and possibly lead to changed encoded proteins (Zhou, Aertsen, & Michiels, 2014). So VNTR loci in specific coding regions could provide high level of discrimination among the bacteria isolated from a factory

In this study, the MLVA generated on average 10,473,774 bases/strain, with a mean of 9,301,495 (88.81%) of bases/strain. The workflow produced on average 45,468 reads/strain (15.32% reads on target). The mean read length was 232 bp. A mean depth of coverage of amplicon was 100x, with a mean target base coverage at 100x of 100% (Table 20). Generally, the minimum depth of coverage required for DNA resequencing is about 20-30x (Trujillano et al., 2015). This value exhibited the great reliability and sensitivity of the NGS method since the coverage represents the amount of a sequenced DNA fragment map to referenced target. Moreover, one of technical challenges in NGS is to have sufficient diversity persisting within library even after NGS target enrichment and not to have the same fragment detected multiple times. However, some amount of duplication is unavoidable when PCR-amplified DNA is randomly sequenced. Therefore, a good protocol must maintain low frequency of duplicated DNA fragments resulting in a sufficient amount of usable sequencing data. Our study showed that performing 2-step PCR strategies could overcome the challenge of generating amplicons libraries suitable for NGS (Boers, Van der Reijden, & Jansen, 2012). The sequencing libraries could be simply prepared by a multiplex amplification and combined with NGS to provide rapid identification of VNTR.

Table 20: Summary of NGS run.

Strains	Base/strain	≥ Q20	Reads/strains	Mapped reads	% reads on target	Mean read length
LM01	7,104,657	6,269,081	29,817	7,859.00	26.36	238
LM02	8,453,501	7,484,663	34,229	8,776.00	25.64	247
LM03	11,174,246	9,765,754	48,333	10,248.00	21.2	231
LM04	6,658,582	5,748,238	29,614	4,383.00	14.8	225
LM05	10,582,270	9,310,307	45,471	2,985.00	6.56	233
LM06	10,616,065	9,247,898	46,432	5,614.00	12.09	229
LM07	11,463,091	10,000,235	50,763	8,223.00	16.2	226
LM08	9,139,299	7,861,123	41,923	8,461.00	20.18	218
LM09	9,899,306	8,713,702	41,362	8,018.00	19.38	239
LM10	10,705,338	9,392,157	45,346	7,116.00	15.69	236
LM11	11,859,377	10,344,118	52,256	9,769.00	18.69	227
LM12	13,806,363	12,143,759	58,540	9,266.00	15.83	236
LM13	9,656,901	8,484,290	40,943	7,414.00	18.11	236
LM14	9,323,033	8,204,115	39,726	6,173.00	15.54	235
LM15	11,849,355	10,336,020	51,044	8,639.00	16.92	232
LM16	13,632,409	11,965,975	59,008	10,614.00	17.99	231

Table 20: Summary of NGS run (continued).

Strains	Base/strain	≥ Q20	Reads/strains	Mapped reads	% reads on target	Mean read length
LM17	15,234,438	13,346,471	66,332	15,302.00	23.07	230
LM18	9,053,382	7,794,087	42,821	5,879.00	13.73	211
LM19	20,218,382	18,078,951	89,403	18,766.00	20.99	226
LM20	15,824,586	13,871,121	70,515	12,269.00	17.4	224
LM21	14,219,679	12,793,919	67,533	14,264.00	21.12	211
LM22	13,186,809	11,540,934	56,993	10,603.00	18.6	231
LM23	9,350,449	8,024,048	43,039	8,987.00	20.88	217
LM24	16,274,174	14,616,087	82,061	3,816.00	4.65	198
LI01	7,810,274	7,018,456	30,270	5,161.00	17.05	258
LI02	8,318,459	7,434,552	36,273	1,037.00	2.86	229
LI03	9,158,437	8,344,166	35,729	4,390.00	12.29	256
LI04	6,524,652	5,900,304	25,459	3,231.00	12.69	256
LI05	13,710,291	12,403,012	52,980	8,661.00	16.35	259
LI06	7,511,882	6,592,405	31,644	3,219.00	10.17	237
LI07	5,778,551	5,072,776	26,427	4,443.00	16.81	219
LI08	6,475,840	5,729,140	31,572	2,717.00	8.61	205
LI09	9,457,858	8,644,853	39,995	6,265.00	15.66	236

Table 20: Summary of NGS run (continued).

Strains	Base/strain	≥ Q20	Reads/strains	Mapped reads	% reads on target	Mean read length
LI10	9,747,585	8,581,447	41,965	4,181.00	9.96	232
LI11	10,166,253	9,243,613	42,258	8,436.00	19.96	241
LI12	9,345,272	8,563,967	38,964	7,438.00	19.09	240
LI13	7,911,312	7,150,617	31,583	4,149.00	13.14	250
LI14	9,415,581	8,680,922	38,698	6,488.00	16.77	243
LI15	18,727,741	17,316,516	81,853	5,068.00	6.19	229
LI16	4,686,853	4,162,410	20,294	4,212.00	20.75	231
LI17	18,922,493	16,719,364	92,663	11,984.00	12.93	204
LI18	13,720,463	12,354,363	55,786	4,194.00	7.52	246
LI19	8,619,098	7,760,568	33,704	3,747.00	11.12	256
LI20	8,996,158	8,166,370	35,354	7,293.00	20.63	254
LI21	8,636,064	7,789,709	34,520	5,485.00	15.89	250
LI22	6,972,072	6,160,481	30,655	2,496.00	8.14	227
LI23	5,657,945	4,975,261	25,776	2,508.00	9.73	220
LI24	7,184,303	6,369,402	34,523	4,285.00	12.41	208
Mean	10,473,774	9,301,494	45,468	6,969	15	232

6.3.2 Identification and discrimination power of MLVA using NGS approach of *L. monocytogenes* and *L. innocua*

Tandem repeat has been recognized to be a suitable target for assessing genetic polymorphisms within bacterial species. In this study, 9 developed VNTR loci of *L. monocytogenes* (24 strains) and 6 developed VNTR loci of *L. innocua* (24 strains) isolated from a food processing plant and from the environment were amplified. The number of repeat units in each of the VNTR in coding regions of all *Listeria* strains were detected by both CE and NGS approaches. To determine the ability of NGS to detect 360 VNTR amplicons in a single read run, % concordance between CE-based and NGS-based detection were analyzed. The results exhibited 100% concordance indicating that both CE and NGS could detect all 360 amplicons. However, NGS could detect isoalleles among the analyzed amplicons. Isoalleles are alleles which are the same in length but different sequence (Gettings et al., 2016). This resulted in 89 different alleles detected by NGS, while 38 different alleles were detected by CE (Table 21). Among 9 VNTR loci of *L. monocytogenes*, locus JLR1 showed the highest discriminatory power when used in MLVA with NGS by differentiating 24 strains of *L. monocytogenes* into 13 different patterns (54%). While locus JLR1 used in MLVA with CE, 4 different patterns (17%) were found. For 24 strains of *L. innocua*, 6 VNTR loci were developed in this study. Locus TR3 was found to show the highest discriminatory power when detected by NGS with 24 different pattarns (100% discrimination). However the discriminatory power of locus TR3 was decreased to 8% when detected by CE. These results revealed that a greater number of the detecting allelic differences were incremented by NGS compared with CE method. NGS method was significantly more likely to provide polymorphic data type in the presence of allelic detection..

Table 21: Comparison of the number of different alleles detected and % of allele identified by CE and NGS approaches.

Locus	Species	Number of different alleles and % of allelic identification[a]			
		MLVA using CE		MLVA using NGS	
JLR1	*L. monocytogenes*	4	17%	13	54%
JLR2		5	21%	11	46%
JLR4		3	13%	5	21%
LM-TR4		1	4%	9	38%
LMV1		4	17%	9	38%
LmLMV6		3	13%	10	42%
LMV9		3	13%	6	25%
Lis-TR1317		2	8%	6	25%
Lis-TR881		2	8%	5	21%
TR1	*L. innocua*	1	4%	4	17%
TR3		2	8%	24	100%
TR5		1	4%	2	8%
TR6		4	17%	11	46%
TR10		2	8%	4	17%
TR13		1	4%	8	33%

[a] The percentage indicates % of allelic idenfication from *L. monocytogenes* 24 strains and *L. innocua* 24 strains, calculated from the number of different alleles divided with 24.

In order to compare the discriminatory power of CE and NGS detection methods, diversity indices were calculated. MLVA with NGS showed higher discriminatory power than MLVA with CE, yielding 48 patterns (DI = 1.0) and 15 patterns (DI = 0.79), respectively (Table 22).

Table 22: MLVA patterns of *L monocytogenes* and *L. innocua* isolates.

Strain	Unit size (bp) of MLVA type (LR1-JLR2-JLR4-LMTR1-LMV1-LMV6-LMV9-LisTR1317-LisTR881)	CE PATTERN	DNA sequence cluster of MLVA type	NGS PATTERN
LM01	231-255-243-168-295-276-231-168-225[a]	CLM01	H-I-A-G-C-F-E-D-D	NLM01
LM02	237-243-243-168-295-276-231-168-225	CLM02	G-H-A-G-C-A-E-D-E	NLM02
LM03	237-231-243-168-295-276-231-168-225	CLM03	J-B-A-F-D-D-D-D-D	NLM03
LM04	237-231-243-168-295-276-231-168-225	CLM03	K-B-A-B-D-G-D-D-E	NLM04
LM05	237-243-243-168-295-276-231-168-225	CLM02	J-C-A-G-D-F-E-D-D	NLM05
LM06	237-267-243-168-277-276-231-168-225	CLM04	I-H-A-E-B-D-D-D-D	NLM06
LM07	237-231-243-168-295-276-231-168-225	CLM03	B-B-A-G-C-F-E-A-E	NLM07
LM08	237-231-243-168-295-276-231-168-225	CLM03	D-B-C-H-F-H-C-D-C	NLM08
LM09	237-255-243-168-295-276-231-168-225	CLM05	A-D-A-G-C-F-E-D-D	NLM09
LM10	237-255-243-168-295-276-231-168-225	CLM05	B-J-A-G-C-F-E-D-D	NLM10
LM11	237-255-243-168-295-276-231-168-225	CLM05	B-E-A-G-C-F-E-D-D	NLM11
LM12	237-243-243-168-295-276-231-168-225	CLM02	B-F-A-G-C-A-E-B-E	NLM12

Table 22: MLVA patterns of *L monocytogenes* and *L. innocua* isolates (continued).

Strain	Unit size (bp) of MLVA type (LR1-JLR2-JLR4-LMTR1-LMV1-LMV6-LMV9-LisTR1317-LisTR881)	CE PATTERN	DNA sequence cluster of MLVA type	NGS PATTERN
LM13	237-243-243-168-277-276-231-168-225	CLM06	B-C-A-G-A-F-E-D-D	NLM13
LM14	237-231-243-168-295-276-231-168-225	CLM03	L-B-A-D-D-G-D-D-E	NLM14
LM15	237-231-243-168-295-276-231-168-225	CLM03	L-B-A-B-D-G-D-D-D	NLM15
LM16	237-231-243-168-295-276-231-168-225	CLM03	I-B-A-C-D-G-D-B-E	NLM16
LM17	237-231-243-168-295-276-231-168-225	CLM03	K-B-B-B-D-D-D-D-D	NLM17
LM18	237-231-243-168-283-276-231-168-225	CLM07	C-B-C-H-E-B-B-D-C	NLM18
LM19	201-231-171-168-295-246-204-ND-270	CLM08	F-A-D-I-H-I-A-ND-A	NLM19
LM20	237-231-243-168-295-276-231-168-270	CLM09	L-B-A-A-D-C-D-D-B	NLM20
LM21	ND-ND-ND-168-295-ND-ND-ND-270	CLM10	ND-ND-ND-A-I-ND-ND-ND-B	NLM21
LM22	237-231-243-168-295-276-231-168-225	CLM03	B-B-A-G-C-A-E-C-E	NLM22
LM23	237-255-243-168-295-276-231-168-225	CLM05	E-G-C-H-F-E-B-D-C	NLM23
LM24	201-231-171-168-301-246-204-168-270	CLM11	F-A-D-I-G-I-A-E-A	NLM24

Table 22: MLVA patterns of *L monocytogenes* and *L. innocua* isolates (continued).

