DETECTION OF TETRACYCLINE-RESISTANCE *ENTEROCOCCUS* SPP. IN READY-TO-EAT THAI TRADITIONAL FERMENTED PRODUCTS

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การตรวจหาเชื้อเอ็นเทอโรคอกคัสที่ดื้อยาเททราไซคลินในอาหารหมักดองพื้นบ้านพร้อมบริโภคของไทย

นางสาวปิยนันท์ เหลืองพูนลาภ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ภาควิชาอาหารและเภสัชเคมี
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ปิยนันท์ เหลืองพูนลาภ : การตรวจหาเชื้อเอ็นเทอโรคอกคัสที่ดื้อยาเททราไซคลินในอาหารหมักดองพื้นบ้านพร้อมบริโภคของไทย (DETECTION OF TETRACYCLINE-RESISTANCE *ENTEROCOCCUS* SPP. IN READY-TO-EAT THAI TRADITIONAL FERMENTED PRODUCTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภญ.ดร. สุญาณี พงษ์ธนานิกร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ภญ.ดร. มณีวรรณ สุขสมทิพย์, 97 หน้า.

งานวิจัยนี้ตรวจหา *Enterococcus* spp. ที่ดื้อยาเททราไซคลิน จากอาหารหมักพื้นบ้านพร้อมบริโภคของไทย 5 ประเภทคือ ปลา หมู กุ้ง ปู และหอย จำนวนทั้งหมด 70 ตัวอย่าง ผลการสำรวจพบว่า อาหารจำนวน 28 ตัวอย่าง (ร้อยละ40.00) มีการปนเปื้อน *Enterococcus* spp. ที่ดื้อยาเททราไซคลิน การปนเปื้อนนี้พบในอาหารประเภทปลา หมู กุ้ง ปู แต่ไม่พบในหอย โดย *Enterococcus* spp. ที่แยกได้จากอาหารประเภทปลาและกุ้งพบการดื้อเททราไซคลินสูงกว่าที่แยกได้จากอาหารชนิดอื่น (ร้อยละ 77.46 และร้อยละ 57.89 ในปลาและกุ้งตามลำดับ) *Enterococcus* spp. ที่ดื้อเททราไซคลินมีค่า minimum inhibitory concentration (MIC) ตั้งแต่ 16-512 µg/ml และส่วนใหญ่มีค่า MIC >64-128 µg/ml ยกเว้นอาหารประเภทปูซึ่งส่วนใหญ่มีค่า MIC >128-256 µg/ml สำหรับ *Enterococcus* spp.ที่ดื้อเททราไซคลินโดยมีค่า MIC ระดับสูง (MIC > 256-512 µg/ml) นั้นพบในอาหารประเภทปลาและปู จากการตรวจสอบสายพันธุ์ที่เกี่ยวข้องทางคลินิก พบ *E. faecalis* ร้อยละ 46.15 และ *E. faecium* ร้อยละ 35.58 โดย *E. faecalis* พบเฉพาะในอาหารประเภทปลาและหมู ส่วน *E. faecium* พบในอาหารทุกประเภท ข้อมูลจากการศึกษาแสดงให้เห็นว่าอาหารหมักพื้นบ้านพร้อมบริโภคของไทยบางชนิดอาจเป็นแหล่งแพร่กระจายการดื้อยาปฏิชีวนะและเชื้อ *Enterococcus* spp. สายพันธุ์ที่อาจก่ออันตรายซึ่งต้องศึกษาต่อไป ดังนั้นเพื่อมิให้เกิดปัญหาการแพร่กระจายเชื้อดื้อยาและคุณสมบัติการดื้อยาที่อาจก่อปัญหาสาธารณสุขจึงควรลดการปนเปื้อนของ *Enterococcus* spp. ในอาหารโดยการปรุงให้สุกด้วยความร้อนและมีกระบวนการผลิตที่สะอาดถูกหลักอนามัย นอกจากนี้ควรมีมาตรการสำหรับควบคุมกำกับการใช้ยาปฏิชีวนะในอุตสาหกรรมการเลี้ยงสัตว์เพื่อป้องกันปัญหาการดื้อยาปฏิชีวนะของเชื้อก่อโรคต่อไป

ภาควิชา อาหารและเภสัชเคมี ลายมือชื่อนิสิต

สาขาวิชา อาหารเคมีและโภชนศาสตร์ทางการแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

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This study detected tetracycline-resistance (Tc^r) *Enterococcus* spp. in 70 samples of 5 different kinds of ready-to-eat Thai traditional fermented foods (fish, pork, crab, shrimp, and shell). It was found that 28 samples (40%) were contaminated with Tc^r *Enterococcus* spp. All five different kinds of food samples were contaminated with *Enterococcus* spp. except for fermented shell. High percentage of Tc^r *Enterococcus* spp. was found in isolates from fish and shrimp (77.46% and 57.89% respectively). The tetracycline minimum inhibitory concentration (TC MIC) of *Enterococcus* spp. was found between 16 and 512 µg/ml. Most of the enterococcal isolates had TC MIC in the range from >64 to 128 µg/ml, except for isolated from crab that had MIC in the range from >128 to 256 µg/ml. The high level of TC MIC (>256-512 µg/ml) was only found in isolated from fish and crab. The clinically relevant *Enterococcus* spp. were also found including 46.15% *E. faecalis* and 35.58% *E. faecium*. *E. faecalis* was found only in fermented fish and pork but *E. faecium* was found in all 4 different kinds of fermented food. The results from this study indicated that some ready-to-eat Thai traditional fermented foods can be reservoir for transmission of antibiotic resistant enterococci or antimicrobial determinant which may cause serious health problem. In order to prevent this problem, the reduction of enterococcal contamination in food should be implemented including heat inactivation, properly cooked before consumption as well as good sanitation and hygiene practice in preparation of foods. Moreover, restriction of using antibiotic in animal feed and aquaculture should be in consideration.

Department : Food and Pharmaceutical Chemistry Student's Signature .....
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bp	base pair
ºC	Degree Celcius
DNA	Deoxy ribonucleic acid
h	hour
g	gram
µg	Microgram (10^{-6} gram)
µl	Microlitre (10^{-6} litre)
M	Molar
mg	Milligram (10^{-3} gram)
min	Minute
ml	Millitre (10^{-3} litre)
mM	Millimolar (10^{-3} molar)
mm	Millimetre (10^{-3} metre)
PCR	Polymerase chain reaction
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background and significance of the study

Food is a basic human need to sustain life and is important for human health. Food starvation, taking uncleaned food, and taking less nutrients cause illness, and disease, and may be a potential life-threatening. The royal Thai government attached significance to the importance of food safety. Therefore, they also assigned food safety to be the principal policy of the country (ศูนย์ปฏิบัติการความปลอดภัยด้านอาหาร, 2548). It was comprehensively implemented by all related sectors at both the central and provincial levels. Then, food safety policy was pursued and action plan was for the promotion of food safety and appropriate food pattern. The objectives of food safety are to assure the safety of food available in Thailand and to assure that the standard of food available in the country and export are comparable to or comply with international standards. Food safety as defined by Codex Alimentarius Food Hygiene Basic Texts (FAO/WHO, 1997), means food that will not cause harm to the consumers when it is prepared and/or eaten according to its intended use. Food safety hazard as defined by Codex, means a biological, chemical or physical agent, or condition of food with the potential to cause an adverse health effect. Food safety hazards include bacteria,

viruses, parasites, hazardous chemicals and foreign materials that can cause an adverse health to a customer (FAO/WHO, 2001). Types of bacteria in biological hazard include many groups such as *Salmonella* spp., *Staphylococcus aureus*, *Enterobacter sakazakii*, *Clostridium perfringens*, *Listeria monocytogenes, Enterococcus* spp., *Mycobacterium avium* subsp *paratuberculosis, Vibrio cholera, Vibrio vulnificus* (Lawley, Cuitis, and Davis, 2008).

Enterococcus spp. is one of the composition of biological hazard. It is the normal flora of the human and animal gastrointestinal tracts. It is expelled to the environment by human faces and animal ejecta. Also, *Enterococcus* spp. can be found in environment such as soil, water, fecal sewage and foods (ภัทรชัย กีรติสิน, 2549). *Enterococcus* spp. has been used in food industries and also could be found in a variety of food products, such as milk and cheese, meat and vegetables because of their role in ripening and flavor development (Giraffa, 2003). Moreover, it has been used as probiotics, especially *E. faecalis* and *E. faecium*. *E. faecium* SF68 is effective in the prevention of antibiotic-associated-diarrhea and has reduction of the duration of diarrhea in humans. In animals, *E. faecium* SF68 has also been used as feed probiotic (Foulquie-Moreno et al. 2006). In addition, *Enterococcus* spp. has ability to inhibit unconsidering bacteria by producing bacteriocins, which are substances that act similarly to antibiotic (Ogier and Serror, 2008). Bacteriocins produced from

Enterococcus spp. are called enterocins (Franz, Holzapfel, and Stiles, 1999). According to several studies, enterocins played the role in inhibition of bacterial growth such as *Listeria monocytogenes, Staphylococcus aureus, Clostidium botulinum, Clostidium perfringen, Vibrio cholera* (Giraffa, 1995; Franz, Schillinger, and Holzapfel, 1996; Nunez *et al*., 1997; Sarantinoupoulos *et al*., 2002b).

Although *Enterococcus* spp. is useful in food industries, in recent time many countries concern about these microbes as it was identified as the cause of a continuously increasing number of hospital and community acquired infections (Franz *et al*., 1999). In 2002, the study of 24 hospitals in Thailand indicated that *Enterococcus* spp. was the cause of diseases (58.2% community-acquired infections and 41.8% hospital-acquired infections) (สมหวัง ด่านชัยวิจิตร และคณะ, 2548). The diseases caused by *Enterococcus* spp. are opportunistic infections. The species of *Enterococcus* spp. that often causes diseases includes *Enterococcus faecalis* (80-90%) and *Enterococcus faecium* (5-15%) (Aarestrup, Butaye, and Witte. 2002; Hancock and Gilmore, 2006). They cause endocarditis, bacteremia, pelvic infection, intra-abdominal infection, urinary infection, and surgery wound infection. The real mechanisms of infection have remained unknown (Lolekha, Ratanaubol, and Manu, 1981; Poyart-Salmeron *et al*, 1992; Dunny, Leonard, and Hedberg, 1995; Peters *et al*, 2003; Esteban and Barbara, 2006). Some mechanisms proposed were ability of the bacteria to adhere to host tissue, invade and

abscess formation, modulation of host inflammatory responses, and secretion of toxic products (Aarestrup *et al*, 2002).

The other important public health problem caused by *Enterococcus* spp. is antibiotic resistance. This could cause bad therapeutic outcomes such as failure to treat infection with antibiotics, limited option in treatment, delayed treatment, increases in mortality, morbidity, and length of hospital stay, and high cost of health care. Therefore, the control and prevention of antibiotic resistance should be concerned. The antibiotic resistance in *Enterococcus* spp. may be caused by unnecessary use of antibiotics in humans, use of low-dose antibiotics in animal feeds, antibiotic misuse by health care professionals, patient failure to follow prescribed course of treatment, and eating raw/undercooked foods or foods that have contaminated with antibiotic-resistance *Enterococcus* spp. The antibiotic-resistance *Enterococcus* spp. not only causes the problem of infection in hospital increasingly, but also transfers the antibiotic-resistance properties to other organisms (Smith and Coast, 2002). According to several studies, it was found that *Enterococcus* spp. could transfer antibiotic resistance genes to other pathogenic bacteria, such as *Listeria monocytogenes* (Courvalin, 1994), *Neisseria* spp., *Escherichia coli* (Clewell, 1990). The antibiotic resistance properties of *Enterococcus* spp. could be divided into two types including intrinsic resistance and acquired resistance. *Enterococcus* spp. exhibits intrinsic resistance to aminoglycosides, β-

lactams, trimethoprim-sulfamethoxazole, quinupristin-dalfopristin, glycopeptides (van c type) while it exhibits acquired resistance to tetracyclines, macrolides, quinolones, glycopeptides (van A type, van B type), aminoglycosides (Dunny *et al*, 1995). Tetracycline is one of antibiotics, which was resistant by *Enterococcus* spp. (Borgo *et al*, 2009). It has been widely used in drug stores and hospitals because it is the broad-spectrum antibiotic (Chopra and Roberts, 2001). It affects against both gram-positive and gram-negative bacteria, as well as chlamydiae, mycoplasma, rickettsia, protozoan. It can also prevent malaria infection by *Plasmodium falciparum* that is resistant to mefloquine (Taylor and Chau, 1996; Thapa *et al*., 2007). Furthermore, it was also used as a growth promoter for feeding animal (Robert, 1996).

Foods can be a potential source for transmission of the resistant phenotype of bacteria to humans. Nowadays, there are several studies which show the occurrence of antibiotic-resistance *Enterococcus* spp. isolated from several kinds of foods in many countries. Huys *et al*. (2004) found tetracycline-resistance *Enterococcus* spp. from pork, chickens, beefs and other poultries. In 2000, Aarestrupa *et al.* found tetracycline-resistant *E. faecalis* and *E. faecium* in human food animals, such as poultry and pigs. In 2005, Wilcks, Andersen, and Licht investigated the raw foods in Denmark, such as chicken meat, poultry meat, beef, pork and found tetracycline-resistant *E. faecalis* in these food samples.

the serious problem of antibiotic-resistance *Enterococcus* spp., many countries begin increasing concerns. In 2004, the government of Canada prohibited the probiotic supplements containing *Enterococcus* spp. in human (Ogier and Serror, 2008). European legislation also has limitation for the level of *Enterococcus* spp. in drinking water (Lawley *et al*., 2008).

In Thailand, according to Food Act B.E. 1979 with ministerial health regulations and announcement (Revised edition 2010) the law has only stipulated the quantity of some pathogenic microorganisms in Thai foods, but *Enterococcus* spp. has not been restricted or prohibited in Thai foods. However, the studies on antibiotic-resistant *Enterococcus* spp. in Thai foods are still limited. A few studies on this area were reported. Tanasupawat *et al* (1992) found *Enterococcus* spp. in Thai fermented foods, but antibiotic susceptibility was not studied. Tansuphasiri, Khaminthakul, and Pandii (2006) found tetracycline-resistance *Enterococcus* spp. isolated from Thai water and frozen foods.

The present study try to expand such information by investigating the occurrence of tetracycline-resistance *Enterococcus* spp. isolated from common ready-to-eat Thai traditional fermented foods. The information on antibiotic-resistance of these microbes will provide the safety information for consumption of some ready-to-eat Thai traditional fermented foods. In addition, it may be a guide for the administrative agency

to plan, and execute the surveillance of antibiotic resistance microbial contaminated in food, and also make consideration for the allowance of some *Enterococcus* spp. in food processing.

1.2 Objective of the Study

The research aimed to investigate the occurrence of tetracycline-resistance *Enterococcus* spp. in some ready-to-eat Thai traditional fermented foods.

1.3 Operational Definition of Terms

According to Food Act B.E. 1979, Food means edible items and those, which sustain life

(1) Substance can be eaten drunk, sucked or gotten into the body either by mouth or by other means, no matter in what form, but not including medicine, psychotropic substances, narcotic under the law as the case may be,

(2) Substance intended for use or to be used as ingredients in the production of food including food additive, colouring matter and flavouring agent.