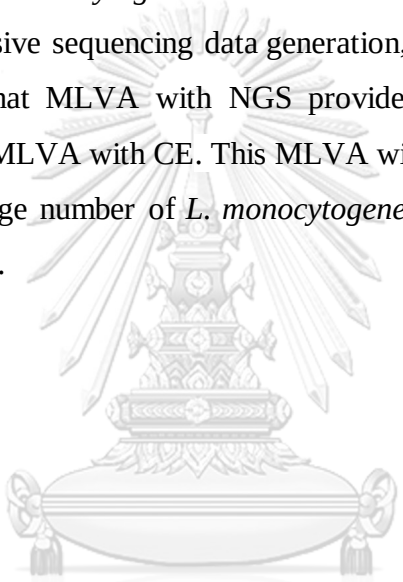
Strain	Unit size (bp) of MLVA type (TR1-TR10-TR13-TR3-TR5-TR6)	CE PATTERN	DNA sequence cluster of MLVA type	NGS PATTERN
LI01	171-255-150-315-216-237[b]	CLI01	C-A-B-I-C-C	NLI01
LI02	171-255-150-315-216-237	CLI01	C-B-B-E-C-D	NLI02
LI03	171-255-150-315-216-237	CLI01	C-C-B-I-C-C	NLI03
LI04	171-255-150-315-216-237	CLI01	C-D-B-J-C-C	NLI04
LI05	171-255-150-315-216-237	CLI01	C-E-B-I-C-C	NLI05
LI06	171-255-150-315-216-237	CLI01	C-F-B-I-C-D	NLI06
LI07	171-255-150-315-216-237	CLI01	A-G-A-C-B-A	NLI07
LI08	171-255-150-303-216-237	CLI02	C-H-B-H-C-D	NLI08
LI09	171-255-150-315-216-237	CLI01	B-I-B-D-C-A	NLI09
LI10	171-255-150-315-216-237	CLI01	C-J-B-I-C-F	NLI10
LI11	171-255-150-315-216-237	CLI01	C-K-B-G-C-F	NLI11
LI12	171-255-150-315-216-237	CLI01	B-L-B-G-C-A	NLI12

Table 22: MLVA patterns of *L monocytogenes* and *L. innocua* isolates (continued).

Strain	Unit size (bp) of MLVA type (TR1-TR10-TR13-TR3-TR5-TR6)	CE PATTERN	DNA sequence cluster of MLVA type	NGS PATTERN
LI13	171-255-150-315-216-237	CLI01	C-M-B-I-C-C	NLI13
LI14	171-255-150-315-216-237	CLI01	C-N-B-B-C-B	NLI14
LI15	171-ND-150-ND-ND-237	CLI03	D-ND-B-ND-ND-H	NLI15
LI16	171-255-150-315-216-237	CLI01	A-O-A-F-A-A	NLI16
LI17	171-255-150-297-216-237	CLI04	A-P-A-A-A-A	NLI17
LI18	171-255-150-315-216-237	CLI01	C-Q-B-I-C-G	NLI18
LI19	171-255-150-315-216-237	CLI01	C-R-B-I-C-E	NLI19
LI20	171-255-150-315-216-237	CLI01	C-S-B-I-C-E	NLI20
LI21	171-255-150-315-216-237	CLI01	C-T-B-E-C-C	NLI21
LI22	171-255-150-315-216-237	CLI01	C-U-B-E-C-D	NLI22
LI23	171-255-150-303-216-237	CLI02	C-V-B-H-C-D	NLI23
LI24	171-255-150-315-216-237	CLI01	A-W-A-F-A-A	NLI24

These results demonstrated a real advantage of the VNTR detection using not only size polymorphism but also sequence polymorphism by MLVA with NGS. Moreover, by obtaining DNA sequence data, NGS also has considerable cost-effective advantage over CE followed by Sanger DNA sequencing. In our laboratory setting, the cost of MLVA with NGS was about 2.5 – 8.6 times lower than that of MLVA with CE and Sanger DNA sequencing. This allowed significant reduction in analytical cost when a large number of samples were examined.

In conclusion, we proposed the integration of MLVA method with NGS platform to subtype *L. monocytogenes* and *L. innocua* which allow an analysis of multiplex mixture, massive sequencing data generation, in spite of cost reduction. The results demonstrated that MLVA with NGS provided greatly high discriminatory power, compared with MLVA with CE. This MLVA with NGS could be an alternative tool for subtyping a large number of *L. monocytogenes* and *L. innocua* isolates with cost-effective operation.



CHAPTER VII
TRACKING SOURCE, RISK ASSESSMENT, AND RISK MANAGEMENT OF *L. monocytogenes* AND *L. innocua* IN FROZEN COOKED CHICKEN PLANT

7.1 Introduction

Thailand is one of the world's biggest producers of food products. Thus, many people worldwide has dubbed Thailand as the "kitchen of the world". In 2016, Thailand exported approximately 30 billion U.S. dollar of food products to major countries such as Japan, the US, China, UK and ASEAN countries with the top 5 exports being rice, sugar, chicken, tuna, and shrimp. For chicken, Thailand is one of the largest cooked chicken meat exporting country in the world. The value of export for cooked chicken meat in Thailand was 2.14 billion U.S. dollars as of 2016. Unfortunately, these products can be contaminated with *Listeria* species, especially *L. monocytogenes*, the species significantly associated with listerosis. This microorganism often affects severe underlying condition such as encephalitis, abortion or blood poisoning in human. Therefore, it directly affects the frozen ready-to-eat food industries.

The regulatory standards of the Department of Livestock Development of Thailand, and various countries including Japan and the EU for ready-to-eat meat and poultry products require a zero tolerance (negative in 25 g sample) for *L. monocytogenes*. Likewise, FDA standards have announced zero tolerance for *L. monocytogenes* contamination in processed foods in the U.S.A (Swaminathan et al., 2001). Strict contamination management for *L. monocytogenes* is therefore necessary at food processing plants (Fox et al., 2011; Vogel et al., 2001). Besides, *Listeria* spp. occurrence, and not only *L. monocytogenes*, in these products is entirely unacceptable by both exporters and importers (Interview data from QA manager of the cooked chicken meat factory in Thailand).

To efficiency control *Listeria*, all potential route of entry and cross-contamination should be taken into consideration. Many authors have demonstrated that *L. monocytogenes*, and other *Listeria* spp. are widely distributed in food processing environments; for example: in chilling and cutting rooms (Van den Elzen & Snijders,

1993), workers' hands (Kerr et al., 1995), conveyor belt rollers (Tompkin, 2002), and processing equipment (Lawrence & Gilmour, 1995). Although, it is strongly suggested that the processing environment represents a significant source of *Listeria* in finished products, they can be recontaminated while being handled, packaged or distributed (Lekroengsin et al., 2007; Tompkin et al., 1999). These should be cleansed and disinfected daily for controlling of *Listeria* in processing plant. However, *Listeria* can persist industrial sanitizers, to colonize the whole environment, forming biofilms that make it even more resistant. Hence, it may remain in the processing environment for months and cause cross-contamination (Carpentier & Cerf, 2011). Effective cleaning and disinfection program for operating procedures with schedules listing the frequency of cleaning and disinfection procedures are needed. Moreover, the effectiveness of the cleaning and disinfection program should be verified through microbiological analysis of both product and environmental samples collected from all areas of processing plant.

The term "persistence" is often used to describe the long-term survival of a pathogen in a particular habitat, e.g. in a food processing plant. The concept often implies that persistent strains harbor a phenotype or an adaptation which enable them to survive in a given environment such as biofilm formation or stress and disinfectant resistance has been detected (Larsen et al., 2014). The observed persistence may be due to resident strains that have become established in niches in the plant such as through raw materials or the surrounding environment. *Listeria* isolates recovered from a food production facility after cleaning and disinfection are more likely to represent resident strains, compared to when sampling is performed during production.

Recent studies have been greatly facilitated by the use of molecular-typing methods with high discriminatory power, including MLVA (Chenal-Francisque et al., 2013; Kimura et al., 2008; Lindstedt et al., 2004a; Sabat et al., 2003). This method has been developed as subtyping methods by employing a DNA sequence analysis. This technique can be used to type bacterial strains by utilizing the number of repeat units in the variable number tandem repeat (VNTR) region varies depending on the strain. The numbers of repeats in multiple VNTR regions can be used to compare and allow highly specific strain classification. Therefore, the purpose of this study was to investigate the prevalence, the transmission routes, and the efficacy of cleansing and disinfection of

Listeria contamination in a representative Thai cooked frozen chicken meat processing plant using MLVA method.

7.2 Material and methods

7.2.1 Sampling

This study was performed in a cooked chicken meat processing plant in Thailand. The 200 processing environmental surfaces and the finished product, were sampled before and after the big cleaning operation (Table 23). All samples were swabbed with sterile cotton swabs moistened with 0.85% NaCl (w/v). After sampling, the swabs were soaked in 10 ml of Dey/Engley (D/E) Neutralizing Broth and kept in a cooler during transport to the laboratory.

7.2.2 *Listeria* identification and DNA extraction

Upon arrival at the laboratory, swab samples were each homogenized for 1 min in 225 ml Half-Fraser (HF) broth (bioMérieux) in a stomacher, and incubated at 30 ^{O}C for 20 to 26 h as a pre-enrichment step. One ml of the suspension was transferred to tubes containing 10 ml of Fraser Broth and incubated at 30 OC for 20 to 26 h. One loop of all positive samples were streaked on *Listeria* selective agar (Oxford; OXOID) and Ottaviani Agosti agar (OAA) plates (bioMérieux), incubated at 37 ^{O}C for 48 $\pm$ 2 h and then observed for the presence of typical *Listeria* colonies according to ISO 11290-1. From each plate, three colonies with morphological characteristics of *Listeria* were picked off, streaked onto TSAYE (Tryptone Soy Agar; OXOID) with 0.6% (w/ v) Yeast Extract (Merck) plates and incubated at 37 ^{O}C for 18 to 24 h. Colonies presumptive for *Listeria* spp. on TSAYE were selected and subjected to Gram staining, catalase test and motility at 25 ^{O}C for 48 h. The API *Listeria* System incubated at 35 ^{O}C for 18 to 24 h was used to confirm the identified species (bioMérieux S.A.). All *L. monocytogenes* and *L. innocua* strains were then individually grown in Trypticase Soy Broth (TSB) (Becton Dickinson, U.S.A.) overnight at 30 °C. Bacterial cells were harvested from 1 mL TSB medium by centrifugation at 8,000×*g* for 3 min and the supernatant was removed. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel, Germany) according to the manufacturer's protocol.

Table 23: Location of environmental surfaces sampling.

No	item	Zone	No	item	Zone
1	air sock	cooking	15	conveyor controller cabinet 4	cooking
2	superine Spiral oven	cooking	16	operating controller cabinet	cooking
3	net conveyor+roller sprocket no.2	cooking	17	male power plug (dicer)	cooking
4	conveyor belt no.1	cooking	18	female power plug (dicer)	cooking
5	net conveyor+roller sprocket no.1	cooking	19	wire	cooking
6	net conveyor+roller sprocket no.3	cooking	20	washingbasin (inside)1	cooking
7	net conveyor+roller sprocket no.4	cooking	21	washingbasin (outside)1	cooking
8	post equipment shelf (storage room)	cooking	22	floor drain nearby dicer	cooking
9	post equipment container (storage room)	cooking	23	floor nearby dicer	cooking
10	equipment cabinet PD (storage room)	cooking	24	bar stool	cooking
11	Table	cooking	25	hose 1	cooking
12	balance no.1	cooking	26	cornice (cooking area)	cooking
13	cooking and packing cart	cooking	27	wall	cooking
14	conveyor controller cabinet 1	cooking	28	net conveyor+roller sprocket No.24	packing

Table 23: Location of environmental surfaces sampling (continued).