Antimicrobial resistance (FAO/WHO, 2001) is the ability of microbes, such as bacteria, viruses, parasites, or fungi, to grow in the presence of a chemical (drug) that would normally kill it or limit its growth.

1.4 Benefits of the study

It is expected that the information obtained from this study will provide safety concern for the consumer to avoid eating undercooked fermented food which may be contaminated with antibiotic-resistant potential pathogenic *Enterococcus* spp. Furthermore, this should provide information to health regulatory authority to restrict the use of antibiotic as a growth promoter or control improper use of antibiotic to prevent the problem of antibiotic resistance among pathogenic bacteria.

CHAPTER II

LITERATURE REVIEWS

2.1 Food Safety

Food safety was assigned by the governmental agencies collaboration with the food industries and consumers in order to control the specific hazards possibly associated with food ingredients or food products (McWilliams, 1997; Gorris, 2005). So it has a constant vigilance throughout the entire sequence by all those involved in raw materials, production, processing, and distribution (ศูนย์ปฏิบัติการความปลอดภัยด้านอาหาร, 2546-2548). Moreover, this scheme also has settled the food safety management international systems, such as Good Manufacturing Practice (GMP), Hazard Analysis and Critical Control Point (HACCP) systems, and International Organization of Standardization (ISO) 9000-22000 (McWilliams, 1997). In Thailand, the vision of food safety was proposed and the national policy has been set since 2004. The objective is to improve the standard of food products that are produced and distributed in the country to have quality and be safe for consumers. The importance of proper food safety management is a decrease in the risk of health illness called foodborne illness.

Food hazard can be caused by physical agents e.g. glass fixtures, wood, stones, chemical agents, and biological agents. The biological agents include microorganisms, virus, parasites, bacteria, especially pathogenic bacteria (FAO/WHO,

2001a), such as *Salmonella* spp., *Staphylococcus aureus*, *Enterobacter sakazakii, Clostridium perfringens*, *Listeria monocytogenes, Enterococcus* spp., *Mycobacterium avium* subsp *paratuberculosis*, *Vibrio cholera*, and *Vibrio vulnificus* (Lawley *et al*., 2008) (Table 1).

Table 1 Bacteria recognized as biological hazard (Lawley *et al*., 2008)

Type of biological hazard bacteria	
Aeromonas	*Pseudomonas aeruginosa*
Arcobacter	Salmonella
Bacillus spp.	Shigella
Campylobacter	*Staphylococcus aureus*
Clostridium botulinum	Streptococci
Clostridium perfringens	Verocytotoxin-producing *Escherichia coli (VTEC)*
Enterobacter sakazakii	*Vibrio cholerae*
Enterococci	*Vibrio parahaemolyticus*
Listeria monocytogenes	*Vibrio vulnificus*
Mycobacterium paratuberculosis	
Plesiomonasshigelloides	

2.2 *Enterococcus* spp.

Enterococcus spp. is a non-spore-forming gram-positive bacteria, facultatively anaerobic cocci that grow in pairs or short chains (Esteban and Barbara, 2006). The enterococcal cells are spherical or ovoid (Hardie and Barbara, 1997).

2.2.1 Physical and biochemical properties of *Enterococcus* spp.

Enterococcus spp. is verified as the most tolerant non-sporing bacteria in environment (Franz *et al*., 1999). It is able to grow as low as 10°C, 45°C and as high as 60°C for 30 min. It can grow in the presence of 6.5% sodium chloride, at pH 9.6, and in 40% bile salts, and it is capable of hydrolyzing esculin in the presence of 40% bile (Brown, Peterson, and de la Maza *et al*., 1983; Hancock and Gilmore, 2006). Furthermore, it has been reported that this genus cannot produce a catalase reaction with hydrogen peroxide (Esteban and Barbara, 2006).

Some physical and biochemical properties of *Enterococcus* spp. which are different from other bacteria can be applied to select *Enterococcus* spp. The differentiation of *Enterococcus* spp. from other gram-positive bacteria based on some properties is presented in Table 2. Moreover, the selection of *Enterococcus* spp. which is gram-positive bacteria from other gram–negative bacteria can be done by selective growing in media that contains 0.01-0.05% of sodium azide (Narang, 2004).

Because *Enterococcus* spp. is being able to tolerate the environment conditions, such as oxidative stress, desiccation, high and low temperatures, a wide range of pH (Meropol and Metlay, 2008) and *Enterococcus* spp. is usual inhabitants of the gastrointestinal (GI) tracts of warm-blooded animals such as human. Therefore, the presence of enterococci in environment could be from the spread through fecal sewage transmission. *Enterococcus* spp. occur in many different habitats, such as soil, water,

fecal sewage and foods, especially foods from animal origins e.g. cow, pig, poultry, fish and fermented foods because the pH of these foods are suitable for the growth of lactic acid bacteria e.g. *Enterococcus* spp.

Table 2 Some conventional methods for differentiating gram-positive organisms from *Enterococcus* spp.

Genus of gram-positive organisms	Condition of growth		
	6.5% NaCl	45°C	10°C
Enterococci	+	+	+
Viridans streptococci	-	V-	-[a]
Lactococci	V+	V-	+
Aerococci	+	-	-
Pediococci	V-	V+	_
Leuconostocs	V+	-	V+
Lactobacilli	V-	V+	+
Gemella	-	-	-

-,most (>90%) strains showed a negative reaction.

+,most (>90%) strains showed a positive reaction.

V-, more than half of the strains showed a negative reaction.

V+, more than half of the strains showed a positive reaction.

-[a], most strains occasionally showed a positive reaction.

Source: adapted from Facklam*et al* (1999); Esteban and Barbara (2006)

Recently, there are more than twenty species of Enterococcus (Gomes, 2010). *Enterococcus faecalis* and *Enterococcus faecium* are common found in GI tract

of human, averaging 10^5-10^7 colony-forming units (CFU/g) and 10^4-10^5 CFU/g respectively (Esteban and Barbara, 2006).

2.2.2 Use of *Enterococcus* spp. in food processing

Enterococcus spp. is one of the lactic acid bacteria and produces typical aroma compounds which contribute to their typical taste, flavor in fermented foods. For this reason, *Enterococcus* spp. has also been used as starters in the manufacture of fermented foods, such as fermented meats (bologna, salami, landjager), cheeses where it often predominate at the end of the ripening period (artisanal cheeses, cebreiro, kefalotyri, manchego, picante da beirabaixa and teleme (Giraffa, 2003; Foulquie Moreno *et al*., 2006). Furthermore, enterococcal products, such as lactic acid, hydrogen peroxide, rutein, especially bacteriocin may considered as a food preservative because they are able to inhibit the growth of certain pathogens and spoilage microorganisms (Ogier and Serror, 2008; Gomes *et al*., 2010) such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium perfringens*, and *Vibrio cholera* (Giraffa, 1995; Franz, Schillinger, and Holzapfel, 1996; Nunez *et al*., 1997; Sarantinoupoulos *et al*., 2002b).

Enterococcus spp. is considered as probiotics. .According to the current definition by the FAO/WHO, probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). *Enterococcus* spp. have the ability to support a beneficial balance in the

microbial population of the gastrointestinal tract (Holzapfel *et al*., 1998) by producing organic elements bacteriocin and hypothiocyanate, which can inhibit unconsidering bacteria e.g. *Listeria monocytogenes* (Ogier and Serror, 2008). *Enterococcus* spp. has also been utilized in production of probiotics in pharmaceutical preparations [e.g. *E. faecium*SF68® (NCIMB 10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland) and *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany)], animal feed additives, and food products (Franz *et al*., 2011).

2.2.3 Hazards from *Enterococcus* spp.

Enterococcus spp. has a variety of benefits, whereas it is not generally recognized as safe (GRAS) like other lactic acid bacteria (Ogier and Serror, 2008). It is classified as one of biological hazards (Lawley *et al*., 2008). *Enterococcus* spp. has become a major cause of hospital and community acquired infections (Franz *et al*., 1999) and can develop antibiotic resistance. The increased resistance of *Enterococcus* spp. to antibiotics poses major therapeutic problem to patients and the treatment of infection could be difficult. Besides, this serious problem could affect the treatment of other pathogenic bacteria such as *Listeria monocytogenes* (Courvalin, 1994), *Neisseria* spp*, Escherichia coli* (Clewell, 1990) because *Enterococcus* spp. has ability to transfer resistance genes to others.

The increasingly continued hazards of *Enterococcus* spp. make several countries watch over the hazards of *Enterococcus* spp. and some countries legislate to

ensure the safety. For example, the government of Canada has prohibited the probiotic supplements in human that contain *Enterococcus* spp. (Ogier and Serror, 2008). European legislation also includes the control for the level of *Enterococcus* spp. in drinking water (Lawley *et al*., 2008).

1) *Enterococcus* spp. as opportunistic pathogens

Enterococcus spp. is usual inhabitants of the gastrointestinal tracts of human. However, *Enterococcus* spp. can become pathogenic bacteria which cause hospital and community acquired infections in kind of opportunistic pathogens. These pathogens cause infection in immunocompromised or intensive care unit patients. The National Nosocomial Infections Surveillance (NNIS) system found that *Enterococcus* spp. was the third most common pathogens causing nosocomial infections (Fraser, 2011), the second most causing urinary tract infection in United States and Europe, and the third most causing bloodstream infections in United States and the fourth in Europe (Ogier and Serror, 2008). Furthermore, *Enterococcus* spp. also cause intra-abdominal infection, endocarditis, infection after cataract surgery. So the source of enterococcal infections in most cases has been thought to be the endogenous flora (Moellering, 1990; Murray, 1990), which may spread through food and water consumption. Several studies found that the most common clinically relevant enterococci species are *E. faecalis* (80-90%) and *E. faecium* (5-15%) (Ogier and Serror, 2008). Both strains have increasingly been studied.

In Thailand, สมหวัง ด่านชัยวิจิตร และคณะ (2005) explored the prevalence of enterococcal infection in 24 hospitals and found that 41.8% of hospital acquired infection and 58.2% of community acquired infection caused by *Enterococcus* spp. Furthermore, Lolekha *et al*. (1981) examined the microorganisms that caused infection in Ramathibodi Hospital and found that *Enterococcus* spp. caused concern to urinary tract infection, surgical site infection, respiratory tract infection, and bacteremia.

In non-severe enterococcal infection, such as urinary tract infection, uncomplicated wound infections, most intra-abdominal infections, the monotherapy was recommended. The antibiotic for treating susceptible enterococcal infection was penicillin or ampicillin. In case of allergic reaction to penicillin, vancomycin was recommended (ภัทรชัย กีรติสิน, 2549). If the enterococcal strain resisted to ampicillin or penicillin, ampicillin plus sulbactam or vancomycin may be used. In a case of vancomycin-resistant enterococcal (VRE) strains, the recommended medicines were nitrofurantoin, linezolid (oxazolidinones group), daptomycin, and tigecycline (Fraser, 2011).

In severe enterococcoal infections, such as sepsis, endocarditis, meningitis, osteomyelitis, the combination therapy with a cell wall–active agent and a synergistic aminoglycoside should be recommended. A cell wall-active agent that is considered are penicillin, ampicillin or vancomycin or quinopristin-dalfopristin (streptogramins group), linezolid, daptomycin, tigecycline. Aminoglycoside that is

considered are gentamicin, streptomycin (ภัทรชัย กีรติสิน, 2549; Fraser, 2011). In very severe enterococcal infection or high level resistance, doxycycline, chloramphenicol, rifampicin should be added together in the treatment regimen (Heuer *et a*l., 2006; Fraser, 2011).

2) Antibiotic resistance

Antibiotic resistance has become a crucial public health concern. It decreases the effectiveness of antibiotic to treat the infections, causes the complications, increases the morbidity and mortality associated with infections, and contributes substantially to rising costs of care resulting from prolonged hospital stays and the need for more expensive medicines. Furthermore, the problem from antibiotic resistance is the difficulty of treating the infections and choosing the good-response medicine. Consequently, pathogenic microorganisms will spread antibiotic resistant genes to the environment (Cohen, 1992; Flaherty and Weinstein, 1996; Gold and Moellering, 1996).

Most of previous studies that investigated antibiotic resistance have focused on the resistance of true pathogen that cause hospital acquired infection. Recently, several studies have increasingly studied on antibiotic resistance of opportunistic pathogen, especially human commensal bacteria, such as *Enterococcus* spp., which is the inhabitants of the gastrointestinal tracts and found that it can be the reservoirs of antibiotic resistance genes (Mathur and Singh, 2005).

The cause of antibiotic-resistant *Enterococcus* spp. in human may originate from unsuitable use of medicines, non-heated foods, non-cooked foods and the use of antibiotic growth promoter (AGP) in animals. AGP may cause animals to be a reservoir of antibiotic-resistant bacteria and it can spread the resistance genes to human from food animals and food of animal origin (Witte, 1997). Most medicine used in animals are the same types as human use, so the therapeutic problems occur.

The transferable pattern of genetic determinants of resistance in *Enterococcus* spp. is transferred between bacteria which is the same species or other species such as the transferable gene transfer from *E. faecalis* to *Staphylococcus aureus*, and *Listeria* spp. (Biavasco *et al*., 1996; Mathur and Singh, 2005). However, these genetic determinants in human only transiently colonize the human gut because *Enterococcus* spp. in general does not colonize for extended periods of time in both intestinal tracts of human and animals (Heuer *et al*., 2006).

In 1940, using of antibiotic as growth promoter in animals such as tetracyclines group was discovered and in 1953, chlortetracycline and oxytetracycline were approved from US-FDA to be used in animal feed additives (มาลินี ลิ้มโภคา, 2540). Using of AGP in animals not only causes a left-over antibiotic in animals, but also causes antibiotic resistance determinant which can cause antibiotic resistance problem in humans. The consequence of using antimicrobial agents in food animals is illustrated by the evidence of an association between the use of gentamicin in food animals

particularly chickens and turkeys in the United States and high level gentamicin resistant *Enterococcus* spp. in humans (Donabedian, Thal, and Hershberger, 2003). In 1969, United Kingdom have prohibited using of AGP in animal feed additives. In 1971, Europion Union have prohibited using of AGP in animal feed additives too.

The antibiotic resistance of *Enterococcus* spp. may be divided into two types, intrinsic and acquired. Intrinsic or inherent resistance refers to naturally occurring, chromosomally encoded characteristics encountered in all or almost all of the strains of a particular species. Acquired resistance is caused by mutation in the existing DNA or acquisition of new DNA. The patterns of antibiotic resistance in *Enterococcus* spp. are shown in Table 3.

Table3 Patterns of antibiotic resistance in *Enterococcus* spp.

Intrinsic resistance	Acquired resistance
- Aminoglycosides(low level) (MICs ≤ 2,000 µg/ml)	- Tetracyclines
- β-Lactams	- Aminoglycosides (high level) (MICs ≥ 2,000 µg/ml)
- Trimethoprim-sulfamethoxazole	- Penicillins
- Quinupristin-dalfopristin	- Quinolones
- Glycopeptides (Van C type)	- Macrolides, lincosamides
	- Streprogramins B(MLS_B group)
	- Glycopeptides
	- Chloramphenicol
	- Oxazolidinones (linezolid)

Source: adapted from Facklam *et al* (1999); Esteban and Barbara (2006)

Tetracycline is an ancient antibiotic group, which composes of many kinds of medicines such as tetracycline, doxycycline, minocycline, oxytetracycline, tigecycline (Chopra and Roberts, 2001). This antibiotic group has broad spectrum and was popularly accepted to be used as an antibiotic growth promoter (AGP) in animals. Also, this group is interesting to study regarding antimicrobial susceptibility, particularly in *Enterococcus* spp. because it is a vital opportunistic pathogen. Sader *et al*. (2005) investigated pathogenic bacteria in intensive care unit patient and they found that *Enterococcus* spp. was the crucial pathogen and resistant to nine out of eleven antibiotics. Tetracycline was the second most antibiotic that found resistance (60.6%).