No	item	Zone	No	item	Zone
29	conveyor belt No.24	packing	46	cart wheel QC Line (in use)	packing
30	net conveyor+roller sprocket No.18	packing	47	cart wheel QC	packing
31	Funnel cone	packing	48	fire extinguisher (inside)	packing
32	Declinding belt	packing	49	shaft set (declinding motor)	packing
33	sprocket declinding belt	packing	50	cornice (Freezer)	packing
34	declinding wheel	packing			
35	male power plug (declinding belt)	packing			
36	female power plug (declinding belt)	packing			
37	wire	packing			
38	floor	packing			
39	floor drain	packing			
40	washingbasin (garbage room)	packing			
41	washingbasin (outside)	packing			
42	bar stool	packing			
43	apron rack	packing			
44	shoe (freezer romm)	packing			
45	staff shoe	packing			

7.2.3 Molecular subtyping of *L. monocytogenes* and *L. innocua* using MLVA

PCR reactions were performed using 15 pairs of primers specific for 15 VNTR loci (Table 19). The PCR amplification was performed in a final volume of 50 µL, containing 10 mMTris-HCl (pH 8.3), 50 mMKCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 100 nM forward primer, 100 nM reverse primer, 25 ng template DNA, and 0.5 U Takara *Taq* DNA polymerase (Takara Bio, Japan). Amplification was performed using the GeneAmp PCR System 9700 thermalcycler (Life Technologies, U.S.A.). The following parameters were used for amplification: 95 ℃ for 5 min, 35 cycles of 95 °C for 30 s, 60 ℃ for 40 s, 72 ℃ for 1 min, and 72 ℃ for 4 min. PCR products were visualized and confirmed by electrophoresis in a 2% agarose gel. To analyze the variants further, the observed amplicons were subjected to capillary electrophoresis (CE; QIAxcel Advanced; Qiagen, Tokyo, Japan) for fragment analysis.

7.3 Result and discussion

7.3.1 MLVA profiling and prevalence of *Listeria* species and strains

Ready-to-eat products (RTE) are defined as a food intended by the producer or the manufacturer for direct human consumption without additional preparation to achieve food safety. However, *L. monocytogenes* appear to be the main cause of the high prevalence in different kinds of refrigerated RTE products (Lianou et al., 2007). The prevalence of pathogen in ready-to-eat meat products has been examined by several researchers. The researches have shown that the prevalence of *L. monocytogenes* in RTE meat products may vary from 0 to 72% (Faber et al., 2007). In this study, a total of 200 of the environmental swab and finished product samples were analyzed. From a total of 100 environmental swab samples, taken from before and after cleaning process, 28 samples were positive for *Listeria* spp. Besides, from a total of 100 finished product samples that were analyzed, 18 were found to be positive for *Listeria* spp. A total of 200 examined samples revealed the prevalence of *Listeria* in 14 and 1.8% of the environmental swab and finished product samples, respectively. The species compositions of these 46 contaminated samples were *L. monocytogenes* (46.9%), *L. innocua* (40%), *L. welshimeri* (10%), and *L. seeligeri* (3.1%). The frequency of

Listeria spp. isolated from environmental surfaces and finished products of chicken meat processing plant, is shown in Table 24.

Table 24: The frequency of *Listeria* spp. isolated from environmental surfaces and finished products of chicken meat processing plant.

Species	Environment		Finish product
	Before cleaning	After cleaning	
L. monocytogenes	49	20	6
L. innocua	29	27	8
L. welshimeri	7	3	6
L. seeligeri	0	5	0
Total	75	97	18

The VNTR has proven to be a suitable target for assessing genetic polymorphisms within bacterial species. Multiple-locus variant-repeat analysis (MLVA) or VNTR-based typing has been developed for typing purposes, together with addressing advantages and drawbacks associated with the use of tandem repeated DNA region as targets for bacterial typing and identification (Lindstedt, 2005). In this study, 15 developed VNTR loci of 213 *L. monocytogenes* and *L. innocua* isolated from the environmental swab and finished product samples were amplified. The number of repeat units in each of the VNTR in coding regions of *L. monocytogenes* (112 isolates) and *L. innocua* (101 isolates) were detected by CE-based MLVA approaches. As a result, the nine VNTR loci of *L. monocytogenes* used in this study, the 112 *L. monocytogenes* isolates were classified into 3 patterns (LM1, LM2, and LM3). While, the six VNTR loci of *L. innocua*, the 101 *L. monocytogenes* isolates were classified into 5 patterns (LI1, LI2, LI3, LI4, and LI5). *L. monocytogenes* strain LM1 was the dominant strain, represented by 69.6% of all the *L. monocytogenes* samples. Other *L. monocytogenes* strains were LM3 (28.6%) and LM2 (1.8%). For *L. innocua*, *L. innocua* strain LI1 was the dominant strain (72.3%) of *L. innocua* found in the processing environment and products. The other contaminated *L. innocua* strains were LI4 (9.9%), LI3 (8.9%), LI5 (5.9%), and LI2 (3%). When focusing on the processing environment, *L. monocytogenes* strain LM1 was also the dominant strain, represented

by 31.3% of all the *L. monocytogenes* samples. Other *L. monocytogenes* strains were LM3 (28.6%) and LM2 (1.8%). The only *L. monocytogenes* found in finished product was strain LM1. For *L. innocua*, *L. innocua* strain LI1 was the dominant strain (38.6%) of *L. innocua* found in the processing environment. The other contaminated *L. innocua* strains were LI3 (8.9%), LI4 (5.0%), LI2 (2.0%), and LI5 (1.0%). A greater number of *L. innocua* strains were found in finished products (LI1, LI1, LI4, and LI5) (Table 25).

Table 25: MLVA patterns of *L.monocytogenes* and *L. innocua* found in the factory.

Species	Examined samples			
	Before cleaning	After cleaning	finish product	Total
L. monocytogenes				
LM1	24	11	43	78
LM2	2		0	2
LM3	23	9	0	32
L. innocua				
LI1	24	15	34	73
LI2	0	2	1	3
LI3	4	5	0	9
LI4	0	5	5	10
LI5	1	0	5	6

The MLVA strains LM1 and LI1 were detected in all parts of the processing line, cooking, packing zone, and processing products, while strains LM1, LM3, LI1, and LI3 were recovered from the cleaned environment. Two additional MLVA strains (LI2 and LI4) were identified in the cooking and packing zone after cleaning of this line. A total of 5 MLVA types were found among the 43 and 45 isolates of *L. monocytogenes* and *L. innocua* from the finished products. Since identical *L. monocytogenes* and *L. innocua* MLVA strains were obtained from both the before and after cleaning processes as well as finished products, it appeared that *L. monocytogenes* and *L. innocua* were able to persist in the processing plant environment. This indicated that persistent *L. monocytogenes* and *L. innocua* which

cannot be eliminated may have been reintroduced at a low level either from the plant environment.

7.3.2 Correlation of *Listeria* MLVA patterns in cooked chicken meat and in the processing environment

As mentioned in 7.3.1, a total of 212 *Listeria* isolates, 6 from *L. monocytogenes* LM1, 8 from *L. innocua* LI1 and one each from *L. innocua* LI2, LI4, and LI5, were obtained from the finished products. The result demonstrated that *L. monocytogenes* LM1 was found on cooking and packing cart, floor drain nearby dicer, bar stool, hose, conveyor belt, roller sprocket, declining wheel, wire, floor of the cooking area, as well as the fire extinguisher, cart wheel, floor of the packing area and its floor drain. For *L. innocua*, examination of the environmental surfaces in the processing area that were contaminated with *L. innocua* LI4 on a nearby day that the finished products B1 were contaminated revealed that *L. innocua* LI4 was mainly found on the cart wheel from packing zone. In addition, the finished product I1 contaminated *L. innocua* LI5 revealed that this *L. innocua* strains was mainly found on shaft set of declining motor at packing zone. Besides, the finished product L1 contaminated L. innocua LI2 demonstrated that the product was contaminated with *L. innocua* LI5 found on declining wheel at the packing zone. For *L. innocua* LI1, the results demonstrated that this strain was found on the floor drain nearby dicer, bar stool, conveyor belt, roller sprocket, declining wheel, wire, floor of the cooking area, as well as the fire extinguisher, cart wheel, staff shoe, floor of the packing area and its floor drain. However, none of finished product was contaminated with *L. monocytogenes* LM2, *L. monocytogenes* LM3, and *L. innocua* LI3 (Table 26).

Table 26: MLVA pattern of *L. monocytogenes* and *L.innocua* isolated from environmental surfaces and finished products.

Item	Zone	MLVA pattern of *L. monocytogenes*			MLVA pattern of *L. innocua*		
		before cleaning	after cleaning	finish product	before cleaning	after cleaning	finish product
conveyor belt no.1	cooking	LM3	LM3				
cooking and packing cart	cooking		LM1				
Washing basin (outside)1	cooking	LM2,LM3					
floor drain nearby dicer	cooking	LM1			LI1		
floor nearby dicer	cooking				LI1	LI1	
bar stool	cooking	LM1			LI1		
hose 1	cooking	LM3					
conveyor belt No.24	packing	LM1			LI1	LI1	
roller sprocket no.18	packing		LM1			LI1	
declinding wheel	packing	LM1	LM1		LI1	LI2	
Wire	packing	LM1			LI1		
Floor	packing	LM1			LI1	LI1	
floor drain	packing	LM1	LM1,LM3		LI1		
washingbasin (garbage room)	packing	LM3	LM1				

Table 26: MLVA pattern of *L. monocytogenes* and *L.innocua* isolated from environmental surfaces and finished products (continued).

Item	Zone	MLVA pattern of *L. monocytogenes*			MLVA pattern of *L. innocua*		
		before cleaning	after cleaning	finish product	before cleaning	after cleaning	finish product
bar stool	packing	LM3					
staff shoe	packing					LI3	
cart wheel QC Line (in use)	packing					LI4	
cart wheel QC	packing	LM1					
fire extinguisher (inside)	packing	LM1			LI1		
shaft set (declinding motor)	packing				LI1		
cornice (Freezer)	packing				LI3,LI5		
product A1	product			LM1			
product B1	product			LM1			LI1
product B2	product						LI4
product C1	product						LI1
product D1	product			LM1			
product E1	product			LM1			
product F1	product			LM1			

Table 26: MLVA pattern of *L. monocytogenes* and *L.innocua* isolated from environmental surfaces and finished products (continued).

Item	Zone	MLVA pattern of *L. monocytogenes*			MLVA pattern of *L. innocua*		
		before cleaning	after cleaning	finish product	before cleaning	after cleaning	finish product
product G1	product			LM1			LI1
product G2	product						LI1
product H1	product						LI1
product I1	product						LI5
product J1	product						LI1
product K1	product						LI1
product L1	product						LI1,LI2
product M1	product						LI1

7.3.3 Risk assessment and practical suggestion

Processing environments in factory are not sterile. *Listeria* are very widespread in the processing environment and are likely to be reintroduced into food processing facilities (Tompkin, 2002). They have the capability to survive and grow in food processing environments. Their ability to grow at refrigeration temperatures gives them a competitive advantage over non-psychrotrophic microbes. Moreover, they can adapt to resist the stresses freezing and high salt and in food production facilities (Yousef, 1999). It is realistic to expect that Listeria can be introduced into the environment in which RTE foods are exposed for further processing and packaging. To prevent contamination caused by *Listeria* in food products, it is very important to detect the contamination routes of *Listeria* in the processing plant. Besides, by controlling the establishment and multiplication of *Listeria* in source of environmental contamination, it is possible to minimize, and in some cases prevent, the risk of product contamination with sanitation procedures. In this study, several corrective and preventive actions were implemented to eliminate the sources of *Listeria* contamination after investigating the *Listeria* in the finished product. These included revision of cleaning and sanitizing procedures (types of chemical and frequency of cleaning and sanitizing), conduct training of staffs against the cleaning procedures, and designing the new supportive equipment for cleaning. After implementing the new procedure, the prevalence of *Listeria* spp. in the finished product and processing environment were not detected.

In conclusion, control of *Listeria* in the processing plant environment is an unending task requiring careful thought, vigilance in sampling, diligence in tracking, and appropriate corrective action. In this study, we applied the developed MLVA for subtyping *L. monocytogenes* and *L. innocua* in the plant. The results demonstrated that this method provided crucial information in the risk assessment and risk management of *Listeria* contamination. Hence, the developed MLVA has the potential to monitor and control the *Listeria* contaminations in the food processing plant. In the future, it is possible that any product recalls caused by *Listeria* could be accurately tracked and identified by utilizing the MLVA data comparison of the *Listeria* strains which have been isolated from the products and those in the food processing environment.

CHAPTER VIII
GENERAL CONCLUSION

Listeria monocytogenes, the species significant widely associated with listerosis directly affects the frozen ready-to-eat food industries. Besides, several research studies showed that *L. innocua* is found to be the most widespread species can be inferred the presence of *L. monocytogenes*. Therefore, the discovery of routes of transmission and emerging of these particular foodborne pathogens is important task for development of better prevention and control in food industries. In our study, we introduced the cost-effective molecular approaches to detect, identify, and subtype *L. monocytogenes* and *L. innocua* for tracking source of bacterial contamination.

Firstly, a comprehensive BE-LisAll biomarker for *Listeria* detection using in silico scheme was developed. Specificity of biomarker BE-LisAll was then evaluated with 17 different *Listeria* species and 58 non-*Listeria* bacteria isolates. The result showed 100% specificity to *Listeria* species, and the biomarker could differentiate *Listeria* species from a variety of non-*Listeria* bacteria.

Secondly, HRMA method using *rar*A and *ldh*, which identified 9 species belonging to the genus *Listeria* was established. Among the 9 species, 7 were identified by HRMA using rarA gene, including 3 new species (*L. innocua*, *L. seeligeri*, *L. rocourtiae*, *L. ivanovii*, *L. grayi*, *L. marthii*, and *L. fleischmannii*). The remaining 2 species were identified by HRMA of *ldh* gene (*L. monocytogenes* and *L. welshimeri*). The method can be considered sufficiently applicable, as evaluation on actual isolates from the food factory identified *Listeria* spp. with a success rate of 92.6%.

Thirdly, a recently validated PCR primer set targeting the VNTR of *L. monocytogenes* based on a literature reviews and a novel PCR primer set targeting the variable number of tandem repeat loci designed using completed genome sequence of *L. innocua* CLIP 11262 for VNTR identification were simultaneously verified and developed. The MLVA protocol developed in this study for *L. innocua* allowed rapid and easy analysis through the use of capillary electrophoresis. The adopted and

discovered MLVA primers has fair amount of reproducibility, typeability, and discriminatory power.

Fourthly, the validated MLVA primers for developing and validating a cost-effective MLVA using Ion Torren Personal Genome Machine (PGM) to simultaneously generate the sequence data set were used. The results show that 10 adopted (JLR1, JLR2, JLR4, LisTR1317, LisTR881, LMTR4, Lm11, LMV1, LMV6, and LMV9) and 6 novel (TR1, TR3, TR5, TR6, TR10, and TR13) VTNR loci provided good differentiation between *L. monocytogenes* (n=24) and *L. innocua* (n=24), respectively. The NGS shows high efficiency for the bacterial subtyping since the approach enabled the ability to distinguish VNTR loci that are the same size but have a different sequence.

Finally, the developed MLVA were applied to investigate the sources and the routes of *Listeria* contamination for reducing the risk of *Listeria* spp. contamination in cooked frozen chicken meat process. The relationships of the *L. monocytogenes* and *L. innocua* in the final product and those in the environment were evaluated. The results demonstrated that the potential source of *L. monocytogenes* and *L. innocua* contamination were cooking and packing cart, floor drain nearby dicer, bar stool, hose, conveyor belt, roller sprocket, declining wheel, wire, floor of the cooking area, as well as the fire extinguisher, cart wheel, floor of the packing area and its floor drain. After implementing the new procedure, the prevalence of *Listeria* spp. in the finished product and processing environment were not detected.

These demonstrated that the developed approaches have the potential to provide new perspectives for large-scale application to investigate and track the sources of *L. monocytogenes* and *L. innocua* contamination in the processing environment and finished products. These approaches will become an attractive and feasible technique for molecular typing of both *Listeria* species in Thai frozen cooked chicken plants.

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APPENDICES

Appendix 1: List of homologous gene of *L. monocytogenes* LMOf2365_0740 found in *Listeria* used in this study.

GI number	DNA reference	Start	End	Strand	Symbol	% Homology	Locus tag	Putative function	Species
16799787	NC_003212	737,024	737,452	+	*L innocua*	86.71	lin0712	Hypothetical protein	*L. innocua Clip11262 uid61567*
347548120	NC_016011	691,205	691,633	+	*L ivanovii*	73.95	LIV_0644	Hypothetical protein	*L. ivanovii PAM55 uid73473*
386731477	NC_017728	749,301	749,729	+	*L mono07PF0776*	97.9	MUO_03865	Hypothetical protein	*L. monocytogenes 07PF0776 uid162185*
284994176	NC_013768	741,970	742,398	+	*L mono08-5578*	93.93	LM5923_0738	Hypothetical protein	*L. monocytogenes 08 5923 uid43727*
386043033	NC_017544	720,080	720,508	+	*L mono10403S*	94.17	LMRG_00393	Hypothetical protein	*L. monocytogenes 10403S uid54461*
405749075	NC_018584	764,519	764,947	+	*L monoATCC19117*	97.43	LMOATCC19117_0740	Hypothetical protein	*L. monocytogenes ATCC19117 uid175109*
226223335	NC_012488	746,227	746,655	+	*L monoCL ip81459*	97.9	Lm4b_00732	Hypothetical protein	*L. monocytogenes Clip80459 uid59317*
16802746	NC_003210	737,743	738,171	+	*L monoEGD-e*	93.93	lmo0704	Hypothetical protein	*L. monocytogenes EGDe uid61583*
550896877	NC_022568	720,089	720,517	+	*L monoEGD*	94.17	LMON_0709	FIG00774686: Hypothetical protein	*L. monocytogenes EGD uid223288*

Appendix 1: List of homologous gene of *L. monocytogenes* LMOf2365_0740 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	% Homology	Locus tag	Putative function	Species
386052970	NC_017547	735,084	735,512	+	*L monoFinL and1998*	94.17	LMLG_0678	Hypothetical protein	*L. monocytogenes Finland 1998 uid54443*
386049632	NC_017546	736,425	736,853	+	*L monoFSL R2-561*	93.93	LMKG_01178	Hypothetical protein	*L. monocytogenes FSL R2 561 uid54441*
217965198	NC_011660	1,907,079	1,907,507	-	*L monoHCC23*	95.33	LMHCC_1921	Hypothetical protein	*L. monocytogenes HCC23 uid59203*
386046365	NC_017545	756,813	757,241	+	*L monoJ0161*	94.4	LMOG_02036	Hypothetical protein	*L. monocytogenes J0161 uid54459*
525733189	NC_021830	522,442	522,870	+	*L monoJ1-220*	97.43	LM220_04852	Hypothetical protein	*L. monocytogenes J1220 uid179735*
525721594	NC_021829	396,945	397,373	-	*L monoJ1816*	97.43	LM1816_00470	Hypothetical protein	*L. monocytogenes J1816 uid179734*
406703491	NC_018642	746,088	746,516	+	*L monoL 312*	97.9	LMOL312_0716	Hypothetical protein	*L. monocytogenes L312 uid175768*
470206393	NC_020557	673,116	673,475	+	*L monoL a111*	78.78	BN418_0817	Myocilin	*L. monocytogenes La111 uid193768*

Appendix 1: List of homologous gene of *L. monocytogenes* LMOf2365_0740 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	% Homology	Locus tag	Putative function	Species
470209526	NC_020558	672,846	673,205	+	*L monoN53*	78.78	BN419_0822	Myocilin	*L. monocytogenes N531 uid193767*
404280269	NC_018587	749,102	749,530	+	*L mono12bSL CC2755*	97.66	LMOSLCC2755_0717	Hypothetical protein	*L. monocytogenes serotype 12b SLCC2755 uid52455*
386007433	NC_017529	748,476	748,904	+	*L mono4aL 99*	95.33	lmo4a_0720	Hypothetical protein	*L. monocytogenes serotype 4a L99 uid161953*
46906955	NC_002973	752,235	752,663	+	*L mono4bF2365*	97.9	LMOf2365_0740	Hypothetical protein	*L. monocytogenes serotype 4b F2365 uid57689*
424713600	NC_019556	752,224	752,652	+	*L mono4bL L 195*	97.9	BN389_07500	Hypothetical protein	*L. monocytogenes serotype 4b LL195 uid182103*
404286127	NC_018591	782,784	783,212	+	*L mono7SL CC2482*	97.66	LMOSLCC2482_0760	Hypothetical protein	*L. monocytogenes serotype 7 SLCC2482 uid174871*

Appendix 1: List of homologous gene of *L. monocytogenes* LMOf2365_0740 found in Listeria used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	% Homology	Locus tag	Putative function	Species
405751940	NC_018585	752,766	753,194	+	*L monoSL CC2378*	97.9	LMOSLCC2378_0736	Hypothetical protein	*L. monocytogenes SLCC2378 uid175105*
405757701	NC_018589	736,373	736,801	+	*L monoSL CC2479*	93.93	LMOSLCC2479_0714	Hypothetical protein	*L. monocytogenes SLCC2479 uid175108*
405754795	NC_018586	737,574	738,002	+	*L monoSL CC2540*	97.43	LMOSLCC2540_0716	Hypothetical protein	*L. monocytogenes SLCC2540 uid175106*
404409945	NC_018592	719,603	720,031	+	*L monoSL CC5850*	94.17	LMOSLCC5850_0706	Hypothetical protein	*L. monocytogenes SLCC5850 uid175110*
404412790	NC_018593	700,877	701,305	+	*L monoSL C7179*	94.4	LMOSLCC7179_0684	Hypothetical protein	*L. monocytogenes SLCC7179 uid175107*
284801034	NC_013766	775,266	775,694	+	*L monouid43671*	93.93	LM5578_0783	Hypothetical protein	*L. monocytogenes uid43671*
289433982	NC_013891	646,467	646,895	+	*L seeL igeri*	73.25	lse_0615	Hypothetical protein	*L. seeligeri serovar 12b SLCC3954 uid46215*
116872093	NC_008555	701,929	702,357	+	*L weL shimeri*	82.51	lwe0673	Hypothetical protein	*L. welshimeri serovar 6b SLCC5334 uid61605*

Appendix 2: List of homologous gene of *L. monocytogenes* LMof2365_0403 found in *Listeria* used in this study.