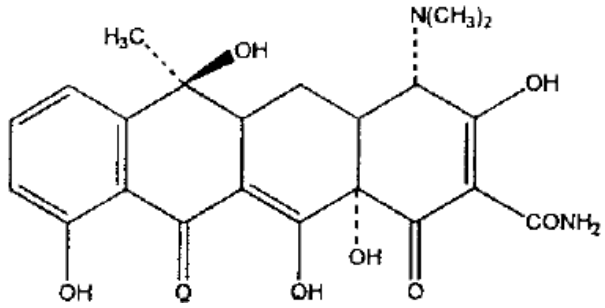
2.3 Tetracycline

In 1953, tetracycline was discovered. It has activity against a wide range of gram-positive and gram-negative bacteria. It was useful for treatment of infection with *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*, which were virulent pathogen by monotherapy or combination with other antibiotic groups. It was used for treating the infection of periodontal disease, acne and preventing malaria infection by *Plasmodium falciparum* which resisted to mefloquin. In clinical practice, tetracycline was the first choice of medicine to treat the infection of atypical organisms such as rickettsiae, mycoplasmas, chlamydiae. Furthermore, it is used for treating the infection of protozoa, ureaplasma.

Tetracycline is a broad-spectrum agent that is effective for inhibiting the growth of bacteria without serious adverse effects. It is used for treatment of human and animal infections, and also used as growth promoters for farm animals (Chopra and Roberts, 2001; ภัทรชัย กีรติสิน, 2549).

2.3.1 Physical properties

The physical appearance of tetracycline is yellow, odorless and crystalline powder. It is stable in air but it is dark when exposes to strong sunlight. The melting point is 170-173°C (with decomposition). The molecular weight of tetracycline is 444.44. It is very slightly soluble in water, freely soluble in dilute acid and in alkali hydroxide solutions, and insoluble in chloroform and ether. The chemical formula of tetracycline is $C_{22}H_{24}N_2O_8$ and the structure is shown in Figure 1.



2-Naphthacenecarboxamide, 4-(dimethylamino) 1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-, [4S-(4a,4aa,5aa,6b,12aa)]

Figure1 Chemical structure of tetracycline (Chopra and Roberts, 2001)

2.3.2 The mode of action of tetracycline

Tetracycline is complexed with a divalent cation in the cytoplasm e.g. Mg^{2+}, then tetracycline complex diffuses into the cells. Tetracycline binds reversibly to 30S sub-unit of the bacterial ribosome at acceptor site (A-site), then the ribosome changes the conformational state. In protein synthesis stage, the aminoacyl-tRNA also can not bind to the acceptor site (A-site). The inhibition of specific binding of aminoacyl-tRNA to ribosomes leads to blocking of protein synthesis by interfering protein elongation cycle in translation process. So, the growth of bacteria was inhibited (Meyers and Salvatore, 2005; ณัฐวุธ สิบหมู่, 2552).

2.3.3 The mechanism of tetracycline resistance

Tetracycline resistance is mediated by two major mechanisms, protection of the binding of ribosome by ribosomal protein and reduction of intracellular concentrations of tetracycline by energy-dependent efflux of tetracycline (Roberts, 2003).

1) Protection against the binding of ribosome by ribosomal protein

The ribosomal proteins, which are produced by tetracycline resistances gene such as *tet*(M), *tet*(O), *tet*(S), *tet*(P), *tet*(Q), *tet* (W), can block protein synthesis by interaction with the ribosome at the base of h34 protein. This interaction cause the primary tetracycline binding site having an allosteric disruption, releasing tetracycline molecules from the ribosome and returning the form of ribosome to normal post

translocational conformational state (Connell *et al*., 2003). So, the protein synthesis restoration is occurred.

2) Reduction of intracellular concentrations of tetracycline by energy-dependent efflux of tetracycline

The second major way to cause tetracycline resistance is the efflux pump. This pump excretes tetracycline out of cell at a rate equal to or greater than its uptake. So the accumulation of tetracycline is decreased, causing limitation of access to bind ribosome. The efflux pump, energy-dependent tetracycline transporter or cytoplasmic membrane protein are generated by the resistance genes such as *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y), *tet*(Z), *tet*(K), *tet*(L), *tet*(O), *tet*A(P), *tet*(30), *tet*(31), *tet*(33), *tet*(35), *tet*(38), *tet*(39) (Speer *et al*., 1992). Almost of the resistance genes found in gram-negative bacteria, except for *tet*(K) and *tet*(L) gene that have been found only in gram-positive bacteria (Meyers and Salvatore, 2005). *Tet*(K) was most found in *Staphylococcus* spp. and *tet*(L) was more likely found in *Streptococcus* spp. and *Enterococcus* spp. (Bismuth *et al*., 1990).

3) Other mechanisms

Other possible antibiotic resistance mechanisms are the enzymatic inactivation and the change of target site of tetracycline.

Tetracycline resistance in *Enterococcus* spp. not only cause the problem of using tetracycline antibiotic, but also affects on other medicines in tetracycline group such as oxytetracycline and doxycycline, which are similar to tetracycline structures. Moreover, Cauwert *et al*. (2007) found that tetracycline-resistant *Enterococcus* spp. was related to the resistance to other antibiotic groups such as erythromycin. This study found that 89% of erythromycin-resistant *Enterococcus* spp. resisted to tetracycline.

2.4 *Enterococcus* spp. and tetracycline resistance

Most tetracycline-resistant *Enterococcus* spp. studies often focuse on *E. faecalis* and *E. faecium*. Because both species are important strains related to infections in human (Huycke, Sahm, and Gilmore, 1998, Franz *et al*., 2003). In 2000, Aarestrupa *et al* found the tetracycline-resistant *E. faecalis* and *E. faecium* in human food animals such as poultry and pigs. In 2005, Wilcks *et al*. investigated the raw foods in Denmark such as chicken meat, other poultry meat, beef, and pork, and they found tetracycline-resistant *E. faecalis* too. Furthermore, Poeta *et al.* (2006) studied on contamination of *Enterococcus* spp. in poultry sewage. They also found the contamination of tetracycline-resistant *Enterococcus* spp. in poultry sewage (97%).

In Thailand, Lertworapreecha, Poonsuk, and Chalermchiakit (2011) studied antimicrobial resistance in *E. faecium* from traditional chicken. This research isolated *E. faecium* from traditional chicken and found that most of the *E. faecium* isolated strains were resistant to tetracycline. This study also found resistance to other antibiotics from

E. faecium, such as erythromycin and cefotaxime. At present, the studies about antibiotic-resistant *Enterococcus* spp. in Thai foods are limited. Tanasupawat *et al* (1992) only found *Enterococcus* spp. in Thai fermented foods, but the antibiotic susceptibility was not studied. Tansuphasiri *et al*. (2006) also found tetracycline resistant *Enterococcus* spp. isolated from water (46.8%) and frozen foods (64.1%).

2.5 The methods for detection and identification of bacterial contamination in foods

There are several methods for the detection of bacteria. All of these methods can be classified into two types, the traditional methods and molecular-based methods. The traditional methods are less sensitive, show viable organisms and are suitable for detecting a lot of organisms. However, they take a relatively long time, labor intensive and the identification is often not as reliable as desired. Molecular-based methods are sensitive, quite specific, relative accurate, less labor intensive and reliable, but often reveal dead or inactivated target organisms after tests (Koster, 2003). However, only one of any methods is not enough to identify the bacteria, so several methods should be used to confirm the accurate identification.

2.5.1 The traditional methods

The traditional or conventional methods for bacterial examination are broadly grouped as quantitative and qualitative methods. The quantitative methods are designed to enumerate directly or indirectly the bacterial load in food samples, while the

qualitative methods are designed to determine the possible bacteria contamination in food samples.

1) Quantitative methods

Quantitative methods are broadly classified as direct enumeration and indirect enumeration. First, direct enumeration methods were suitable for the food samples that have a less numbers of bacterias. The example of direction enumeration methods are microscopic counts, colony-forming units (CFUs) in nonselective agar media, nonselective differential media, selective agar media, and selective-differential agar media. Second, indirection enumeration methods were used widespread. The example of indirect enumeration methods are dilution to extinction in nonselective broths, most probable number (MPN) in selective broth, dye reduction test, and enumeration of bacterial groups by selective media. The selection of methods relies on the objective of the research, the group of bacteria and the type of food.

2) Qualitative methods

The objective of qualitative method is only to find out the specific bacteria in food samples without determination of the number of bacteria. The isolation of specific bacterial generally contains several steps such as nonselective pre enrichment, followed by selective enrichment, and then testing on a selective media or differential selective media.

2.5.2 Molecular-based methods

The molecular-based methods can overcome disadvantage of the traditional methods. There are many different rapid methods and many of which are automated. They are specific, and suitable for finding delicate information of bacteria more than the traditional methods. The detection of species of bacteria is more accurate when detect the target genes which specify the species of bacterial strain are known by molecular-based methods. In addition, the mechanisms of antibiotic resistance can be detected from antibiotic resistance genes, which are stretches of DNA coding for an altered antibiotic target, antibiotic efflux, antibiotic inactivation or others.

The molecular-based methods that detect the target genes include hybridization, restriction, and amplification. Hybridization is the reaction that nucleic acid fragments in the reaction bind together to form a double-stranded molecule. One fragment is composed of the denatured bacterial DNA target and the other is a probe that is the nucleic acid fragment. The probe is a synthetic oligonucleotide which is a cloned DNA fragment. The hybridization is visualized by colorimetric, fluorescent, or luminescent methods. Restriction is a reaction that uses the restriction endonucleases as a principle. The restriction endonucleases are enzymes that recognize short specific DNA sequence and cleave double stranded DNA. The function of the enzymes is to digest a specific DNA molecule to a DNA fragments. In the detection of target genes, many of the specific restriction endonuclease enzymes may be used depending on the

specific target genes. The visualization of these fragments is electrophoresis, hybridizing with a specific probe (Southern method).

Amplification is a reaction which the chosen nucleic acid sequence (DNA or RNA) is repeatedly copied. The polymerase chain reaction (PCR) is a techinique widely used in molecular biology. PCR reaction is composed of DNA templates, two oligonucleotide primers that bind to the target sequence, PCR ingredients such as PCR buffer, dNTP, $MgCl_2$, and DNA polymerase enzyme. PCR amplification of DNA consists of three steps: denaturation, annealing and extension. The visualization of target PCR product often uses electrophoresis method. The advantages of PCR method are high sensitivity, selectivity, specificity, rapidity, and ability to detect several genes in suitable PCR condition at one time (duplex PCR, multiplex PCR) (Koster, 2003). Several methods can also be combined, for example, amplified fragment length polymorphism (AFLP) which is the method using the selective amplification of restriction fragments (Koster, 2003).

2.6 Antimicrobial susceptibility methods

Antimicrobial susceptibility test is the measurement of ability of antibiotics or other antimicrobial agents to inhibit bacterial growth or kill the test organisms *in vitro*. The test is interpreted by comparing the result with international standard criteria defined by the government sector. Clinical and Laboratory Standards Institute (CLSI) or The National Committee for Clinical Laboratory Standards (NCCLS) is the standards-

developing organization in USA which was set up more than forty years ago. The CLSI gauge was applied widespread. Europian Committee on Antimicrobial Susceptibility Testing (EUCAST), another standard government sector, was established in 2002 and used in Europe area such as France, Germany, Sweden, Netherlands, and the United Kingdom (Kahlmeter *et al*., 2006). The standard criteria to interpretation of these two government sectors are similar, but the methods for test may be different. There are several methods for antimicrobial susceptibility testing. The standard methods used now are divided into two types, diffusion and dilution. The disk diffusion and broth microdilution are the methods that are popularly accepted to test various microorganisms and antimicrobial agents because both methods are available, convenient and rapid.

2.6.1 Disk diffusion testing

The disk diffusion method is a qualitative susceptibility tests (Craig, 1993). This method categorized most bacterial isolates as susceptible, intermediate, or resistant to an antimicrobial agents (Jorgensen and Turnidge, 2007). It depends on the diffusion of antimicrobial agent from disk through the agar. Initially, the standardized test organism suspension was inoculated in the agar medium by rubbing with the swab containing the inoculums. Following, the antimicrobial discs were applied to the surface of the agar. The drugs in the disk diffused through the agar. As increasing of distance from the disk, the concentration of drugs also decrease logarithmically. After incubation at 37°C, the

results were reported by the diameter of zone inhibition (Figure 2) and the interpretation of susceptibility or resistance were determined by references on the basis of guideline published by the CLSI.

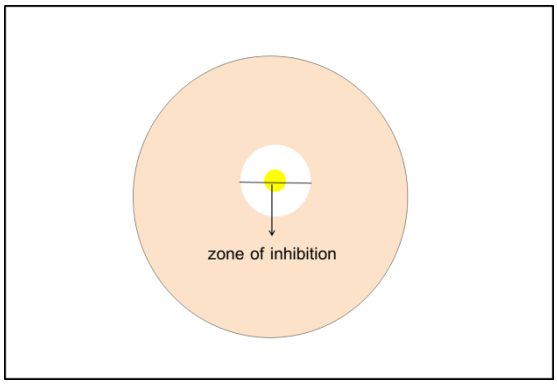


Figure 2 Zone of inhibition

In order to achieve the precision and accuracy of the procedures, the good performance of the persons who do the testing, read, interpret, and report the results, and the good performance of the reagents (medium and disks), the recommended reference strains were used. The kind of reference strains which are used depending on the test organisms. For example, according to the quality control of CLSI recommended test, *Staphylococcus aureus* ATCC 25923 is reported as the reference strain for testing *Enterococcus* spp. (ภัทรชัย กีรติสิน, 2549; Jorgensen and Turnidge, 2007; CLSI, 2011).

2.6.2 Broth micro dilution testing

The broth micro dilution method is one of the dilution methods, which is a quantitative susceptibility tests (Craig, 1993). This method is now considered the

international reference susceptibility testing method. Because the microdilution trays used in this method are availability of dilution susceptibility testing and affordable (Jorgensen and Turnidge, 2007). The objective of this method is to determine the minimal concentration of antimicrobial agent that can inhibit or kill the selected microorganisms. The antimicrobial agents are usually tested at twofold serial dilutions before the test organisms are inoculated. The lowest concentration of antimicrobial agent to inhibit or kill a microorganism is defined as the minimum inhibitory concentration (MIC). The result is determined by interpreting the MIC on the basis of guideline published by the CLSI. This method is also usually used to test the reference strains, which is reported in each test organism to achieve the quality control. For *Enterococcus* spp., *Enterococcus faecalis* ATCC 29213 is defined as the reference strain testing (ภัทรชัย กีรติสิน, 2549; Jorgensen and Turnidge, 2007; CLSI, 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Samples

Ready-to-eat Thai traditional fermented foods were collected from different markets in Bangkok during March to June 2011. Seventy food samples were divided into five kinds of fermented food including fermented fish (pla-ra, pla-jom, som-fak), fermented pork (nham-moo), fermented crab (poo-dong), fermented shrimp (koung-jom), and fermented shell (hoi-dong). The recipes of each fermented food were shown in Appendix.