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
577757825	AOCG01000008	166,926	167,222	+	*L aquatica*	MAQA_08418	Hypothetical protein	*L. aquatica FSL S10-1188*
696204037	JNFA01000007	7,390	7,695	+	*L booriae*	EP57_03190	Hypothetical protein	*L. booriae FSL A5-0281*
577764864	AODE01000034	11,880	12,185	-	*L corneL L ensis*	PCORN_15886	Hypothetical protein	*L. cornellensis FSL F6-969*
577798585	AODM01000021	554	850	+	*L fL eischmanniiFSL*	MCOL2_06712	Hypothetical protein	*L. fleischmannii FSL S10-1203*
494736582	NZ_ALWW01000002	8,535	8,831	+	*L fL eischmanniiL U2006-1*	LFLEISCH_00870	Hypothetical protein	*L. fleischmannii LU2006-1*
494807766	NZ_AGUG01000005	16,920	17,216	-	*L fL eischmanniiCoL oradonensis*	KKC_01157	Hypothetical protein	*L. fleischmannii subsp. coloradonensis*
577766994	AODF01000030	46,870	47,166	-	*L fL oridensis*	MFLO_12841	Hypothetical protein	*L. floridensis FSL S10-1187*
577762464	AODD01000009	146,237	146,542	+	*L grandensis*	PGRAN_08253	Hypothetical protein	*L. grandensis FSL F6-971*
577766486	AODG01000011	64,143	64,427	+	*L grayi*	LMUR_08399	Hypothetical protein	*L. grayi FSL F6-1183*
16799486	NC_003212	420,923	421,222	+	*L innocua*	lin0409	Hypothetical protein	*L. innocua Clip11262*
347547809	NC_016011	353,202	353,501	+	*L ivanovii*	LIV_0318	Hypothetical protein	*L. ivanovii subsp. ivanovii PAM 55*

Appendix 2: List of homologous gene of *L. monocytogenes* LMof2365_0403 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
313615457	ADXF01000300	3,056	3,355	+	*L marthii*	NT05LM_0553	conserved Hypothetical	*L. marthii FSL S4-120*
386731147	NC_017728	422,482	422,781	+	*L mono07PF0776*	MUO_02110	Hypothetical protein	*L. monocytogenes 07PF0776*
284800678	NC_013766	429,179	429,478	+	*L mono08-5578*	LM5578_0425	Hypothetical protein	*L. monocytogenes 08-5578*
284993864	NC_013768	429,200	429,499	+	*L mono08-5923*	LM5923_0424	Hypothetical protein	*L. monocytogenes 08-5923*
386042730	NC_017544	414,246	414,554	+	*L mono10403S*	LMRG_00084	Hypothetical protein	*L. monocytogenes 10403S*
405748744	NC_018584	437,369	437,668	+	*L monoATCC19117*	LMOATCC19117_0409	Hypothetical protein	*L. monocytogenes ATCC 19117*
226223016	NC_012488	416,338	416,637	+	*L monoCL ip81459*	Lm4b_00409	Hypothetical protein	*L. monocytogenes Clip81459*
550896570	NC_022568	414,266	414,565	+	*L monoEGD*	LMON_0400	Hypothetical protein	*L. monocytogenes EGD*
16802436	NC_003210	414,928	415,227	+	*L monoEGD-e*	lmo0391	Hypothetical protein	*L. monocytogenes EGD-e*
386052667	NC_017547	422,550	422,858	+	*L monoFinL and1998*	LMLG_2035	Hypothetical protein	*L. monocytogenes Finland 1998*

Appendix 2: List of homologous gene of *L. monocytogenes* LMof2365_0403 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
217965519	NC_011660	2,224,649	2,224,948	-	*L monoHCC23*	LMHCC_2245	Hypothetical protein	*L. monocytogenes HCC23*
386046051	NC_017545	439,523	439,831	+	*L monoJ0161*	LMOG_02689	Hypothetical protein	*L. monocytogenes J0161*
525732920	NC_021830	195,293	195,592	+	*L monoJ1-220*	LM220_05247	Hypothetical protein	*L. monocytogenes J1-220*
525721850	NC_021829	716,629	716,928	-	*L monoJ1816*	LM1816_16500	Hypothetical protein	*L. monocytogenes J1816*
406703167	NC_018642	416,357	416,656	+	*L monoL 312*	LMOL312_0392	Hypothetical protein	*L. monocytogenes L312*
470206046	NC_020557	383,384	383,683	+	*L monoL a111*	BN418_0443	E3 ubiquitin-protein ligase sspH2	*L. monocytogenes La111 complete*
470209188	NC_020558	383,321	383,620	+	*L monoN53*	BN419_0453	E3 ubiquitin-protein ligase sspH2	*L. monocytogenes N53-1 complete*

Appendix 2: List of homologous gene of *L. monocytogenes* LMof2365_0403 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
404285756	NC_018591	419,713	420,012	+	*L mono7SLCC2482*	LMOSLCC2482_0389	Hypothetical protein	*L. monocytogenes serotype 7 str. SLCC2482*
404282825	NC_018588	413,643	413,942	+	*L monoSLCC2372*	LMOSLCC2372_0395	Hypothetical protein	*L. monocytogenes SLCC2372*
404406860	NC_018590	416,407	416,706	+	*L monoSLCC2376*	LMOSLCC2376_0377	Hypothetical protein	*L. monocytogenes SLCC2376*
405751605	NC_018585	425,012	425,311	+	*L monoSLCC2378*	LMOSLCC2378_0401	Hypothetical protein	*L. monocytogenes SLCC2378*
405757380	NC_018589	413,660	413,959	+	*L monoSLCC2479*	LMOSLCC2479_0393	Hypothetical protein	*L. monocytogenes SLCC2479*
405754482	NC_018586	426,196	426,495	+	*L monoSLCC2540*	LMOSLCC2540_0403	Hypothetical protein	*L. monocytogenes SLCC2540*

Appendix 3: List of homologous gene of *L. monocytogenes* LMof2365_0404 found in *Listeria* used in this study.

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
577757826	AOCG01000008	167,223	168,179	+	*L aquatica*	MAQA_08423	Hypothetical protein	*L. aquatica FSL S10-1188*
696204038	JNFA01000007	7,692	8,627	+	*L booriae*	EP57_03195	Hypothetical protein	*L. booriae FSL A5-0281*
577764863	AODE01000034	10,948	11,883	-	*L corneL L ensis*	PCORN_15881	Hypothetical protein	*L. cornellensis FSL F6-969*
577798586	AODM01000021	851	1,804	+	*L fL eischmanniiFSL*	MCOL2_06717	Hypothetical protein	*L. fleischmannii FSL S10-1203*
494736584	NZ_ALWW01000002	8,832	9,785	+	*L fL eischmanniiL U2006-1*	LFLEISCH_00875	Hypothetical protein	*L. fleischmannii LU2006-1*
494807764	NZ_AGUG01000005	15,966	16,919	-	*L fL eischmanniiCoL oradonensis*	KKC_01152	Hypothetical protein	*L. fleischmannii subsp. coloradonensis*
577766993	AODF01000030	45,901	46,869	-	*L fL oridensis*	MFLO_12836	Hypothetical protein	*L. floridensis FSL S10-1187*
577762465	AODD01000009	146,539	147,474	+	*L grandensis*	PGRAN_08258	Hypothetical protein	*L. grandensis FSL F6-971*
577766487	AODG01000011	64,441	65,382	+	*L grayi*	LMUR_08404	Hypothetical protein	*L. grayi FSL F6-1183*
16799487	NC_003212	421,219	422,163	+	*L innocua*	lin0410	Hypothetical protein	*L. innocua Clip11262*
347547810	NC_016011	353,498	354,442	+	*L ivanovii*	LIV_0319	Hypothetical protein	*L. ivanovii subsp. ivanovii PAM 55*

Appendix 3: List of homologous gene of *L. monocytogenes* LMof2365_0404 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
313615458	ADXF01000300	3,352	4,296	+	*L marthii*	NT05LM_0554	Conserved hypothetical protein	*L. marthii FSLS4-120*
386731148	NC_017728	422,778	423,722	+	*L mono07PF0776*	MUO_02115	Hypothetical protein	*L. monocytogenes 07PF0776*
284800679	NC_013766	429,475	430,419	+	*L mono08-5578*	LM5578_0426	Hypothetical protein	*L. monocytogenes 08-5578*
284993865	NC_013768	429,496	430,440	+	*L mono08-5923*	LM5923_0425	Hypothetical protein	*L. monocytogenes 08-5923*
386042731	NC_017544	414,551	415,495	+	*L mono10403S*	LMRG_00085	Hypothetical protein	*L. monocytogenes 10403S*
405748745	NC_018584	437,665	438,609	+	*L monoATCC19117*	LMOATCC19117_0410	Hypothetical protein	*L. monocytogenes ATCC 19117*
226223017	NC_012488	416,634	417,578	+	*L monoCL ip81459*	Lm4b_00410	Hypothetical protein	*L. monocytogenes Clip81459*
550896571	NC_022568	414,562	415,506	+	*L monoEGD*	LMON_0401	DUF1432 domain-containing protein	*L. monocytogenes EGD*
16802437	NC_003210	415,224	416,168	+	*L monoEGD-e*	lmo0392	Hypothetical protein	*L. monocytogenes EGD-e*
386052668	NC_017547	422,855	423,799	+	*L monoFinL and1998*	LMLG_2034	Hypothetical protein	*L. monocytogenes Finland 1998*
386049320	NC_017546	413,903	414,847	+	*L monoFSL R2-561*	LMKG_01519	Hypothetical protein	*L. monocytogenes FSL R2-561*

Appendix 3: List of homologous gene of *L. monocytogenes* LMof2365_0404 found in *Listeria* used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
386046052	NC_017545	439,828	440,772	+	*L monoJ0161*	LMOG_02688	Hypothetical protein	*L. monocytogenes J0161*
525732921	NC_021830	195,589	196,533	+	*L monoJ1-220*	LM220_17980	Hypothetical protein	*L. monocytogenes J1-220*
525721849	NC_021829	715,688	716,632	-	*L monoJ1816*	LM1816_01427	Hypothetical protein	*L. monocytogenes J1816*
406703168	NC_018642	416,653	417,597	+	*L monoL 312*	LMOL312_0393	Hypothetical protein	*L. monocytogenes L312*
386007118	NC_017529	431,089	432,033	+	*L monoL 99*	lmo4a_0405	Hypothetical protein	*L. monocytogenes L99*
470206047	NC_020557	383,680	384,624	+	*L monoL a111*	BN418_0444	UPF0365 protein lin0410	*L. monocytogenes La111 complete*
386025706	NC_017537	429,375	430,319	+	*L monoM7*	LMM7_0418	Hypothetical protein	*L. monocytogenes M7*
470209189	NC_020558	383,617	384,561	+	*L monoN53*	BN419_0454	UPF0365 protein lin0410	*L. monocytogenes N53-1 complete*
46906623	NC_002973	424,776	425,720	+	*L mono4bF2365*	LMOf2365_0404	Hypothetical protein	*L. monocytogenes serotype 4b str. F2365*
424713259	NC_019556	424,765	425,709	+	*L mono4bL L 195*	BN389_04090	UPF0365 protein lin0410	*L. monocytogenes serotype 4b str. LL195*
404285757	NC_018591	420,009	420,953	+	*L mono7SLCC2482*	LMOSLCC2482_0390	Hypothetical protein	*L. monocytogenes serotype 7 str. SLCC2482*
404282826	NC_018588	413,939	414,883	+	*L monoSLCC2372*	LMOSLCC2372_0396	Hypothetical protein	*L. monocytogenes SLCC2372*

Appendix 3: List of homologous gene of *L. monocytogenes* LMof2365_0404 found in Listeria used in this study (continued).