3.2 Experimental design

This study focused on the detection of tetracycline resistance in *Enterococcus* spp. isolated from ready-to-eat Thai traditional fermented foods. *Enterococcus* spp. were isolated from food samples by growing on selective media and the confirmation was performed by physical appearance, such as color of isolates and biochemical methods. All isolates identified as *Enterococcus* spp. were tested for tetracycline susceptibility. The isolates with tetracycline resistant phenotype were randomly selected to identify for potential clinically relevant *Enterococcus* spp. (*Enterococccus faecalis*

and *Enterococcus faecium*) by PCR assay. The steps of this study are shown in Figure 3.

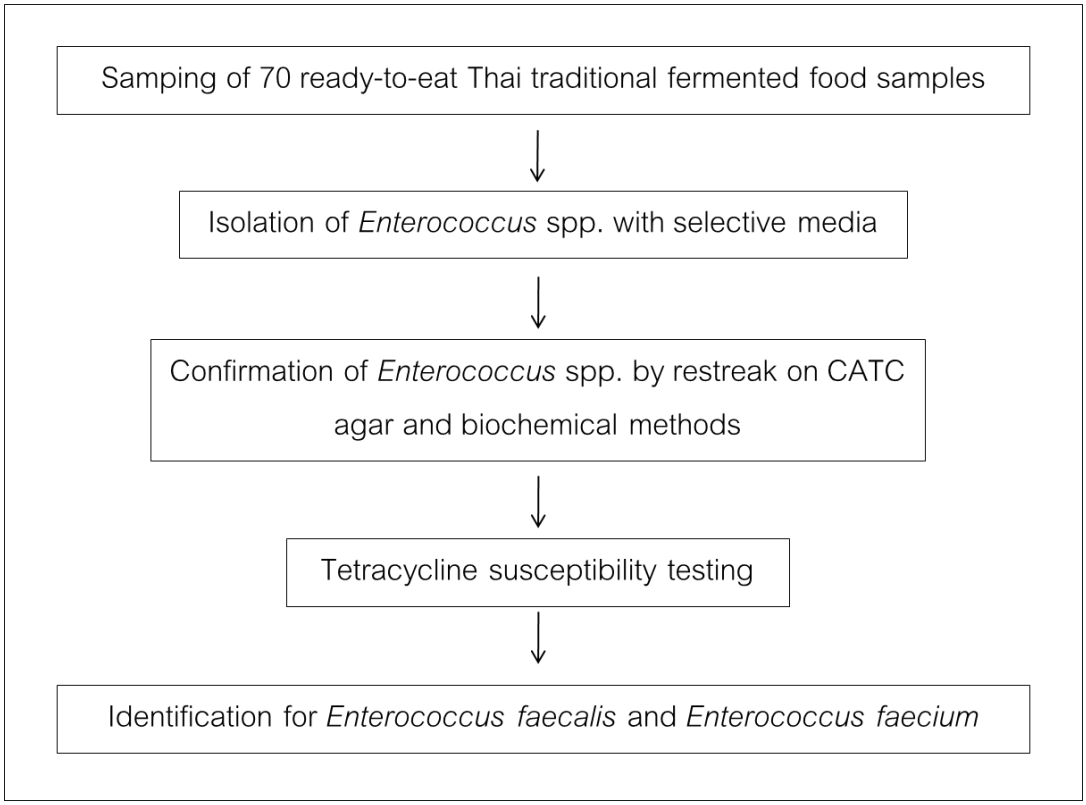


Figure 3 The steps of this study

3.3 Instruments and chemical substances

3.3.1 Instruments

Incubator (POLAR 1000C Incubator, Australia), PCR mastercycler gradient thermal cycler (Eppendorf, Germany), laminar air flow (Astec Microflow ATC 1800N, United Kingdom), autoclave (HA-300MD, Hirayama, United Kingdom), hot air oven (YCO-No 1, Gemmy, Taiwan), centrifuge (SCR 20B Himac Centrifuge, Hiyachi, Japan), pH meter (Mettler Toledo S40 SevenMulti, Switzerland), analytical balance (Mettler

Toledo PL602-s, Switzerland), vortex mixer (Votex-2 Genic, Scientific Industries, USA), spin down (Mini Centrifuge C-1200,National Labnet, USA), water bath (Memmert®, England), gel documentation (Gel Doc XR, Bio-Rad, USA), micropipette (Gilson, France), micropipette tip (Axygen Scientific Inc, USA), microcentrifuge tube (Axygen Scientific Inc, USA), laboratory blender stomacher (Seward Model 400, England).

3.3.2 Chemical substances

Pancreatic digest of casein (Criterion, USA), yeast extract (Lab M, England), potassium dihydrogen phosphate (Merck, Germany), sodium citrate (Merck, Germany), polyoxyethylene sorbitanmonooleate (tween® 80) (Ajax Finechem, Australia), agar-agar (Scharlau Chemie S.A., Spain), sodium carbonate (Univar, New zealand), 2,3,5-triphenyltetrazolium chloride (Fluka, Switzerland), sodium azide (Labchem, Australia), sodium chloride (Univar, New zealand), chemical substances for PCR amplification (Taq polymerase, PCR buffer, $MgCl_2$, dNTP (invitrogen®,Brazil), primer (operon biotechnologies, Germany)), dna ladder (SibEnzyme Ltd., Russia; Invitrogen®,Brazil), agarose gel (Research organics, USA), ehidium bromide 10mg/ml (Fluka, Switzerland), EDTA (Ajax Finechem, Australia), tris (hydroxymethyl)aminomethane (Sigma Algrich Inc., USA), boric acid (Univar, New zealand), lysozyme (Sigma Algrich Inc., USA), tetracycline hydrochloride powder (USB Corporation Cleveland, USA), MRS (Man, Rogosa and Shape) broth powder (Lab M, England), peptone water powder (Criterion, USA), plate count agar (Britania, Argentina), mueller hinton II broth (cation-adjusted)

powder (BD, France), muller hinton agar (oxoid, UK), bromophenol blue (J.T. Baker Inc., USA), tetracycline discs (Oxoid, UK).

3.4 Methods

3.4.1 Collection of food samples and Isolation of *Enterococcus* spp.

3.4.1.1 Collection of food samples

All food samples were kept in tight container in refrigerator (2-8°C) and were not kept more than 48 hours before experiment.

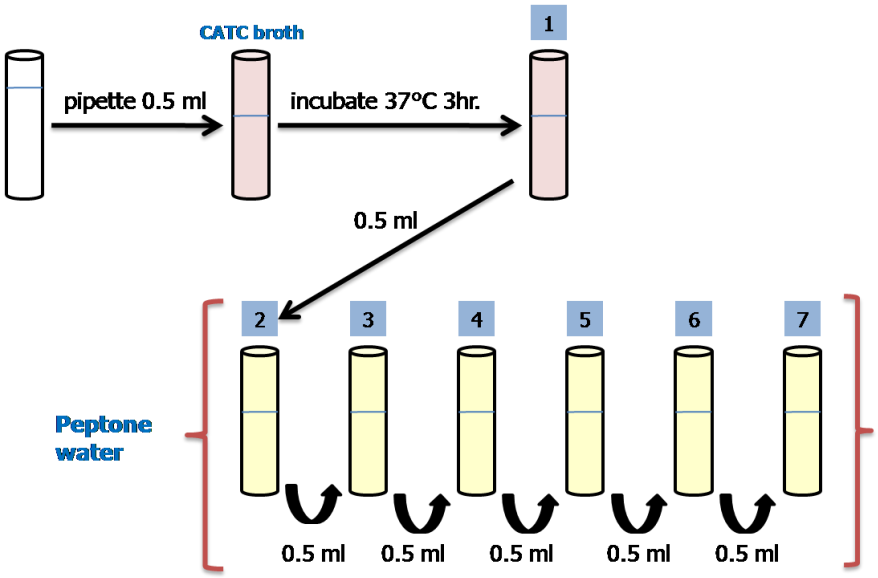
3.4.1.2 Preparation of food samples

25 gram of each food sample was weighed into a sterile stomacher bag. Then, 225 ml of the primary broth (0.1% peptone water) was added. The mixture was blended in a stomacher for approximately 3 minutes. So, the initial concentration of food sample was 0.1 g/ml.

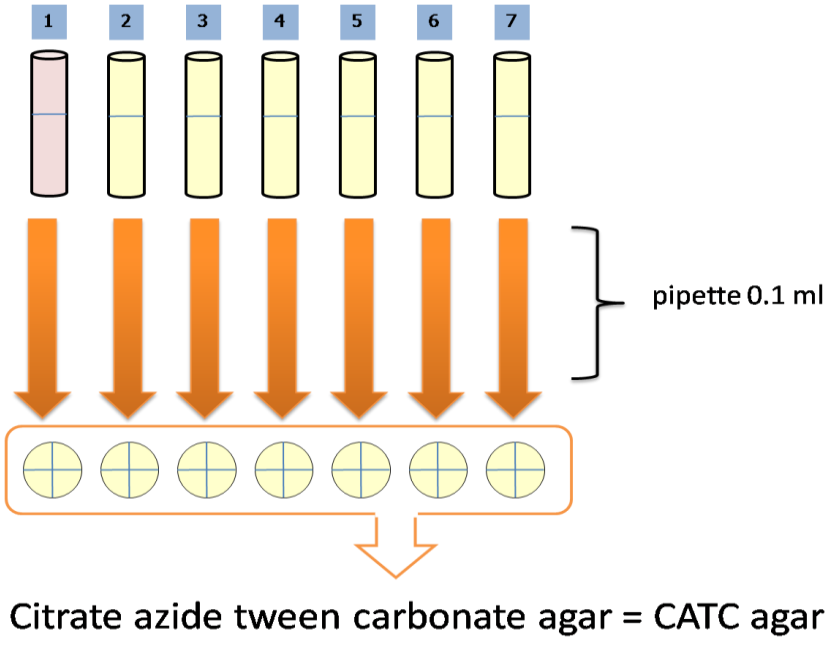
3.4.1.3 Isolation and enumeration of *Enterococcus* spp. from food samples

A half (0.5) ml of blended mixture of each food sample was added into 4.5 ml of selective broth (citrate azide tween carbonate broth; CATC broth) and incubated at 37°C for 3 hours. After incubation, the 0.5 ml of culture was further serial diluted with 4.5 ml of peptone water and mixed together. The solution was further serial diluted until the final concentration of the dilution was 10^{-8} g/ml. After that, 100 µl of each dilution was spread onto citrate azide tween carbonate agar (CATC agar) and was incubated at 37°C for 48 hours. The number of *Enterococcus* spp. in food samples were

recorded as CFU (colony forming unit) per gram of food. The isolation and enumeration of *Enterococcus* spp. from food sample was shown in Fig 4.



(a)



(b)

Figure 4 Isolation and enumeration of *Enterococcus* spp. from food samples

3.4.2 Confirmation of *Enterococcus* spp. by physical appearance of isolates on CATC agar and biochemical methods

After recording the number of *Enterococcus* spp., 20 randomly selected colonies from each food sample were confirmed by CATC agar and biochemical methods.

1) Observation the physical appearance (color) on CATC agar

The randomly selected colonies were inoculated in MRS (Man Rogosa and Shape) broth and were incubated at 37°C for 16 hours. Next, the broth culture was streaked thinly on the surface of the CATC agar again and incubated at 37°C for 24 hours. The color of the isolates (red or pink color) were represented the species of Enterococcus. The color of the isolates (red or pink color) were observed after incubation for differentiate roughly between *Enterococcus faecalis* and *Enterococcus faecium*. The result of red and pink colonies will be expected to be *E. faecalis* and *E. faecium*, respectively.

2) Biochemical test for *Enterococcus* spp.

After observation for the color of *Enterococcus* spp., all isolates were further confirmed by biochemical methods including determination of the growth ability in plate count agar with the presence of 0.04 % sodium azide, plate count agar with the presence of 6.5 % sodium chloride, plate count agar which incubated at 10°C and plate

count agar which incubated at 45°C. All isolates with positive results for all biochemical tests were kept in stock media for further test.

3.4.3. Tetracycline Susceptibility Testing of *Enterococcus* spp.

All isolates identified as *Enterococcus* spp. by the previous step were tested for tetracycline susceptibility that were consisted of three steps including the screening test for tetracycline resistance in *Enterococcus* spp., the standard disk diffusion method and the standard broth microdilution method.

3.4.3.1 The screening test for tetracycline-resistant *Enterococcus* spp.

The method for screening of tetracycline resistance in *Enterococcus* spp. was modified from replica plating method. The principle of this method was the growth ability of *Enterococcus* spp. on agar media with tetracycline concentration of 16 µg/ml. The concentration of 16 µg/ml was chosen because it is the breakpoint of resistance according to CLSI standard (CLSI, 2011).

The preparations of plate count agar, plate count agar with 16 µg/ml tetracycline, starting culture (*Enterococcus* spp.), and the instruments for replica plate preparations were described in Appendix. The procedures of screening test was as follows. First, the sterile skewer (blunt end) was used to touch the bacterial colonies slightly on the surface of starting culture. Then, the bacterial cells attached to the surface of sterile skewer were transferred to the surface of agar medium on plate count

agar with 16 µg/ml tetracycline as well as plate count agar without tetracycline respectively. Both agar plates were incubated at 37°C for 16 hours.

After incubation, the results were recorded bacterial cells can grow on both media, it was suggested that *Enterococcus* spp. was resistant to tetracycline. In other case, if bacterial cells can grow only on agar plate but can not grow on plate with tetracycline, the result was recorded as susceptible to tetracycline. The potential tetracycline-resistant colonies were further tested for the susceptibility of tetracycline by the standard method of Clinical and Laboratory Standards Institute (disk diffusion and broth micro dilution) (CLSI, 2011).

3.4.3.2 Disk diffusion method

Disk diffusion is the method for antimicrobial susceptibility testing by the diffusion of antimicrobial agent from disk through the agar. Disk diffusion method used in this study was performed according to CLSI, 2011. *Enterococcus faecalis* ATCC 29212 was used as control strain for each resistant test.

All of the Muller-Hinton Agar (MHA), inoculums, and tetracycline discs preparations were displayed in Appendix. The procedure of disk diffusion method was as followed. First, a sterile cotton swab was dipped into the inoculums suspension, rotated several times, and gently pressed onto the inside wall of the tube above the fluid level to remove excess inoculums from the swab. Second, the swab containing the inoculums was rubbed on the entire surface of the agar plate at three directions over the

surface of the agar. A final sweep was made around the rim of the agar. Within 15 minutes after the plates were inoculated, tetracycline discs were distributed evenly onto the surface. Third, the plates are inverted and incubated at 37°C for 16-18 hours within 15 minutes after discs were applied.

The interpretation was performed by measurement of the diameters of the zones of inhibition. The diameters of the zones read with mechanical caliper. Each zone read twice (at right angles) and the average result recorded to the nearest millimeter (averages of 0.5 mm rounded up). If the result of the control strain (*Staphylococcus aureus* ATCC® 25923) was in the breakpoint criteria of CLSI (Table 4), the result of the inoculums strain would be accurate and the diameters of the zones of inhibition could be used to compare the results in acceptability assay. If the results generated from the control strain were outside the acceptability limit, all data generated by the particular assay would be rejected.

The interpretation of susceptibility or resistance of the inoculums strains were determined by references on the basis of guideline published by the CLSI, 2011 (Table 5).

3.4.3.3 Broth microdilution method

This study used broth microdilution method to confirm the resistance of tetracycline in *Enterococcus* spp. isolates. This method was performed according to CLSI, 2011 so as to determine minimal inhibitory concentrations (MICs) of tetracycline

against all tested isolates. The found MIC was used to compare with standard guideline. *Enterococcus faecalis* ATCC 29212 was used as control strain for each resistant test.

The preparations of the cation supplemented mueller-hinton broth (CSMHB), inoculums, and tetracycline stock solution were displayed in appendix. The steps of broth microdilution was shown in Figure 6. Twelve wells on micro dilution tray (96 well plate) were prepared for each dilution. The first well was empty but the second to ninth well were for 0.1 ml of CSMHB. The 0.1 ml of tetracycline stock solution (1,024 µg/ml) was added into the first and the second well. Then the 0.1 ml of the solution in the second well was added into the third well, and then the 0.1 ml of the solution in third well was added into the fourth well. The twofold dilution of the solution were done until the ninth well. After mixing, the 0.1 ml solution in the ninth well was thrown away.

After dilution, the 0.1 ml of culture (1×10^6 cfu/ml) were added into the first to the ninth well. So, the final volume of each well was 0.2 ml and the concentration of culture in each well was 5×10^5cfu/ml (5×10^4cfu/well). There were three control wells including the tenth, eleventh, and twelfth. The tenth well was added by the 0.1 ml of CSMHB and 0.1 ml of culture but was not added with the tetracycline stock solution. The eleventh well was added by the 0.1 ml of CSMHB and 0.1 ml of tetracycline stock solution but was not added with the culture. The twelfth well was added by the 0.2 ml of CSMHB but was not added with the culture and the tetracycline stock solution. After that, the micro dilution tray was incubated at 37°C for 16 hours.

Table 4 Disk diffusion and MIC: Quality Control Ranges for *Enterococcus* spp. (CLSI, 2011)

Reference strain	Interpretation by	The standard range
Staphylococcus aureus ATCC® 25923	Zone diameter breakpoints (mm.)	24-30
Enterococcus faecalis ATCC® 29212	MIC interpretive standard (µg/ml)	8-32

Table 5 Interpretative standards for diffusion susceptibility and dilution susceptibility testing (CLSI., 2011)

Interpretation by	level of susceptibility to tetracycline		
	susceptible	intermediate	resistance
Zone diameter breakpoints, nearest whole mm	≥ 19	15-18	≤ 14
MIC interpretive standard (µg/ml)	≤ 4	8	≥ 16

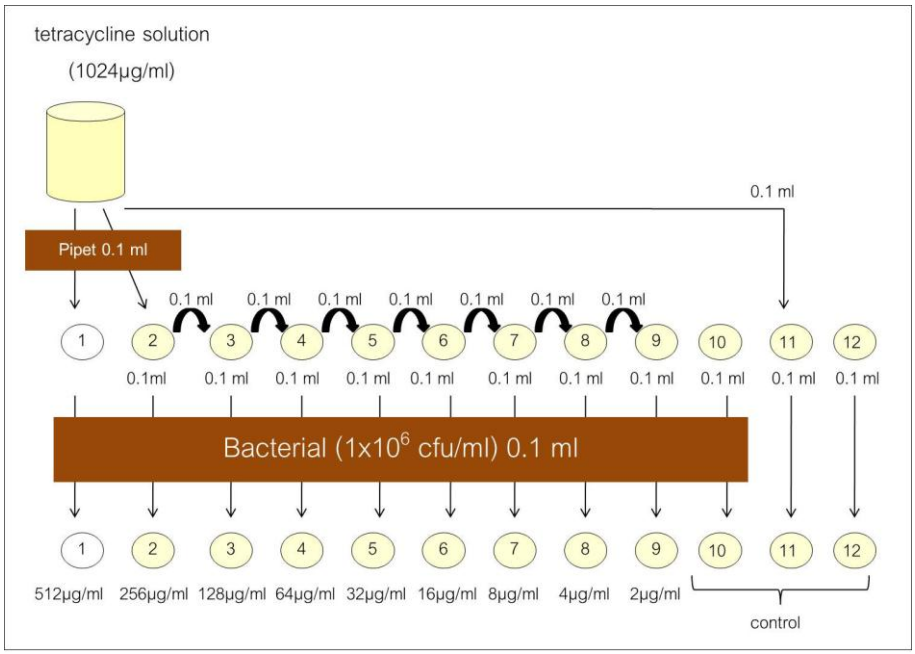


Figure 6 Broth microdilution method

After incubation, observation for the color and turbidity of the growth media was done. The turbidity indicated that the concentration of tetracycline in that well could not inhibit the growth of bacteria or the bacteria was resisted to tetracycline. The clear well indicated that the concentration of tetracycline in that well could inhibit the growth of bacteria or the bacteria was susceptible. The control well (10^{th}) must be turbid because this hole contained only the media and the culture. The eleventh and twelfth wells were prepared for detection the sterility of media. If there was contamination, these wells would be turbid. Reading assays, the end-point was defined as the lowest concentration for which there was no growth apparent to the unaided eye. This concentration should be reported as the MIC.

Each time of testing for tetracycline resistance in *Enterococcus* spp., if the result of the control strain (*Enterococcus faecalis* ATCC® 29212) was in the breakpoint criteria of CLSI (Table 4), the result of the inoculums strain would be accurate and the MIC could be used to compare the results in acceptability assay. But, if the results generated from the control strain were outside the acceptability limits, all data generated by the particular assay would be rejected.

3.4.4 The identification as *E. faecalis* and *E. faecium* from tetracycline resistant *Enterococcus* isolates

The potential clinically relevant *Enterococcus* spp. that this study focused on were *E. faecalis* and *E. faecium*. This study used molecular identification by polymerase chain reaction (PCR) assay targeting the D-alanine: D-alanine ligase gene (*ddl* gene) that is the specific gene for each species to identify. As the number of tetracycline-resistant *Enterococcus* spp isolates from fermented shrimp and fermented crab were only 11 and 6 colonies respectively, so all colonies (100%) were further identified the species. But the isolates from fermented fish and pork were randomly selected at a ratio 1:3 (approximately 33%). Therefore, 104 out of 302 isolates were the representatives of the tetracycline-resistant *Enterococcus* spp. The tetracycline-resistant isolates that gave red color on CATC agar were identified for *E. faecalis* and the isolates that gave pink color were identified for *E. faecium*. The red isolates that was not positive as *E. faecalis* were further used to identify for *E. faecium* and the pink isolates that were not positive for *E. faecium* were further used to identify for *E. faecalis*.

3.4.4.1. DNA extraction by boiling lysis method

One colony of tetracycline-resistance *Enterococcus* spp. was inoculated into 5 ml of MRS broth and then incubated at 37°C for 16 hours. The bacterial culture (1.8 ml for identifying *E. faecalis* and 2 ml for identifying *E. faecium*) was added into a 1.5 ml of micro centrifuge tube and centrifuged at 9000 rpm for 3 minutes. The

supernatant was discarded, then 20 µl of sterile deionized water and 30 µl of lysozyme (5mg/ml) were added and mixed together with bacterial cells to resuspend and digest the gram-positive bacterial cell walls. The suspension was incubated at 37°C for 30 minutes and then was lysed by heating in a boiling water bath (100°C) for 5 minutes. After that, the suspension was centrifuged at 12,000 rpm for 2 minutes. The DNA samples were stored at 2-8 °C before using as the template in all PCRs described below.

3.4.4.2 PCR detection for $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ gene

PCR amplification of $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ gene was performed using the primers for amplifying the *ddl* gene of both species reported by Kariyama *et al*. (2000) which produce the target of 941 and 658 bp fragments from *E. faecalis* and *E. faecium*, respectively upon amplification. Details of these primers were presented in Table 6. The PCR reaction mixture was performed in a total volume of 25 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, and 0.625 U of *Taq* DNA polymerase, a 5 µl volume of the supernatant lysis and 0.4 µM of *ddl E. faecalis* primer (or 0.6 µM of *ddl E. faecium* primer) as shown in Table 7. PCR amplification was carried out in PCR Thermocycler, Eppendorf Mastercycler Personel. The PCR cycling program started with an initial denaturation step, followed by strand denaturation, annealing, extension, and final extension. PCR conditions to detect both genes were shown in Table 8. In this study *E. faecalis* NRIC 379 was used as a positive

control to identify *E. faecalis* and *E. faecium* NRIC 1145 was used as a positive control to identify *E. faecium.*

3.4.4.3 Detection of PCR product

First, after PCR reactions, the PCR products were resolved by electrophoresis on a 1% w/v agarose gel. Second, a 5 µl of each PCR product was mixed with 3 µl of loading dye (bromophenol blue). Third, a 8 µl of the mixtures (each sample) were loaded into the submerged slot and the 4 µl of DNA ladder into the first slot. Fourth, the lid of gel tank was closed and attached the electrical leads. So, the DNA will migrate toward the anode and applied a voltage of 100 volt for 60 minutes. Then, the current was turned off, the lid and the leads were removed. Fifth, the agarose gel was stained into ethidium bromide solution for 30 seconds and into the deionized water for 3 minutes. Finally, this agarose gel was visualized under a UV transilluminator by gel documentation.

Table 6 PCR amplification primers of $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ gene used in PCR method

Gene	Primer	Sequence	Size of PCR product (bp)
$ddl_{E.\ faecalis}$	$ddl_{E.\ faecalis}$ R	5'- ATCAAGTACAGTTAGTCTTTATTAG-3'	941
	$ddl_{E.\ faecalis}$ F	5'-ACGATTCAAAGCTAACTGAATCAGT-3'	
$ddl_{E.\ faeciuum}$	$ddl_{E.\ faecium}$ R	5'- TTGAGGCAGACCAGATTGACG-3'	658
	$ddl_{E.\ faecium}$ F	5'- TATGACAGCGACTCCGATTCC-3'	

Table 7 PCR reaction mixtures for detection of $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ gene

PCR reaction mixtures	Volume (µl) of PCR mixture	
	Detection of *E. faecalis*	Detection of *E. faecium*
10x PCR buffer	2.5	2.5
dNTPs mixed	2	2
$MgCl_2$	0.75	0.75
Taq DNA polymerase	0.125	0.125
DNA template	5	5
Primer		
$ddl_{E.\ faecalis}$ or $ddl_{E.\ faecium}$ R	1	1.5
$ddl_{E.\ faecalis}$ *or* $ddl_{E.\ faecium}$ F	1	1.5
DI water	12.625	11.625
Total volume (µl)	25	25

Table 8 PCR condition for detection of $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ gene

PCR condition	$ddl_{E.\ faecalis}$ gene (temperature, time)	$ddl_{E.\ faecium}$ gene (temperature, time)
1. Initial denaturation	94ºC 3 min	95ºC 6 min
2. Denaturation	94ºC 1 min	94ºC 1 min
Annealing	52ºC 1 min	55ºC 1 min
Extension	72ºC 1 min	72ºC 1 min
Repeat 30 cycles		
3. Final extension	72ºC 10 min	72ºC 10 min

CHAPTER IV

RESULTS

4.1 Occurrence of *Enterococcus* spp.

The number of collected food samples and the contamination of *Enterococcus* spp. in the samples are shown in Table 9. A total of 70 ready-to-eat Thai traditional fermented food samples included fermented fish, fermented pork, fermented shrimp, fermented crab, and fermented shell.

From the data obtained, it was found that 28 samples (40%) were contaminated with *Enterococcus* spp. Fermented fish, pork, shrimp, and crab were found to be contaminated with *Enterococcus* spp. but fermented shell were not found to be contaminated with *Enterococcus* spp. All samples of nham-moo and som-fak were contaminated with *Enterococcus* spp. The contamination rate in pla-jom, pla-ra, koung-jom, and poo-dong were 33.33%, 31.25%, 12.50%, and 9.09% respectively. The total *Enterococcus* spp. contamination in food samples ranged between 10^3-10^7 CFU/g (Table 10).

4.2. Confirmation of *Enterococcus* spp. by physical appearance and biochemical methods

The physical appearance of 560 isolates that were randomly selected from food samples showed that 539 (96.25%) isolates were red and 21 (3.75%) isolates were pink. The result from biochemical methods indicated that 508 (90.71%) isolates showed positive reaction for all four methods, so they were identified as *Enterococcus* spp.

Table 9 The contamination of *Enterococcus* spp. in ready-to-eat Thai traditional fermented food samples

Types of food		Number of samples	Number of samples contaminated with *Enterococcus* spp. (%)	
Fish	pla-ra	16	5	(31.25)
	pla-jom	6	2	(33.33)
	som-fak	4	4	(100.00)
	all	26	11	(42.31)
Pork	nham-moo	15	15	(100.00)
Shrimp	koung-jom	8	1	(12.50)
Crab	poo-dong	11	1	(9.09)
Shell	hoi-dong	10	0	(0.00)
	Total	70	28	(40.00)

Table 10 The number of *Enterococcus* spp. found in ready-to-eat Thai traditional fermented food samples

Types of food		The number of *Enterococcus* spp. (CFU/g.)
Fish	pla-ra	10^3
	pla-jom	10^3
	som-fak	10^4-10^6
Pork	nham-moo	10^3-10^7
Shrimp	koung-jom	10^4
Crab	poo-dong	10^3
Shell	hoi-dong	ND*

ND*, not detected (<10^3 CFU/g)

4.3. Tetracycline susceptibility testing

The results from screening test for tetracycline resistance showed that 302 out of 508 isolates (59.44%) were resisted to tetracycline. Among 70 food samples, 28 (40%) samples were contaminated with tetracycline-resistance (Tc^r) *Enterococcus* spp. The contamination was found in fermented fish, pork, shrimp, and crab but not found in fermented shell. Fermented fish was contaminated with the highest number of Tc^r *Enterococcus* spp. (77.46%), especially in pla-jom (100.00%) and fermented shrimp was contaminated with the second most (57.89%). The results are shown in Table 11. The results from disk diffusion showed that all 302 isolates had zone diameter breakpoints ≤ 14 mm which were represented resistant to tetracycline. The results are shown in Table 12. The tetracycline minimum inhibitory concentration (TC MIC) for *Enterococcus* spp. are from 16 to 512 µg/ml. Most of the Tc^r *Enterococcus* spp. isolated

from all fermented food samples had TC MIC ranging from 64 to 128 µg/ml, except for the fermented crab that had MIC ranging from 128 to 256 µg/ml (Table 13). The high level of TC MIC (>256-512 µg/ml) for *Enterococcus* spp. was only found in the fermented fish (pla-jom and som-fak) and fermented crab.