GI number	DNA reference	Start	End	Strand	Symbol	Locus tag	Putative function	Species
404406861	NC_018590	416,703	417,647	+	*L monoSLCC2376*	LMOSLCC2376_0378	Hypothetical protein	*L. monocytogenes SLCC2376*
405751606	NC_018585	425,308	426,252	+	*L monoSLCC2378*	LMOSLCC2378_0402	Hypothetical protein	*L. monocytogenes SLCC2378*
405757381	NC_018589	413,956	414,900	+	*L monoSLCC2479*	LMOSLCC2479_0394	Hypothetical protein	*L. monocytogenes SLCC2479*
405754483	NC_018586	426,492	427,436	+	*L monoSLCC2540*	LMOSLCC2540_0404	Hypothetical protein	*L. monocytogenes SLCC2540*
404279942	NC_018587	420,408	421,352	+	*L monoSLCC2755*	LMOSLCC2755_0390	Hypothetical protein	*L. monocytogenes SLCC2755*
404409634	NC_018592	414,074	415,018	+	*L monoSLCC5850*	LMOSLCC5850_0395	Hypothetical protein	*L. monocytogenes SLCC5850*
404412486	NC_018593	406,693	407,637	+	*L monoSLC7179*	LMOSLCC7179_0380	Hypothetical protein	*L. monocytogenes SLCC7179*
696204723	JNFB01000012	159,140	160,075	-	*L newyorkensis*	EP58_05455	Hypothetical protein	*L. newyorkensis FSL M6-0635*
577781877	AODL01000039	7,791	8,726	+	*L riparia*	PRIP_16247	Hypothetical protein	*L. riparia FSL S10-1204*
577786724	AODK01000024	74,401	75,336	+	*L rocoutiae*	PROCOU_10351	Hypothetical protein	*L. rocourtiae FSL F6-920*
289433706	NC_013891	364,665	365,609	+	*L seeligeri*	lse_0339	Hypothetical protein	*L. seeligeri serovar 1/2b str. SLCC3954*
116871760	NC_008555	361,453	362,397	+	*L welshimeri*	lwe0340	Hypothetical protein	*L. welshimeri serovar 6b str. SLCC5334*

Appendix 4: The number of repeats of *L. monocytogenes* inferred from the size of the PCR product by capillary electrophoresis.

Isolate	JLR1		JLR2		1869		881		TR1317		JLR4		LM-TR4		LMV1		LMV9	
	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit
1	383	17	258	4	469	8	235	0	194	1	248	4	488	3	384	3	510	2
2	377	16	258	4	469	8	238	0	194	1	248	4	487	3	384	3	510	2
3	359	13	258	4	439	6	273	2	194	1	228	3	487	3	390	4	519	3
4	383	17	258	4	469	8	239	0	195	1	266	5	488	3	384	3	NA	NA
5	377	16	246	3	469	8	221	0	200	1	240	4	478	2	378	2	501	1
6	353	12	270	5	439	6	221	0	193	1	228	3	472	2	378	2	539	3
7	383	17	258	4	469	8	239	0	193	1	248	4	486	3	384	3	501	1
8	383	17	246	3	469	8	220	0	199	1	238	4	480	2	384	3	539	3
9	383	17	258	4	469	8	238	0	193	1	246	4	485	3	384	3	501	1
10	383	17	258	4	469	8	237	0	193	1	246	4	488	3	384	3	510	2
11	383	17	258	4	469	8	237	0	193	1	247	4	487	3	384	3	501	1
12	353	12	270	5	439	6	218	0	194	1	228	3	468	1	384	3	548	4
13	353	12	270	5	439	6	219	0	194	1	230	3	468	1	384	3	548	4
14	347	11	234	2	415	4	288	3	169	0	171	0	482	2	402	6	501	1
15	347	11	234	2	427	5	288	3	169	0	172	0	482	2	402	6	501	1

Appendix 4: The number of repeats of *L. monocytogenes* inferred from the size of the PCR product by capillary electrophoresis (continued).

Isolate	JLR1		JLR2		1869		881		TR1317		JLR4		LM-TR4		LMV1		LMV9	
	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit
16	347	11	234	2	415	4	288	3	169	0	173	0	482	2	396	5	501	1
17	359	13	246	3	469	8	220	0	200	1	240	4	481	2	378	2	539	3
18	383	17	258	4	469	8	238	0	193	1	248	4	490	3	384	3	510	2
19	377	16	246	3	469	8	220	0	199	1	239	4	481	2	378	2	501	3
20	383	17	246	3	469	8	221	0	199	1	238	4	481	2	378	2	539	3
21	377	16	246	3	469	8	221	0	200	1	238	4	475	2	378	2	510	3
22	377	16	246	3	469	8	220	0	200	1	239	4	478	2	384	3	519	3
23	377	16	246	3	469	8	220	0	201	1	239	4	476	2	384	3	539	3
24	377	16	246	3	469	8	220	0	200	1	238	4	472	2	384	3	519	3
25	383	17	258	4	469	8	239	0	194	1	248	4	487	3	384	3	519	2
26	377	16	246	3	469	8	220	0	200	1	239	4	475	2	378	2	539	2
27	347	11	234	2	439	6	291	3	170	0	172	0	482	2	402	6	519	1
28	377	16	246	3	469	8	222	0	201	1	239	4	479	2	384	3	510	3
29	353	12	270	5	439	6	222	0	194	1	230	3	474	2	384	3	510	3
30	377	16	258	4	469	8	237	0	196	1	244	4	487	3	384	3	501	1

Appendix 5: DNA sequences of VNTR of *L. monocytogenes.*

>JLR1 (10 repeat units)

GCGCTATAACCTGAGGAAAGCATTGTTCCTGAACCATTTGAGCTCATGTTTGTAA
CGGTTACGCCAGTTCCTCCGTTAGAAGTTCCAGTAGTTCCTCCATTGGTAGAAGG
ATTTGGTCCACCATCCGCATCAGCGTCAGCATCCGCGTCAGCATCGGCGTCAGCA
TCGGCATCCGCATCAGCGTCTGAGTCTACTAAAGGCAGAACTGTTACGGATACAT
CATCACTTGTTTTTACTTCATCGCCGTATTTAGCTGTTACTTGAAGATGAATAACA
TCTCCAGCTTTCAAATTATAATTAGGAATATTGATTGTATAGGTTCCATCTGCATG
GATTAAGAC

>JLR1 (11 repeat units)

GCGCTATAACCTGAGGAAAGCATTGTTCCTGAACCATTTGAACTCATGTTCGTAA
CGGTTACGCCAGTTCCTCCGTTAGAAGTTCCAGTAGTTCCTCCATTGGTAGAAGG
ATTTGATCCACCATCCGCATCCGCATCCGCATCAGCGTCGGCATCGGCATCGGCA
TCAGCGTCGGCATCAGCGTCGGCATCAGAATCTGCTAATGGTAGGACAGTTACGG
CTACATCTTCACTCGTTTTAACTTCATTACCATATTTAGCTGTTACTTGAAGATGA
ATAACATCTCCAGCTTTCAAATTATAATTAGGAATATTAATAGTATAGGTTCCATC
TGCATGGATTAAGAC

>JLR1 (16 repeat units)

GCGCTATAACCTGAGGAAAGCATTGTTCCTGAACCATTTGAACTCATGTTCGTAA
CGGTTACGCCAGTTCCTCCGTTAGAAGTTCCAGTAGTTCCTCCATTGGTAGAAGG
ATTTAATCCACCATCCGCATCGGCATCCGCATCGGCATCGGCGTCGGCATCAGCA
TCGGCATCGGCATCCGCATCAGCATCCGCATCAGCGTCGGCATCTGCGTCGGCAT
CAGAATCTGCTAATGGTAGGACAGTTACGGCTACATCTTCACTCGTTTTAACTTCA
TTACCATATTTAGCTGTTACTTGAAGATGAATAACATCTCCAGCTTTCAAATTATA
ATTAGGAATATTAATAGAATAGGTTCCATCTGCATGGATTAAGAC

Appendix 5: DNA sequences of VNTR of *L. monocytogenes* (continued).

>JLR1 (17 repeat units)

GCGCTATAACCTGAGGAAAGCATTGTTCCTGAACCATTTGAACTCATGTTCGTAA
CGGTTACGCCAGTTCCTCCGTTAGAAGTTCCAGTAGTTCCTCCATTGGTAGAAGG
ATTTAATCCACCATCCGCATCGGCATCCGCATCGGCGTCGGCATCAGCATCCGCA
TCAGCATCCGCATCAGCATCCGCATCAGCATCCGCATCAGCGTCGGCATCTGCGT
CGGCATCAGAATCTGCTAATGGTAGGACAGTTACGGCTACATCTTCACTCGTTTT
AACTTCATTACCATATTTAGCTGTTACTTGAAGATGAATAACATCTCCAGCTTTCA
AATTATAATTAGGAATATTAATAGAATAGGTTCCATCTGCATGAATTAAGAC

>JLR2 (2 repeat units)

TGATGCTAATAACCAAGATTATCGTTACTCCGTTGCTGAAATAGCAGTTCCAGGC
TACAAAACAACGACAGATGGAATGAACTTAACCAACACAAAAGTAACCGATGAG
AAAAATGTAACTTCAGCGAGCGGCACAAAAACTTGGGTGGGAGACAATGAAAAA
ACACGTCCAGACTCCATCGAAGTACAACTTTTACAAAATGGGAAAGCATACGGA
ACACCAATCAAAGTAACAGCAAAATCGAATTGGAAATACAGCTTTACCAACCTTC
CAGAGAAAGACAAAACAGGTGAAAAATACAGCTATACCGTATCTGAAAAACAAG
TATCTGGCTATAGTGTAAAAGTAAAAGGCATGGACTTAACGAATACAAAAGTAA
CAAAAACGACACCAAAAGATACACCAAAAGATACACATTCTACCAATAAACCAA
GTAAAACGAAGAAATTACCTGGAACTGGTGATACGAAT

>JLR2 (3 repeat units)

TGATGCTAATAACCAAGATTATCGTTACTCTGTTACTGAAATAGCAGTTCCAGGC
TACAAAACAACCACAGATGGAATGAACTTAACCAATACAAAAGTAACCGATGAG
AAAAATGTAACTACAGCGAGCGGCACAAAAACTTGGGTGGGAGACAATGAAAA
AACACGTCCCGCATCCATCGAAGTTCAACTTTTACAAAACGGTAAAGCATACGGA
ACACCAATCAAAGTAACAGCAAAATCGAATTGGAAATACAACTTTACCAACCTTC
CAGAGAAAGACAAAACAGGTGAAAAATACAGCTATACTGTATCTGAAAAACAAG
TATCTGGCTATAGTGTAAAAGTAAAAGGTATGGACTTAACGAATACAAAAGTAA
CAAAAACGACACCAAAAGATACACCAAAAAATACACCAAAAGATACACATTCTA
CCAATAAACCAAGTAAAACGAAAAAATTACCTGGAACTGGTGATACGAAT

Appendix 5: DNA sequences of VNTR of *L. monocytogenes* (continued).

>JLR2 (4 repeat units)

TGATGCTAATAACCAAGATTATCGTTACTCTGTTACTGAAATAGCAGTTCCAGGC
TACAAAACAACCACAGATGGAATGAACTTAACCAATACAAAAGTAACCGATGAG
AAAAATGTAACTACAGCGAGCGGCACAAAAACTTGGGTGGGAGACAATGAAAA
AACACGTCCCGCATCCATCGAAGTTCAACTTTTACAAAACGGTAAAGCATACGGA
ACACCAATCAAAGTAACAGCAAAATCGAATTGGAAATACAACTTTACCAACCTTC
CAGAGAAAGACAAAACAGGTGAAAAATACAGCTATACTGTATCTGAAAAACAAG
TATCTGGCTATAGTGTAAAAGTAAAAGGTATGGACTTAACGAATACAAAAGTAA
CAAAAACGACACCAAAAGATACACCAAAAAATACACCAAAAGATACACCAAAA
GATACACATTCTACCAATAAACCAAGTAAAACGAAAAAATTACCTGGAACTGGT
GATACGAAT

>JLR2 (5 repeat units)

TGATGCTAATAACCAAGATTATCGTTACTCTGTTACTGAAATAGCAGTTCCAGGC
TACAAAACAACCACAGATGGAATGAACTTAACCAATACAAAAGTAACCGATGAG
AAAAATGTAACTACAGCGAGCGGCACAAAAACTTGGGTGGGAGACAATGAAAA
AACACGTCCCGCATCCATCGAAGTTCAACTTTTACAAAACGGTAAAGCATACGGA
ACACCAATCAAAGTAACAGCAAAATCGAATTGGAAATACAACTTTACCAACCTTC
CAGAGAAAGACAAAACAGGTGAAAAATACAGCTATACTGTATCTGAAAAACAAG
TATCTGGCTATAGTGTAAAAGTAAAAGGTATGGACTTAACGAATACAAAAGTAA
CAAAAACGACACCAAAAGATACACCAAAAAATACACCAAAAGATACACCAAAA
GATACACCAAAAGATACACATTCTACCAATAAACCAAGTAAAACGAAAAAATTA
CCTGGAACTGGTGATACGAAT

>JLR4 (4 repeat units)

AGAAATTCCAGTCCGCCAGCCGTGATTTTATTTTGGTTCATTGTTATCTTTTGGAG
CTTCTGGTGTTTCAGGAGCTTCCGGTACTACTGGAGTTTCGGGTTCTTTTGCTGCA
TCTGGTCCAAAGCCTTCTGGATGTGTATCCTCTGCAAATGGATCAGCTTCTGTTGC
TCC

Appendix 5: DNA sequences of VNTR of *L. monocytogenes* (continued).