Table 11 The number of *Enterococcus* spp. and Tc^r *Enterococcus* spp. isolates

Types and kinds of food		Number of *Enterococcus* spp. isolates	Number of Tc^r *Enterococcus* spp. isolates (%)	
Fish	pla-ra	94	60	(63.83)
	pla-jom	37	37	(100.00)
	som-fak	71	55	(18.21)
	all	202	152	(77.46)
Pork	nham-moo	270	133	(49.26)
Shrimp	koung-jom	19	11	(57.89)
Crab	poo-dong	17	6	(35.29)
	Total	508	302	(59.44)

TC= tetracycline, Tc^r = tetracycline resistance

Table 12 The number of *Enterococcus* spp. from susceptibility test to tetracycline by disk diffusion

The number of *Enterococcus* spp.	Zone diameter value (mm)	level of susceptibility to tetracycline*
89	6-8	resistance
54	8-10	resistance
73	10-12	resistance
86	12-14	resistance

*zone diameter at ≤ 14 are the breakpoint of resistance according to CLSI standard (CLSI, 2011)

Table 13 The number of Tc^r *Enterococcus* spp. isolated from different types of Thai fermented foods classified by level of MIC value

Type of fermented food		Number of Tc^r *Enterococcus* spp. (%) classified by level of MIC VALUE (μg/ml)											
		16-32		>32-64		>64-128		>128-256		>256-512		total	
Fish	pla-ra	4		14		26		16		0		60	
	pla-jom	2		4		15		14		2		37	
	som-fak	3		4		29		17		2		55	
	all	9	(5.92)	22	(14.47)	70	(46.05)	47	(30.92)	4	(2.63)	152	(100.00)
Pork	nham-moo	3	(2.26)	13	(9.77)	86	(64.66)	31	(23.31)	0	(0.00)	133	(100.00)
Shrimp	koung-jom	4	(36.36)	2	(18.18)	5	(45.45)	0	(0.00)	0	(0.00)	11	(100.00)
Crab	poo-dong	0	(0.00)	2	(33.33)	0	(0.00)	3	(50.00)	1	(16.67)	6	(100.00)
	Total	16	(5.30)	39	(12.91)	161	(53.31)	81	(26.82)	5	(1.65)	302	(100.00)

TC= tetracycline, Tc^r = tetracycline-resistance, MIC = minimum inhibitory concentration

4.4 The identification for the presence of *E. faecalis* and *E. faecium* from tetracycline-resistance *Enterococcus* isolates

Tc^r *Enterococcus* spp. isolates (n=104) that were randomized from the total of 302 isolates were further identified for the clinically relevant *Enterococcus* spp. (*E. faecalis* and *E. faecium*) by PCR analysis targeting the *ddl* gene specific for each species. It was found that from the total of 104 Tc^r *Enterococcus* spp. isolates, 48 (46.15%) isolates were *E. faecalis*, 37 (35.58%) isolates were *E. faecium*, and 19 (18.27%) isolates were other *Enterococcus* spp. In the fermented fish and pork, most of the Tc^r *Enterococcus* spp. isolates were *E. faecalis* (58.33% and 51.28%, respectively). On the other hand, *E. faecalis* was not found in the fermented shrimp and crab. Almost all isolates in fermented shrimp and crab were *E. faecium* (90.91% and 50.00%, respectively.) The data are shown in Table 14 and Figure 6. The example picture of *ddl* gene of both species in isolates are shown in Figure 7 and 8.

Table 14 The identification for the presence of *E. faecalis* and *E. faecium* from Tc^r *Enterococcus* isolates

Type of fermented food	Number of Tc^r *Enterococcus* spp. isolates	*E. faecalis* (%)	*E. faecium* (%)	Others (%)
Fish	48	28 (58.33)	14 (29.17)	6 (12.50)
Pork	39	20 (51.28)	10 (25.64)	9 (23.08)
Shrimp	11	0 (0.00)	10 (90.91)	1 (9.09)
Crab	6	0 (0.00)	3 (50.00)	3 (50.00)
Total	104	48 (46.15)	37 (35.58)	19 (18.27)

TC= tetracycline, Tc^r = tetracycline resistance

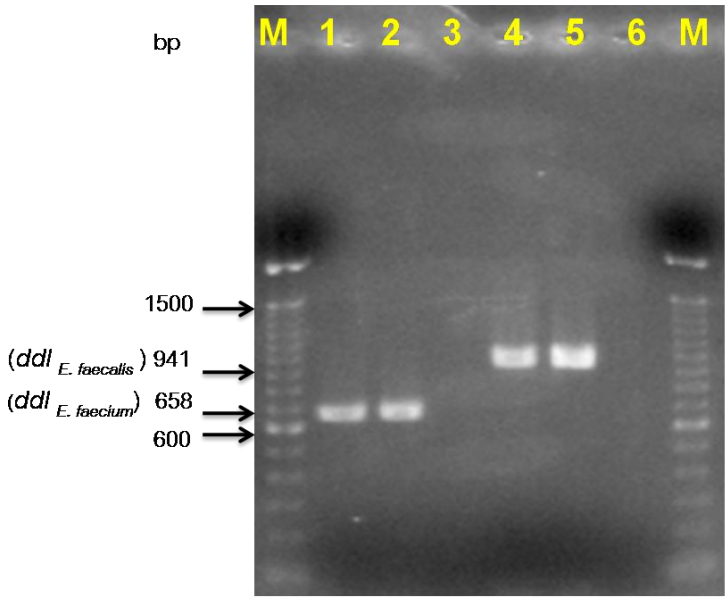


Figure 6 Agarose gel electrophoresis of amplified *ddl*$_{E.\ faecalis}$, and *ddl*$_{E.\ faecium}$ genes by the PCR assay containing two primer sets. lane M: 100-bp DNA ladder, lane 1: an *E. faecium* NRIC 1145 *ddl*$_{E.\ faecium}$ isolate (positive control), lane 2: an *E. faecium ddl*$_{E.\ faecium}$ food isolate, lane 3: a negative control of *ddl*$_{E.\ faecium}$ isolate, lane 4: an *E. faecalis* NRIC 379 *ddl*$_{E.\ faecalis}$ isolate (positive control), lane 5: an *E. faecalis ddl*$_{E.\ faecalis}$ food isolate, lane 6: a negative control of *ddl*$_{E.\ faecalis}$ isolate

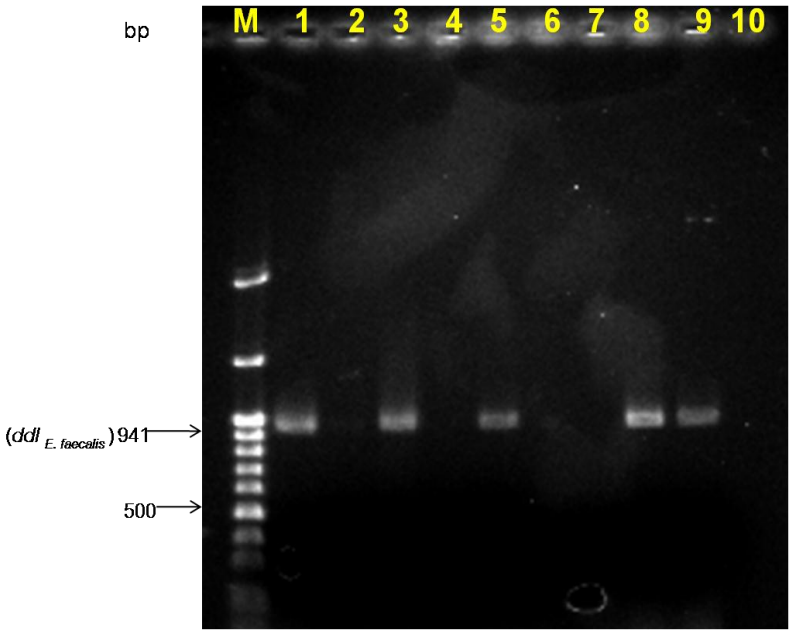


Figure 7 Agarose gel electrophoresis of amplified $ddl_{E.\ faecalis}$ genes by the PCR assay using specific primer to $ddl_{E.\ faecalis}$. lane M: 100-bp DNA ladder; lane1: an *E. faecalis* NRIC 379 $ddl_{E.\ faecalis}$ isolate (positive control), lane 2-9: fermented pork (sample) isolates, lane 10: a negative control

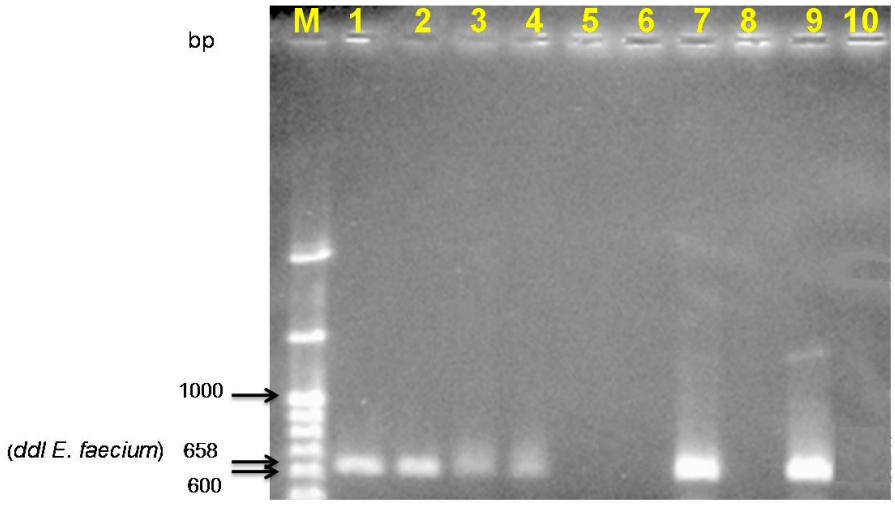


Figure 8 Agarose gel electrophoresis of amplified $ddl_{E.\ faecium}$ genes by the PCR assay using specific primer to $ddl_{E.\ faecium}$. Lane M: 100-bp DNA ladder, lane 1: an *E. faecium* NRIC 1145 $ddl_{E.\ faecium}$ isolate (positive control), lane 2-9: fermented pork (sample) isolates, lane 10: a negative control

The results in Table 15 and 16 showed that most Tc^r *E. faecalis* and Tc^r *E. faecium* isolates had TC MIC in the range of 64-128 µg/ml (45.83% for Tc^r *E. faecalis* and 45.95% for Tc^r *E. faecium*). A high TC MIC level (>256-512 µg/ml) was not found in Tc^r *E. faecalis* whereas it was found in Tc^r *E. faecium* isolated from the fermented fish.

Most Tc^r *E. faecalis* isolated from fermented fish (42.86%) had TC MIC level in the range of 128-256 µg/ml, while 57.14% Tc^r *E. faecium* isolates had TC MIC level in the range of 64-128 µg/ml. The TC MIC level in both species isolated from fermented pork was between 64 and 128 µg/ml. Most Tc^r *E. faecium* isolated from fermented shrimp had TC MIC in the range of 64-128 µg/ml and 16-32 µg/ml, while Tc^r *E. faecium* isolated from fermented crab had TC MIC in the range of 32-64 µg/ml.

Table 15 The number of Tc^r *E. faecalis* isolated from different types of Thai fermented foods classified by level of TC MIC value

Type of fermented food	Number of Tc^r *E. faecalis* (%) classified by level of MIC value (μg/ml)									
	16-32		>32-64		>64-128		>128-256		total	
Fish	1	(3.57)	5	(17.86)	10	(35.71)	12	(42.86)	28	(100.00)
Pork	1	(5.00)	1	(5.00)	12	(60.00)	6	(30.00)	20	(100.00)
Total	2	(4.17)	6	(12.50)	22	(45.83)	18	(37.50)	48	(100.00)

TC= tetracycline, Tc^r = tetracycline resistance, MIC = minimum inhibitory concentration

Table 16 The number of Tc^r *E. faecium* isolated from different types of Thai fermented foods classified by level of TC MIC value

Type of fermented food	Number of Tc^r *E. faecium* (%) classified by level of MIC value (μg/ml)											
	16-32		>32-64		>64-128		>128-256		>256-512		total	
Fish	0	(0.00)	3	(21.43)	8	(57.14)	2	(14.29)	1	(7.14)	14	(100.00)
Pork	0	(0.00)	2	(20.00)	5	(50.00)	3	(30.00)	0	(0.00)	10	(100.00)
Shrimp	4	(40.00)	2	(20.00)	4	(40.00)	0	(0.00)	0	(0.00)	10	(100.00)
Crab	0	(0.00)	2	(66.67)	0	(0.00)	1	(33.33)	0	(0.00)	3	(100.00)
Total	4	(10.81)	9	(24.32)	17	(45.95)	6	(16.22)	1	(2.70)	37	(100.00)

TC= tetracycline, Tc^r = tetracycline resistance, MIC = minimum inhibitory concentration

CHAPTER V

DISCUSSION

Some *Enterococcus* spp. as well-recognized as nosocomial pathogens that can cause infections in hospitalized patients. Furthermore, antibiotic-resistant *Enterococcus* spp. may increase serious health problem. This study tried to expand the knowledge about potential pathogenic enterococcal as well as antibiotic-resistance *Enterococcus* spp. contamination in food, which is a possible reservoir for transferring *Enterococcus* spp. to human body.

In this study, the occurrence of *Enterococcus* spp. contamination in some ready-to-eat Thai traditional fermented foods as well as the occurrence of tetracycline-resistance (Tc^r) in these isolates were reported. Five different kinds of Thai traditional fermented foods were chosen as follows; fermented fish (pla-ra, pla-jom, som-fak), fermented pork (nham-moo), fermented crab (poo-dong), fermented shrimp (koung-jom), and fermented shell (hoi-dong). The data about the number of enterococcal contamination in each type of food, the amount of *Enterococcus* spp. and the tetracycline resistance among these isolates were compared to evaluate the prevalence of *Enterococcus* spp. in each type of foods and the subsequent high risk of enterococcal contamination in food. Furthermore, some tetracycline-resistant

Enterococcus spp. were randomly selected to identify for potential clinically relevant *Enterococcus* spp. strains (*E. faecalis* and *E. faecium*) in order to find out the risk of foods contaminated with these strains.

The results from this study showed that fermented fish (pla-ra, pla-jom, som-fak), fermented pork (nham-moo), fermented crab (poo-dong), and fermented shrimp (koung-jom) were contaminated with *Enterococcus* spp. *Enterococcus* spp. found in foods may come from *Enterococcus* spp. used as growth promoter in animal food or from the contamination of *Enterococcus* spp. in environments e.g. soil, water, feces, plant, air, equipment. Furthermore, these fermented foods may provide suitable environment for the growth of *Enterococcus* spp. such as concentration of sodium chloride and pH. A high concentration of sodium chloride in these fermented foods could inhibit the growth of most microbial excepted for halophilic bacteria such as *Enterococcus* spp. because it can tolerate high concentration of sodium chloride. Because, water will leave the bacterial cell when there is a high concentration of sodium chloride in the food matrix, then the plasma membrane will shrink away from the cell wall (plasmolysis) and the microbial cell will stop growing and eventually dies (นาถสุดา วิศวงศ์, 2522). This is also the reason of using high concentration of sodium chloride as preservative in fermented foods. Moreover, the low-intermediate acid (pH > 4.5) of these foods is suitable for growing of *Enterococcus* spp. which can grow in a pH range from 4.4-10.6 while most bacteria can grow at a pH range of 6-8 (Lawlet *et al*., 2008). Last, the good practice in

sanitation and hygiene during processing or handling is important too. The contamination of *Enterococcus* spp. in this study indicated that the procedure for processing of most fermented foods could not kill or inhibit the growth of *Enterococcus* spp. The results of this study was in agreement with the previous study (Tanasupawat *et al*, 1992) which found enterococcal contamination in pla-ra, and pla-jom. However, this was the first study to discover enterococcal contamination in nham-moo, som-fuk, and poo-dong. Furthermore, this was also the first study to find the potential clinically-relevant antibiotic-resistance enterococcal contamination in Thai traditional fermented foods.

In this study, it was found that the amount of *Enterococcus* spp. contaminated in ready-to-eat Thai traditional fermented food samples ranged between 10^3 to 10^7 CFU/g. The number of *Enterococcus* spp. in pla-ra, pla-jom, koung-jom, and poo-dong was between 10^3 to 10^4 CFU/g, whereas the number in som-fak was between 10^4 to 10^6 CFU/g., and in nham-moo the number was between 10^3 to 10^7 CFU/g. The amount of enterococcal contaminations in different kinds of fermented food are high difference. This may reflex the different period of time to ferment each foods and different recipes in each sample. However, the amount of *Enterococcus* spp. in hoi-dong can not be detected in this study. The reasons for this can be two possibilities; first, it may be possible that this type of food was not contaminated with *Enterococcus* spp. or the second, it may be possible that the number of enterococcal contamination in this food

was less than 10^3 CFU/g which is beyond the limitation of detection in this study (the method used in this study can detect *Enterococcus* spp. at ≥ 10^3 CFU/g). Furthermore, the number of enterococcal contamination in different kinds of fermented food found in this study may be different from other studies and cannot be comparable because this study used different method for enumeration of bacterial contamination. In this study, the pre-enrichment method was utilized for isolation and enumeration of enterococci. The short-period (3 hours) pre-enrichment method utilized in this study may increase the number of bacterial contamination in food. So, the number of enterococcal contamination in food found in this study may be higher than other studies which utilize direct isolation method in selective CATC agar.

In this study tetracycline-resistance *Enterococcus* spp. was found as high as 59.44% of all *Enterococcus* spp. isolates. This study was in agreement with the study of Tansuphasiri *et al* (2006) that found 66% of *Enterococcus* spp. isolated from frozen foods was resistant to tetracycline. Furthermore, this study also found that fermented fish was contaminated with the highest number of tetracycline-resistance (Tc^r) *Enterococcus* spp. (77.46%) and the second most was found in fermented shrimp (57.89%). Tetracycline and tetracycline derivatives such as chlortetracycline and oxytetracycline, which are widely used antibiotics (Flaherty, Vandergeest, and Miller, 1999), have been used to treat infection and prevention of animal diseases (as a growth promoter). These applications may promote antibiotic resistance in *Enterococcus* spp. The high number

of tetracycline resistance (Tc^r) *Enterococcus* spp. in fermented fish and fermented shell was consistent with the widespread use of oxytetracycline in the aquatic animals, especially in fish and shrimp (Kummerer, 2009). This resistance can contaminate with animal carcasses during slaughtering inevitably or contaminate to environment by unclean sanitary process. Therefore, the data from this study indicated that fermented foods may serve as a vehicle to transport resistant bacteria and genes between animals and humans.

In this study, the occurrence of potential clinically relevant enterococci with tetracycline resistance isolated from four different kinds of fermented foods was also found (*E. faecalis* (46.15%) and *E. faecium* (35.58%)). Fermented fish was found to be high contamination with both species. A high percentages of Tc^r *E. faecalis* was also found in fermented fish, but a high percentages of Tc^r *E. faecium* was found in fermented shrimp. The finding of these two species contamination in food in this study was in agreement with the previous study (Huy *et al*., 2004) which found Tc^r *E. faecalis* and Tc^r *E. faecium* isolated from several kinds of food e.g. meat, fish, milk, cheese. The clinical isolates of these two species can cause infection such as urinary tract infection, bloodstream infections, intra-abdominal infection, infection after cataract surgery (Moellering, 1990; Murray, 1990). However, there is a study showed that *E. faecalis* and *E. faecium* isolated from foods can be potential pathogenic bacteria by determining virulence determinants (Franz *et al*., 2001). Therefore, the result of contamination with

potential clinically relevant *Enterococcus* spp. in some Thai fermented foods found in this study should be concerned. Further study should determine for pathogenicity of these contaminated strains before strict regulations can be stipulated.

The result from this study may reflex the effect of extensive agricultural use of tetracycline or tetracycline derivatives in animal feeding, especially in aquatic animals. It may create an animal reservoir of resistant *Enterococcus* spp. which may lead to increase antibiotic resistance problem in human. Therefore, prevention of spreading enterococcal from foods by heat inactivation before taken, especially in these ready-to-eat foods should be suggested. For example, this kind of food should be cooked thoroughly and heated with high temperature up to 70°C. The serving food should not be left at room temperature for more than 2 hours (preferably below 5°C). Moreover, the kitchen, the hands and equipment used for food preparation should keep clean and sanitize before and often during food preparation (Marriot and Gravini, 2006). Furthermore, use of antibiotics in agriculture should be avoided to prevent the development of antibiotic resistance in microorganism and humans.

CHAPTER VI

CONCLUSION

The present study investigated the occurrence of tetracycline-resistance *Enterococcus* spp. in some ready-to-eat Thai traditional fermented foods. The results showed that most types of fermented food samples were contaminated with *Enterococcus* spp. and tetracycline-resistance (Tc^r) *Enterococcus* spp. These indicated that food processing and preservative methods in ready-to-eat Thai traditional fermented foods could not kill or prevent the growth of *Enterococcus* spp. The isolates with high TC MIC value (>256-512μg/ml) were also found in fermented fish and fermented crab. The clinically relevant enterococci, *E. faecalis* and *E. faecium* with Tc^r phenotype were also found with high percentages in some Thai traditional fermented foods. Among five different fermented foods, the highest percentage of Tc^r *E. faecalis* and Tc^r *E. faecium* were found in fermented fish and fermented shrimp, respectively.

The prevention of enterococcal contamination in Thai traditional fermented foods during production may be difficult because *Enterococcus* spp. were generally found in environment. Therefore, the prevention should be done by appropriated processing before consumption such as heat inactivation, food irradiation. Moreover, the good practice in sanitation and hygiene should be concerned during preparation of these kinds of food.

Finally, the use of tetracycline group in agriculture, especially the widespread use of oxytetracycline hydrochloride in aquatic animals, should be concerned. The replacement of antibiotic with other developing alternatives such as probiotics, oligosaccharides, plant extracts, and fermented mash as a growth promoter should be considered in order to prevent the promotion of the development of antibiotic resistance in microorganism and humans.

Recommendation

Because the studies of antibiotic resistance in *Enterococcus* spp. isolated from Thai food has been limited. Further study should increase more information by determining antibiotic-resistance *Enterococcus* spp. from other types of food e.g. other fermented foods, cooked foods and from other antibiotic groups. Moreover, the virulence determinants of clinically relevant *E. faecalis* and *E. faecium* isolated from foods should be determined in order to strengthen the information needed for consumer's safety concern.

REFERENCES

ภาษาไทย

ณัฐวุธ สิบหมู่. 2552. เภสัชวิทยา : เนื้อหาสำคัญและแบบฝึกหัด. กรุงเทพฯ : โฮลิสติก.

นาถสุดา วิศวงศ์. 2522. การศึกษาจุลชีววิทยาของอาหารหมักพื้นเมือง: ปลาเจ่า, ปลาส้ม และส้มฟัก, วิทยานิพนธ์ปริญญามหาบัณฑิต, สาขาวิชาจุลชีววิทยา ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์.

พระราชบัญญัติอาหาร พ.ศ. 2522 พร้อมกฎกระทรวงและประกาศกระทรวงสาธารณสุข ฉบับปรับปรุงปี 2553. 2553. ราชกิจจานุเบกษา ฉบับกฎษฎีกา, 126 (ตอนที่ 41ง), หน้า 443-452.

ภัทรชัย กีรติสิน. 2549. ตำราวิทยาแบคทีเรียการแพทย์. พิมพ์ครั้งที่ 1. กรุงเทพฯ : วี.เจ.พริ้นติ้ง.

มาลินี ลิ้มโภคา. 2540. การใช้ยาต้านจุลชีพในสัตว์: สัตว์บกและสัตว์น้ำ. กรุงเทพฯ : โรงพิมพ์จรัลสนิทวงศ์.

วิลาวัณย์ เจริญจิระตระกูล. 2539. จุลินทรีย์ที่มีความสำคัญด้านอาหาร. กรุงเทพฯ : โอเดียนสโตร์.

สุภาพ อัจฉริยศรีพงศ์. 2522. การศึกษาจุลชีววิทยาของอาหารหมักพื้นเมือง: กุ้งจ่อม และหอยแมลงภู่ดอง, วิทยานิพนธ์ปริญญามหาบัณฑิต, สาขาวิชาจุลชีววิทยา ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์.

สุมาลี เหลืองสกุล. 2527. จุลชีววิทยาทางอาหาร. กรุงเทพ: โรงพิมพ์ชัยเจริญ.

สมหวัง ด่านชัยวิจิตร, เชิดศักดิ์ ธีระบุตร, ดวงพร จินตโนทัยถาวร, ไพฑูรย์ บุญมา, มนทกานติ ตระกูลดิษฐ์, มาลัย วรจิตร และคณะ. 2548. การดื้อยาของแบคทีเรียก่อโรคในชุมชนและในโรงพยาบาล. ในโครงการวิจัยการป้องกันและควบคุมโรคติดเชื้อในโรงพยาบาล.

ศูนย์ปฏิบัติการความปลอดภัยด้านอาหาร. ผลสำเร็จการดำเนินงานตามยุทธศาสตร์ความปลอดภัยด้านอาหาร (Food Safety). กระทรวงสาธารณสุขปีงบประมาณ 2546 – 2548, หน้า 2.

ศูนย์ทางด่วนข้อมูลการเกษตร *1677 สถานีวิทยุร่วมด้วยช่วยกัน จ. นครศรีธรรมราช. 2552. วิธีการทำปูเค็ม. [online]. Available from: http://www.rakbankerd.com/agriculture/page?id=1054&s=tblanimal [2012, April 20].

ภาษาอังกฤษ

Aarestrup, F.M., Agerso, Y., Madsen, M., Smith, P.G., Jensen, L.B. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers and pigs in Denmark. Diagn Microbiol Infect Dis 37: 127–137.

Aarestrup, F.M., Butaye, P., Witte, W. 2002. Non-human reservoirs of enterococci. In M.S. Gilmore (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance, pp. 55-99. Washington, DC: ASM Press.

Aarestrup, F.M., Hasman, H., Jensen, L.B., Moreno, M., Herrero, I. A., Dominguez, L., et al. 2002. Antimicrobial resistance among enterococci from pigs in three European countries. Appl Environ Microbiol 68: 4127-4129.

Biavasco, F., Giovanetti, E., Miele, A., Vignaroli, C., Varoldo, P.E. 1996. In vitro conjugative transfer of VanA vancomycin resistance between enterococci and listeriae of different species. Eur J Clin Microbiol Infect Dis 15: 50–59.

Bismuth, R., Zilhao, R., Sakamoto, H., Guesdon, J. L., Courvalin P. 1990. Gene heterogeneity for tetracycline resistance in Staphylococcus spp. Antimicrob Agents Chemother 34: 1611-1614.

Borgo, F., Ricci, G., Arends, K., Schiwon, K., Grohmann, E., Fortina, M.H. 2009. Evaluation of plasmid content and tetracycline resistance conjugative transfer in *Enterococcus italicus* strains of dairy origin. Curr microbial 55: 261–266.

Brown, L.H., Peterson, E.M., de la Maza, L.M. 1983. Rapid identification of enterococci. J Clin Microbiol 17(2): 369–370.

Cauwerts, K., Decostere, A., De Graef, E.M., Haesebrouck, F., Pasmans, F. 2007. High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm* (B) gene. Avian Pathol 36: 1-2.

Chopra, I., Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65: 232-260.

Clewell, D. 1990. Movable genetic elements and antibiotic resistance in enterococci. Eur J Clin Microbiol Infect Dis 9: 90-102.

Clinical and Laboratory Standards Institute (CLSI). 2011. Performance standards for antimicrobial susceptibility testing, 21st informational supplement. CLSI document M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA.

Cohen, M.L. 1992. Epidemiology of drug resistance implications for a post antimicrobial era. Science 257: 1050-1055.

Connell, S.R., Tracz, D.M., Nierhaus, K.H. Taylor, D.E. 2003. Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother 47: 3675– 3681.

Courvalin, P. 1994. Transfer of antibiotic resistance genes between Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 38: 1447– 1451.

Craig, W.A. 1993. Qualitative susceptibility tests versus quantitative MIC tests. Diagn Microbiol Infect Dis 16(3): 231–236.

Donabedian, S.M., Thal, L.A., Hershberger, E., Perri, M.B., Chow, J.W., Bartlett, P., et al. 2003. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. J Clin Microbiol 41: 1109-1113.

Dunny, G.M., Leonard, B.A., Hedberg, P.L. 1995. Pheromone-inducible conjugation in *Enterococcus faeca*lis: interbacterial and host-parasite chemical communication. J Bacteriol 177(4): 871–876.

Esteban, C., Barbara, E. 2006. *Enterococcus* spp. In: Stephen H, Peter M, editors. Principles and Practice of Clinical Bacteriology (Second Edition). New York: John Wiley & Sons Ltd: 59-71.

FAO/WHO. 1997. Codex Alimentarius Food Hygiene Basic Texts. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission. Pub. # M-83.

FAO/WHO. 2001. Definitions, Section II. In: Recommended International Code of Practice General Principles of Food Hygiene, Codex.

FAO/WHO. 2002. Guidelines for the evaluation of probiotics in food. [online]. Available from: ftp://ftp.fao.org/es/esn/food/wgreport2.pdf [2012, February 9].

Faser, S.L. 2011. Enterococcal Infection [Online]. Available from : http://emedicine.medscape.com/article/216993-overview#a0199 [2011, November 1].

Flaherty, J.P, Weinstein, R.A. 1996. Nosocomial infection caused by antibiotic-resistant organisms in the intensive care unit. Infect Cont Hosp Ep 17: 236 – 248.

Flaherty, M., Vandergeest, P., Miller, P. 1999. Rice paddy or shrimp pond: tough decisions in rural Thailand. World Dev, 27 (12): 2045–2060.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L. 2006. The role and application of enterococci in food and health. Int J Food Microbiol 106: 1-24.

Franz, C., Holzapfel, W., Stiles, M. 1999. Enterococci at the crossroads of food safety. Int J Food Microbiol. 47: 1-24.

Franz, C.M.A.P., Schillinger, U., Holzapfel, W.H. 1996. Production and characterisation of enterocin 900, a bacteriocin produced by Enterococcus faecium BFE 900 from black olives. Int J Food Microbiol. 29: 255–270.

Franz C.M.A.P, Muscholl-Silberhorn, A.B., Yousif, N.M., Vancanneyt, M., Swings, J., Holzapfel, W.H. 2001. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Appl Environ Microbiol. 67: 4385-4389.

Franz, C.M.A.P., Stills, K.H. Schleifer, Holzapfel, W.H. 2003. Enterococci in foods-a conundrum for food safety. Int J Food Microbiol. 88:105-122.

Franz, C.M., Huch, M., Abriouel, H., Holzapfel, W, Gálvez, A. 2011. Enterococci as probiotics and their implications in food safety. Int J Food Microbiol. 2: 151(2):125-140.