>JLR4 (2 repeat unit)

AGAAATTCCAGTCCGCCAGCCGTAATTTTATTTTGGTTCATTGTTATCTTTT
GGAGCTTCCGGTGTTTCGGGTGCTTCCGGAGTCTCGGGTGCTTCCGGAGTC
TCGGGTGCCTCAGGAGTTTCAGGAACTTCCGGCGTTTCTGGTGTTTCAGGA
GCCTCAGGAGCTTCTGGTACTACAGGCGTTTCGGGTTCTTTTGTTGCTTCT
GGTCCGAAGCCTTCTGGATGAGTATCTTCAGCAAATGGATCAGCTTCTGTT
GCTCC

>LMV1 (14 repeat units)

CGTATTGTGCGCCAGAAGTACGTGGAAGGGAGATTCCCGCTTTAGCAAAT
ACATATTTAGTGTAACCAGAGCAATCAAATGTAGTTGGTCCGTTACCACCC
CATGAATAAGCTTTTCCAAGGTGTTTTTGAGCTTCAGCAATAATAGCACTT
GCACTTGAATTGCTGTTATTGTTAGAAGAACCTTGATTAGCATTCGTATTT
GAGTTTGTATTCGTATTGGAGTTTGTATTAGTATTGGTATTTTTAGATGGTG
TACTTGTATTAGTATTGTTTGTATTCGTATTCGTTTTATTAGCATTTGTGTTT
GTAGATGGTGCGGGAGCTGGTTTTGCAGCTTCTGTTGGTGCTTTAGGTGCT
GTTTGTTGTTGCGTTGTTTA

>LMV9 (2 repeat units)

AACGGTGGCTGATTTACTTCCAGGTGAGTATCAATTTGTTGAAACAAAAG
CACCAACAGGTTATATTTTAGACACTACACCATTGAAATTTAAAATCAGC
ACAGAAGCATTAAACGTAACTGTAACAAAAGAAAATACGAAAAAACCAG
AAATACCAAAAGTGCCAGTACCACCAAAAAAACCAGAAAAACCGGATAA
AATAATAAGTGAAGACAGCAAACAGACAGCTTTACCAAAAACAGGAGAT
TCGCCACTTGTTAATGGATGGGGACTGTTACTCGTAGCCATTTCAGCGAGC
GGCTTAATTGCACTTAGAAGAAAATAATAAAAAACCGTAAGCGATAATAC
AATCGCTTACGGTTTTTCTTATATAGTTCTAGTTTGATCTTGCAAAACGGC
CTTTAACGAATGATAGGCTGGAATAGATAAGTTTAAGTCAACGCGATTAA
TTTGTTGCGCCATTTTTGGTTGGATACCAGAAATAAATGCCTCGACGCCAA
G

Appendix 5: DNA sequences of VNTR of *L. monocytogenes* (continued).

>LMV9 (3 repeat units)

AACGGTGGCTGATTTACTTCCAGGCGAGTATCAATTTGTCGAAACAAAAG
CGCCAACAGGCTATATTTTAGATGCTACTCCAGTCAAATTTAAAATCAGCA
CAGAGGCACTAAACGTAACCGTAACAAAAGAGAACACGAAAAAACCAGA
AATACCAAAAGTACCAGTACCACCAAAAAACACCAGAACAACCGGATAAA
CCGGATAAACCGGATAAACCAGAACAACCAGATAAAATAATAAGCGCAG
ATAGCAAACGGACGACTTTACCAAAAACAGGGGATACACCACTTGTTAAT
GGTTGGGGAATACTGCTCGTAGCCATTTCAGCGAGTGGATTAATTGCGCTT
AGAAGAAAATAATTAAAAAAACCGTAAGCGATAATACAATCGCTTACGGT
TTTTCTTATATAGTTCTAGTTTGATCTTGTAAGACAGCCTTTAACGAATGAT
AGGCAGGAATAGATAAGTTTAATTCAACACGATTAATTTGTTGTGCCATTT
TTGGTTGGATGCCAGAAATAAATGCCTCGACACCAAG

> TR881 (1 repeat unit)

TGTAAATAAAGCTGGTACGTACAAAGTGATTTATACGTATGATCCGAATG
AAGGAACAGCGGATGCAGGTAAGAAAGAACTTTCTGTTACAGCTAATATT
CAAGTAGAAGCAGAATTTGTAAAACCTATAAAACCAGTTGACCCATCAAA
ACCAACAGATCCTAAAAAACCATCAACAGAAAAAACACCATTGAAAGTA
GTTGATAACAAGCAACATAC

>TR881 (2 repeat units)

TGTAAATAAAGCTGGTACGTACAAAGTGATTTATACGTATGATCCGAATG
AAGGAACAGCGGATGCAGGTAAGAAACAACTTTCTGTCACAGCTAATATT
CAAGTAGAAGCAGAATTTGTAAAACCTATAAAACCTATAAAACCTATAAA
ACCAGTTGACCCATCAAAACCAACAGATCCTAAAAAACCATCAACAGAA
AAAACACCATTGAAAGTAGTTGATAACAAGCAACATAC

Appendix 5: DNA sequences of VNTR of *L. monocytogenes* (continued).

>LMTR4 (3 repeat units)

TCCGAAAAAGACGAAGAAGTAGCAGGTAAATCTTTTGAAGAAGAAAAAG
AAGAGTTAAAAGAAGAAGAAAAAGTCGAAGAACCAAAAGAAGAACCAA
AAGAAGAACCAAAAGAAGTAACTTCGGAAGATGCTCCAAACATTGAGCA
AACACCAAACGATAAAAAAGACGAATAAAATCAAAGAGGCTGGGCTTTC
CCAGTCTCTTTTTTGAAGCTATATTATTCGGATTTTCCTATAAAACATGTTA
TGATACGTTAGAAAAATCTTCTAAAGGACGGTATTTAAACTTATGATACTT
GTAATAGACGTTGGAAATACTAACTGTACTGTCGGAGTTTATGAAAAACA
AAAACTTCTGAAACATTGGCGCATGACAACAGATCGTCACCGCACATCCG
ATGAATTAGGAATGACAGTCTTGAACTTTTTTTCTTATGCGAATTTAACTC
CTTCTGATATTCAAGGAATTATTATTTCGTCCGTCGTTCCA

>TR1317 (1 repeat units)

TGATTTACAAAAAGCTTTGCCAGTGCAAGTGATGGTTATTGTTGAAAAAG
AAACACCAATACCAGACCCGACGCCTACACCAACACCGGACCCAACACC
AACACCAGATCCAAGTCCAACACCTAATCCGGTTATCAACCCAAACGTAA
ATAAACCAGAAGTGCCAAGT

>TR1317 (2 repeat units)

TGATTTACAAAAAGCTTTGCCAGTGCAAGTGACGGTTATTGTTGAAAAAG
AAACACCAATACCAGACCCAACGCCAAATCCAACACCAGATCCGACGCC
AAATCCAACACCAACACCAACACCAGATCCAACACCGGACCCAAGCCCA
ACACCTAATCCGGTTATCAAACCAAACGTCAATAAACCAGAAGTGCCAAG

Appendix 5: DNA sequences of VNTR of *L. monocytogenes* (continued).

>LMV1 (19 repeat units)

CGTATTGTGCGCCAGAAGTACGTGGAAGGGAGATTCCCGCTTTAGCAAAT
ACATATTTAGTGTAACCAGAGCAATCAAATGTAGTTGGTCCGTTACCACCC
CATGAATAAGCTTTTCCAAGGTGTTTTTGAGCTTCAGCAATAATAGCACTT
GCACTTGAATTGCTGTTATTGTTGGAAGAACCTTGATTAGCATTCGTATTT
GAGTTTGTATTCGTATTAGTATTTGAGTTTGTATTAGTATTTTTAGATGGTG
TATTTGTATTAGTATTGTTTGTATTTGTATTTGTATTTGTATTTGTATTTGTA
TTTGTTTTATTAGCATTTGTGTTTGTAGATGGTGCAGGAGCTGGTTTTGCA
GCTTCTGTTGGTGCTTTAGGTGCTGTTTGTTGTTGCGTTGTTG

>TR1869 (9 repeat units)

CCGCGCTATAACCTGAGGAAAGCATTGTTCCTGAACCATTTGAACTCATGT
TCGTAACGGTTACGCCAGTTCCTCCGTTAGAAGTTCCAGTAGTTCCTCCAT
TGGTAGAAGGATTTAATCCACCATCCGCATCGGCATCCGCATCGGCGTCG
GCATCAGCATCCGCATCAGCATCCGCATCAGCATCCGCATCAGCATCCGC
ATCAGCGTCGGCATCTGCGTCGGCATCAGAATCTGCTAATGGTAGGACAG
TTACGGCTACATCTTCACTCGTTTTAACTTCATTACCATATTTAGCTGTTAC
TTGAAGATGAATAACATCTCCAGCTTTCAAATTATAATTAGGAATATTAAT
AGAATAGGTTCCATCTGCATGGATTAAGACATTTCCGATAACAGTACCGT
CCGGAAGAGTCAAATTAATATAGAAAGTTGTTCCAGCC
GGTGCATCTGATTGCAATGATTTCAG

Appendix 6: The number of repeats of *L. innocua* inferred from the size of the PCR product by capillary electrophoresis.

Isolate	TR1		TR3		TR6		TR13		Isolate	TR1		TR3		TR6		TR13	
	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)		Size (bp)	Repeat Unit		Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit
1	250	3	485	14	244	10	149	10	18	250	3	494	15	244	10	167	12
2	250	3	485	14	244	10	149	10	19	250	3	485	14	244	10	149	10
3	250	3	485	14	244	10	149	10	20	250	3	485	15	244	10	149	10
4	250	3	485	14	244	10	149	10	21	250	3	485	14	244	10	149	10
5	250	3	458	11	244	10	149	10	22	250	3	485	14	244	10	149	10
6	250	3	458	11	244	10	149	10	23	250	3	485	14	244	10	149	10
7	250	3	485	14	244	10	149	10	24	250	3	485	14	244	10	149	10
8	250	3	485	14	244	10	149	10	25	250	3	485	14	244	10	149	10
9	250	3	485	14	244	10	149	10	26	250	3	485	14	244	10	149	10
10	250	3	485	14	244	10	149	10	27	250	3	485	14	244	10	149	10
11	250	3	485	14	244	10	149	10	28	250	3	485	14	244	10	167	12
12	250	3	485	14	244	10	149	10	29	250	3	485	14	244	10	149	10
13	250	3	485	14	244	10	167	12	30	250	3	485	14	244	10	149	10
14	250	3	485	14	244	10	149	10	31	250	3	485	14	244	10	149	10
15	250	3	485	14	244	10	149	10	32	250	3	485	14	244	10	149	10
16	250	3	485	14	244	10	167	12	33	250	3	485	14	244	10	149	10
17	250	3	485	14	244	10	149	10	34	250	3	485	14	244	10	149	10

Appendix 6: The number of repeats of *L. innocua* inferred from the size of the PCR product by capillary electrophoresis (continued).

Isolate	TR1		TR3		TR6		TR13		Isolate	TR1		TR3		TR6		TR13	
	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)		Size (bp)	Repeat Unit		Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit
35	250	3	485	14	244	10	149	10	52	250	3	485	14	244	10	167	12
36	250	3	485	14	244	10	149	10	53	250	3	485	14	244	10	149	10
37	250	3	485	14	244	10	149	10	54	250	3	485	14	244	10	167	12
38	250	3	485	14	244	10	149	10	55	250	3	485	14	244	10	77	2
39	250	3	485	14	244	10	149	10	56	250	3	485	14	244	10	149	10
40	250	3	485	14	244	10	149	10	57	250	3	485	14	244	10	167	12
41	250	3	485	14	244	10	149	10	58	250	3	485	14	244	10	149	10
42	250	3	485	14	244	10	149	10	59	256	4	395	4	226	7	104	5
43	250	3	485	14	244	10	149	10	60	250	3	431	8	256	12	167	12
44	250	3	485	14	244	10	149	10	61	250	3	485	14	244	10	149	10
45	250	3	485	14	244	10	149	10	62	250	3	485	14	244	10	149	10
46	256	4	395	4	226	7	104	5	63	250	3	485	14	244	10	149	10
47	250	3	485	14	244	10	149	10	64	250	3	485	14	244	10	149	10
48	250	3	485	14	244	10	149	10	65	250	3	485	14	244	10	167	12
49	250	3	485	14	244	10	149	10	66	250	3	485	14	244	10	149	10
50	250	3	485	14	244	10	167	12	67	250	3	449	10	244	10	149	10
51	250	3	485	14	244	10	149	10	68	250	3	485	14	244	10	167	12

Appendix 6: The number of repeats of *L. innocua* inferred from the size of the PCR product by capillary electrophoresis (continued).

Isolate	TR1		TR3		TR6		TR13		Isolate	TR1		TR3		TR6		TR13	
	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)		Size (bp)	Repeat Unit		Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit	Size (bp)	Repeat Unit
69	250	3	485	14	244	10	149	10	86	250	3	485	14	244	10	149	10
70	250	3	485	14	244	10	149	10	87	250	3	485	14	244	10	149	10
71	250	3	485	14	244	10	149	10	88	250	3	485	14	244	10	167	12
72	250	3	485	14	244	10	149	10	89	250	3	485	14	244	10	149	10
73	250	3	485	14	244	10	149	10	90	250	3	485	14	244	10	149	10
74	250	3	485	14	244	10	149	10	91	250	3	485	14	244	10	140	9
75	250	3	485	14	244	10	149	10	92	250	3	485	14	244	10	149	10
76	250	3	494	15	244	10	167	12	93	250	3	485	14	244	10	149	10
77	256	4	395	4	244	10	104	5									
78	250	3	485	14	244	10	149	10									
79	250	3	485	14	244	10	149	10									
80	250	3	485	14	244	10	149	10									
81	250	3	485	14	244	10	167	12									
82	250	3	485	14	244	10	167	12									
83	250	3	485	14	244	10	167	12									
84	250	3	485	14	244	10	167	12									
85	250	3	485	14	244	10	149	10									

Appendix 7: DNA sequences of VNTR of *L. innocua*.

>TR1 (3 repeat units)

AAACAACAGATAAAACAACAAAAGATGAAACGAAAAAACAAGAAGAAA
AAAACAACGAACAATCAGAAACAAATACAAATACAAATCAAGCTACAAA
TGATAATACAAATAAGGCAGAGGGAGCAAACGGATCTGCTAATGGCGGG
GACACTGGAACAACTACTAAGGGTAACGCAAATGAAGGTGGAACCACAA
AAGACAACCAAAATACTAC

>TR3 (7 repeat units)

TATGCAAATGGCGAGATTACATGGGAAGGGTTGCAAAGTAATTATATTCT
AAATTACGAGTATAATTTACCCGTAGCAATTGGTTCGTTAACAACAACGT
ATTCTGGTAAAATTACCCAGCCACTGTTGGAGAAGCCTGTTGATCCGATTA
CTCCGGTAGACCCAGTAGACCCGGTAGATCCAGTAGACCCGGTAGACCCG
GTAGACCCGGTAGACCCGGTAGACCCGGTAGACCCGGTAGACCCGGTAG
ACCCAATAGATCCAGTAAACCCGGTAGATCCAATCACTCCAGTAGACCCG
TCAAATCAAGTAAATTCAACTGATCCAGTGAAGTCAGTTCTTCAAGCAAC
TGAAACTTTAAT

>TR3 (13 repeat units)

TATGCAAATGGCGAGATTACATGGGAAGGGTTGCAAAGTAATTATATTCT
AAATTACGAGTATAATTTACCCGTAGCAATTGGTTCGTTAACAACAACGT
ATTCTGGTAAAATTACCCAGCCACTGTTGGAGAAGCCTGTTGATCCGATTA
CTCCGGTAGACCCAGTAGACCCGGTAGATCCAGTAGACCCGGTAGACCCG
GTAGACCCGGTAGACCCGGTAGACCCGGTAGACCCGGTAGACCCGGTAG
ACCCGGTAGACCCGGTAGACCCGGTAGACCCGGTAGACCCGGTAGACCCG
GTAGACCCAATAGATCCAGTAAACCCGGTAGATCCAATCACTCCAGTAGA
CCCGTCAAATCAAGTAAATTCAACTGATCCAGTGAAGTCAGTTCTTCAAG
CAACTGAAACTTTAAT

Appendix 7: DNA sequences of VNTR of *L. innocua* (continued).

>TR6 (10 repeat units)

GGAGCCGTTTGTACTCATATTAGAAACGGTTACGCCCGTACCTCCATTTGC
TGTTCCAGTAGTTCCACCGTTATTAGAAGAGTTAGTGCCTCCTCCATCTGA
ATCAGCGTCAGCATCGGCGTCAGCATCGGCATCGGCATCGGCGTCGGCGT
CAGCATCTGCGTCAGCATCGGCATCAGCGTCAGCATCCGAATCAACTAAT
GGTTGAACTGTTACTGCTACATCATCACTTGTTTTTACTTCGTCACCATATT
TAGCAGTTACTTGAAGGT

>TR13 (5 repeat units)

TGTGCTGGATCTGCTGGTTGAATCGGATTTACTGGGTTTACTGGGTTTACT
GGGTTTACTGGGTTTACTGGGTTTACTGGGTTTATCGGATTTACTGGATTT
ACTGGATTTACCGGGTCTACCGGAACTACTGGATTTTGGGTAAAGCGCGC
ATATAAGGCT

>TR13 (9 repeat units)

TGTGCTGGATCTGCTGGTTGAATCGGATTTACTGGGTTTACTGGGTTTACT
GGGTTTACTGGGTTTACTGGGTTTACTGGGTTTACTGGGTTTACTGGGTTT
ACTGGGTTTATCGGATTTACTGGATTTACTGGATTTACCGGGTCTACCGGA
ACTACTGGATTTTGGGTAAAGCGCGCATATAAGGCT

Appendix 8: Fusion primers used in this study. Nucleotides in black and blue represent respectively Ion torrent sequencing-specific adapter nucleotides and the illumina key sequence; MID sequences are shown in red; and universal tails are depicted in green or blue.

Fusion primer	Primer sequence (5' to 3')
PGM-Findex-1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACTCGTCGGCAGCGTC
PGM-Findex-2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACTCGTCGGCAGCGTC
PGM-Findex-3	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCTCGTCGGCAGCGTC
PGM-Findex-4	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCTCGTCGGCAGCGTC
PGM-Findex-5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACTCGTCGGCAGCGTC
PGM-Findex-6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCTCGTCGGCAGCGTC
PGM-Findex-7	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCTCGTCGGCAGCGTC
PGM-Findex-8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACTCGTCGGCAGCGTC
PGM-Findex-9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACTCGTCGGCAGCGTC
PGM-Findex-10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACTCGTCGGCAGCGTC
PGM-Findex-11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCTCGTCGGCAGCGTC
PGM-Findex-12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCTCGTCGGCAGCGTC
PGM-Findex-13	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACTCGTCGGCAGCGTC
PGM-Findex-14	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCTCGTCGGCAGCGTC
PGM-Findex-15	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCTCGTCGGCAGCGTC
PGM-Findex-16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACTCGTCGGCAGCGTC

Appendix 8: Fusion primers used in this study. Nucleotides in black and blue represent respectively Ion torrent sequencing-specific adapter nucleotides and the illumina key sequence; MID sequences are shown in red; and universal tails are depicted in green or blue (continued).

Fusion primer	Primer sequence (5' to 3')
PGM-Findex-17	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCTCGTCGGCAGCGTC
PGM-Findex-18	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCTCGTCGGCAGCGTC
PGM-Findex-19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACTCGTCGGCAGCGTC
PGM-Findex-20	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCTCGTCGGCAGCGTC
PGM-Findex-21	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACTCGTCGGCAGCGTC
PGM-Findex-22	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCTCGTCGGCAGCGTC
PGM-Findex-23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACTCGTCGGCAGCGTC
PGM-Findex-24	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTCTCGTCGGCAGCGTC
PGM-Findex-25	CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACTCGTCGGCAGCGTC
PGM-Findex-26	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCTCGTCGGCAGCGTC
PGM-Findex-27	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCTCGTCGGCAGCGTC
PGM-Findex-28	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCTCGTCGGCAGCGTC
PGM-Findex-29	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCTCGTCGGCAGCGTC
PGM-Findex-30	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTATCTCGTCGGCAGCGTC
PGM-Findex-31	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCTCGTCGGCAGCGTC
PGM-Findex-32	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACTCGTCGGCAGCGTC

Appendix 8: Fusion primers used in this study. Nucleotides in black and blue represent respectively Ion torrent sequencing-specific adapter nucleotides and the illumina key sequence; MID sequences are shown in red; and universal tails are depicted in green or blue (continued).

Fusion primer	Primer sequence (5' to 3')
PGM-Findex-33	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTCATTGAACTCGTCGGCAGCGTC
PGM-Findex-34	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCATCGTTCTCGTCGGCAGCGTC
PGM-Findex-35	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCCATTGTCTCGTCGGCAGCGTC
PGM-Findex-36	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGAATCGTCTCGTCGGCAGCGTC
PGM-Findex-37	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGAGAATGTCTCGTCGGCAGCGTC
PGM-Findex-38	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGGACGGACTCGTCGGCAGCGTC
PGM-Findex-39	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAATCGGCTCGTCGGCAGCGTC
PGM-Findex-40	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACATAATCTCGTCGGCAGCGTC
PGM-Findex-41	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCACTTCGCTCGTCGGCAGCGTC
PGM-Findex-42	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACGAATCTCGTCGGCAGCGTC
PGM-Findex-43	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGACACCGCTCGTCGGCAGCGTC
PGM-Findex-44	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGGCCAGCTCGTCGGCAGCGTC
PGM-Findex-45	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGCTTCCTCTCGTCGGCAGCGTC
PGM-Findex-46	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGTCCGAACTCGTCGGCAGCGTC
PGM-Findex-47	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGCAACCACTCGTCGGCAGCGTC
PGM-Findex-48	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTAAGAGACTCGTCGGCAGCGTC
PGM-R	CCTCTCTATGGGCAGTCGGTGATGTCTCGTGGGCTCGG

VITA

Mr. Chirapiphat Phraephaisarn was born on March 22nd, 1981 in Bangkok, Thailand. He attended Samakkhi Wittayakom School, Chiang Rai, Thailand before joining the Chiang Mai University for his undergraduate studies in 2000. He majored in animal science earning a B.Sc. Agriculture degree in 2003. In the same year, he enrolled in Department of Animal science, Faculty of Agriculture at Chiang Mai University, and graduated with a M.Sc. Agriculture in 2006. From 2013 to 2017, he was a PhD student at Program in biotechnology, Faculty of Science, Chulalongkorn University.

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