Giraffa, G. 1995. Enterococcal bacteriocins: their potential use as anti-Listeria factors in dairy technology. Food Microbiol 12: 551–556.

Giraffa, G. 2003. Functionality of enterococci in dairy products. Int J Food Microbiol. 88: 215-222.

Gold, H.S., Moellering, R.C. 1996. Antimicrobial-drug resistance. N Engl J Med. 335: 1445-1453.

Gomes, B.C., Franco, Bernadette D.G.M., Martinis, E.C.P. 2010. Dualistic aspects of *Enterococcus* spp. in foods. In: Antonio Mendez Vilas. (Org.). Current Research Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, pp. 1119-1125. Badajoz, Espanha: Formatex Research Center, v. 2.

Gorris, L.G.M. 2005. Food safety objective: An integral part of food chain management. Food Control 16(9): 801-809.

Hancock, L.E., Gilmore, M.S. 2006. Pathogenicity of Enterococci. In V. A. Fischetti, R.P., Novick, J.J., Ferretti, Portnoy, D.A., Rood, J.I. (ed.), Gram-Positive Pathogens, 2nd ed, pp. 299-311. Washington, DC : ASM Press.

Hardie, J.M., Whiley, R.A. 1997. Classification and overview of the genera *Streptococcus* and *Enterococcus*. J Appl Microbiol Symp. Suppl. 83:1S-11S.

Heuer, O.E., Hammerum, A.M., Collignon, P.C., Wegener, H.C. 2006. Human health hazard from antimicrobial resistant enterococci in animals and food. Clin Infect Dis 43(7): 911-916.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U., Huis in't Veld, J.H. 1998. Overview of gut flora and probiotics. Int J of Food Microbiol 41:85-101.

Huycke, M.M., Sahm, D.F., Gilmore, M.S. 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis 4: 239-249.

Huys, G., D'Haene, K., Collard, J.-M., Swings, J. 2004. Prevalence and molecular characterization of tetracycline resistance in Enterococcus isolates from food. Appl Environ Microbiol 70: 1555–1562.

Jorgensen, J.H., Turnidge, J.D. 2007. Susceptibility test methods: Dilution and disk diffusion method. In: Baron, E.J., Jorgensen, J.H., Pfaller, M.A., Landry, M.L. (ed.), The Manual of Clinical Microbiology, (ninth edition), pp 1152-1172. ASM Press: Washington, D.C.

Kahlmeter, K., Brown, D.F.J., Goldstein, F.W., Macgowan, A.P., Mouton, J.W., Odenholt, I., et al. 2006. European Committee on Antimicrobial Susceptibility Testing (EUCAST) Technical Notes on antimicrobial susceptibility testing. Clin Microbiol Infec 12: 501-503.

Kariyama, R., Mitsuhata, R., Chow, J. W., Clewell, D. B, Kumon, H. 2000. Simple and reliable multiplex PCR assay for surveillance isolates of Vancomycin-Resistant Enterococci. J Clin Microbiol 38(8): 3092-3095.

Koster W., Egli, T., Ashbolt, N., Botzenhart, K., Burlion, N., Endo, P., et al. 2003. Analytical methods for microbiological water quality testing. In: Assessing Microbial Safety of Drinking Water: Improved approaches and methods, Chapter 8, pp. 237-257. Paris: OECD and World Health Organization.

Kummerer, K. 2009. Antibiotics in the aquatic environment – A review – Part I . Chemosphere 75: 417–434.

Lawley, R., Cuitis, L., Davis, G. 2008. The food safety hazard guidebook. Cambridge, UK : RSC Publishing.

Lertworapreecha, N., Poonsuk, K., Chalermchiakit, T. 2011. Selection of potential *Enterococcus faecium* isolated from Thai native chicken for probiotic use according to the *in vitro* properties. Songklanakarin J Sci Techno 33(1): 9-14.

Lolekha, S., Ratanaubol, B., Manu, P. 1981. Nosocomial Infection in a Teaching Hospital in Thailand. Phil J Microbiol Infect Dis 103-114.

McWilliams, M. 1997. Foods: Experimental perspectives. Upper Saddle River NJ: Merrill.

Marriot, N.G., Gravini, R.B. 2006. Principle of Food Sanitation. 5th ed. New York.: Van Nostrand Reinhold.

Mathur, S., Singh, R. 2005. Antibiotic resistance in food lactic acid bacteria-A review. Int J Food Microbiol 105: 281-295.

Meropol, S.B., Metlay, J.P. 2008. Antibiotic Resistance: Modern Principles and Management Strategies to Optimize Outpatient Use of Antibiotics. Chapter 22. In Owens RC, Lautenbach E (Eds.). Antimicrobial Resistance: Problem Pathogens and Clinical Countermeasures, pp.461-478. New York, NY: Inform a Healthcare USA.

Meyers, B., Salvatore, M. 2005. Tetracyclines and Chloramphenicol. In: G.L. Mandell, J.E. Bennett, and R. Dolin (eds.), Mandell, Douglas, and Bennett's, Principles and Practice of Infectious Diseases, pp. 356-373. Philadelphia : Elsevier Churchill Livingstone.

Moellering, R.C. 1990. The enterococci: an enigma and a continuing therapeutic challenge. Eur J Clin Microbiol Infect Dis 9: 73–74.

Murray, B.E. 1990. The life and times of the enterococcus. Clin Microbiol Rev 3: 46–65.

Narang, S.P. 2004. Important selective media. In*:* food Microbiology. (1st Edition), pp. 9. New-Delhi.India : publishing Cooperation.

Nunez, M., Rodriguez, J.L., Garcia, E., Gaya, P., Medina, M. 1997. Inhibition of Listeria monocytogenes by enterocin 4 during the manufacture and ripening of Manchego cheese. J Appl Microbiol 83: 671– 677.

Ogier, J.C., Serror, P. 2008. Safety assessment of dairy microorganisms: the Enterococcus genus. Int J Food Microbiol 126: 291-301.

Peters, J., Mac, K., Wichmann-Schauer, H., Klein, G., Ellerbroek, L. 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int J Food Microbiol 88: 311-314.

Poeta, P., Costa, D., Rodrigues, J., Torres, C. 2006. Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. Int J Antimicrob Ag 27: 131-137.

Poyart-Salmeron, C., Trieu-Cuot, P., Carlier, C., MacGowan, A., McLauchlin, J., Courvalin, P. 1992. Genetic basis of tetracycline resistance in clinical isolates of *Listeria monocytogenes*. Antimicrob Agents Chemother 36: 463-466.

Robert, M.C. 2003. Tetracycline therapy: Update. Clin Infect Dis 36: 462-467.

Sader, H.S., Jones, R.N., Dowzicky, M.J., Fritsche, T.R. 2005. Antimicrobial activity of tigecycline tested against nosocomial bacterial pathogens from patients hospitalized in the intensive care unit. Diagn Microbiol Infect Dis 52: 203– 208.

Sarantinoupoulos, P., Leroy, F., Leontopoulou, E., Georgalaki, M., Kalantzopoulos, G., Tsakalidou, E., et al. 2002b. Bacteriocin production by Enterococcus faecium FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. Int J Food Microbiol 72: 125– 136.

Smith, R.D., and Coast, J. 2002. Antimicrobial resistance: a global response. Bulletin of the World Health Organisation 80:126-133.

Speer, B.S., Shoemaker, N.B., Salyers, A.A. 1992. Bacterial resistance to tetracycline: mechanism, transfer, and clinical significance. Clin Microbiol Rev 5: 387-399.

Steinkraus, K.H. 1989. Industrialization of Indigenous Fermented Foods. Marcel Dekker, New York.

Tanasupawat, S., Okada, S., Suzuki, K., Kozaki, M., Komagata, K. 1992. Identification of *Enterococcus hirae, E. faecalis, E. faecium, and E. casseliflavus* strains from fermented foods. Bull JFCC 8: 86-94.

Taylor, D.E, Chau, A. 1996. Tetracycline resistance mediated by ribosomal protection. Antimicrob Agents Chemother 40: 1–5.

Thapa, B., Tattawasart, U., Manjai, A., Chantarasuk, Y. 2007. Antimicrobial resistance and species prevalence of enterococcalisolates in srinagarind hospital, Northeastern Thailand. KKU Res J (GS) 7: 97-108.

Tansuphasiri, U., Khaminthakul, D., Pandii, W. 2006. Antibiotic resistance of enterococci isolated from frozen foods and environmental water. Southeast Asian J Trop Med Public Health 37(1): 162-170.

Wilcks, A., Andersen, S.R., Licht, T.R. 2005. Characterization of transferable tetracycline resistance genes in *Enterococcus faecalis* isolated from food. FEMS Microbiology Letters 243: 15-19.

Witte, W. 1997. Impact of antibiotic use in animal feeding on resistance of bacterial pathogens in humans. Ciba Foundation Symposium 207: 61-75.

Appendix

1. Media for culture

1.1. MRS broth (de Man, Rogasa and Sharpe Broth)

A selective medium for the enumeration of lactic acid bacteria in foods

Typical composition (g/L)

Mixed Peptones	10.0	g
Yeast Extract	5.0	g
Beef Extract	10.00	g
Glucose	20.00	g
Potassium phosphate	2.00	g
Sodium acetate	5.00	g
Magnesium sulphate	0.20	g
Manganese sulphate	0.05	g
Tween 80	1.08	g
Ammonium citrate	2.00	g

Directions

1. Weigh 55 grams of MRS powder into the flask
2. Add 1 litre of deionized water into the flask. Allow to soak for 10 minutes, swirl to mix, then warm to completely dissolve solids
3. Disperse into final containers and sterilize by autoclaving at 121ºC for 15 minutes.

1.2. CATC agar (Citrate azide tween carbonate agar)

A selective agar for the identification of enterococci in meat, meat products, dairy products, and other food stuffs.

Typical Composition (g/litre)

Part 1

peptone from casein (pancreatic digest of casein)	15.0	g
yeast extract	5.0	g
potassium dihydrogen phosphate	5.0	g
sodium citrate	15.0	g
polyoxyethylene sorbitanmonooleate (Tween® 80)	1.0	g
agar-agar	15.0	g

Part 2

sodium carbonate	2.0	g
2,3,5-triphenyltetrazolium chloride	0.1	g
sodium azide	0.4	g

Directions

1. Weigh 56 grams of part 1 powder into the flask
2. Add 966 milllitre of deionized water into the flask. Allow to soak for 10 minutes, swirl to mix, then warm to completely dissolve solids
3. Sterilize by autoclaving at 121ºC for 15 minutes.

4. Then, cool the flask in the waterbath (50 °C)

5. Prepare the part 2 ingredients to the solution

   - 20 ml of a 10 % sodium carbonate solution
   - 10 ml of a 1 % 2,3,5-triphenyltetrazolium chloride solution
   - 4 ml of a 10 % sodium azide solution
   - Pour plates. The plates are clear and yellow.

6. Bring part 2 solution to sterile by filter (pore size 0.2 micron)

7. When the temperature of the flask is 50 °C, add sterile part 2 solution into the flask by aseptic technique and swirling slowly

8. Pour into the sterile plates. The plates are clear and yellow.

1.3. CATC broth (Citrate azide tween carbonate broth)

The same ingredient and direction of CATC agar preparation except for agar-agar.

1.4. Peptone water

The diluents for the homogenization of samples.

Typical Composition (g/litre)

Peptone	1.0	g

Directions

1. Weigh 15 grams of peptone water powder into the flask

2. Add 1 litre of deionized water into the flask. Allow to soak for 10 minutes, swirl to mix, then warm to completely dissolve solids
3. Disperse into final containers and sterilize by autoclaving at 121ºC for 15 minutes.

1.5. Plate count agar

Typical Composition (g/litre)

Casein enzymic hydrolysate	5.0	g
Yeast extract	2.5	g
Dextrose	1.0	g
Agar	15.0	g

Directions

1. Weigh 23.5 grams of plate count agar powder into the flask
2. Add 1 litre of deionized water into the flask. Allow to soak for 10 minutes, swirl to mix, then warm to completely dissolve solids
3. Disperse into final containers and sterilize by autoclaving at 121ºC for 15 minutes.
4. Pour into the sterile plates. The plates are clear and yellow.

1.6. Cation-adjusted Mueller-Hinton broth (CAMHB)

Muller Hinton II Broth is intended for use in quantitative procedures for susceptibility testing of facultative anaerobic bacteria. It is formulated to have a low

thymine and thymidine contents and is adjusted to the calcium and magnesium ion concentrations recommended in the CLSI standard M7-A7.

Typical Composition (g/litre)

beef extract	3.0	g
acid hydrolysate of casein	17.5	g
starch	1.5	g

Directions

1. Weigh 22 grams of Muller Hinton II Broth powder into the flask
2. Add 1 litre of deionized water into the flask. Allow to soak for 10 minutes, swir to mix, then warm to completely dissolve solids
3. Disperse into final containers and sterilize by autoclaving at 121ºC for 15 minutes.
4. Pour into the sterile plates. The plates are clear and yellow.

2. Preparation of substances for gel electrophoresis

2.1 EDTA (pH 7.8)

Typical Composition

EDTA	93.05	g
DI water	400	ml
NaOH q.s. to pH 8.0		
DI water q.s.to	500	ml

Directions

1. Weigh 93.05 grams of EDTA into a flask

2. Add 400 millitre of deionized water into a flask

3. Adjust pH to 8.0 by NaOH solution and pH meter

4. Add deionized water into a flask q.s. 500 millitre

2.2 Tris-borate-EDTA buffer solution (5X TBE)

Typical Composition(g/L)

Tris base	54	g
Boric acid	27.5	g
EDTA (pH 8.0)	20	ml
DI water q.s.to	1000	ml

Directions

1. Weigh 54 grams of Tris base and 27.5 grams of boric acid into the flask
2. Add 20 millitre of EDTA (pH 8.0) into the flask
3. Add deionized water qs. 1000 millitre
4. Sterilize by autoclaving at 121ºC for 15 minutes.

0.5x TBE buffer solution were prepared by dilute 5x TBE buffer solution with deionized water 10 folds dilution

2.3 1% agarose gel (50ml) of volume

Typical Composition(g/L)

agarose	0.5	g
0.5X TBE buffer	50	ml

Direction

1. Weigh 0.5 grams of agarose into the conical flask
2. Add 0.5x TBE buffer solution into the flask and swir to mix
3. Heat with microwave for about 1 minute to dissolve agarose
4. Leave it to cool for 5 minutes down to about 60°C
5. While the agarose is cooling, prepare the gel tank ready, on a level surface and insert the comb.
6. Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip.
7. Pour 0.5X TBE buffer into the gel tank to submerge the gel to 2–5mm depth.

2.4 6X Gel loading dye, blue (bromophenol blue and sucrose)

1. Weigh 25 mg of bromophenol blue and 4 g of sucrose into a beaker
2. Add distilled water to 10 ml to beaker
3. Store at 4ºC

3. Preparation of media, starting culture, and instruments using in the screening test of tetracycline resistance in *Enterococcus* spp.

3.1 Plate count agar, plate count agar containing 16 µg/ml of tetracycline

Plate count agar (PCA)

The 23.5 grams of plate count agar powder were weighed into the flask. The one litre of deionized water was added into the flask. It was swirled and warmed to be completely dissolved. Then, the solution of media was sterilized by autoclaving at 121ºC for 15 minutes, and then was poured onto the sterile plates (20 ml each). After that, drawing the bottom of plate as the table was done (Figure 3-4).

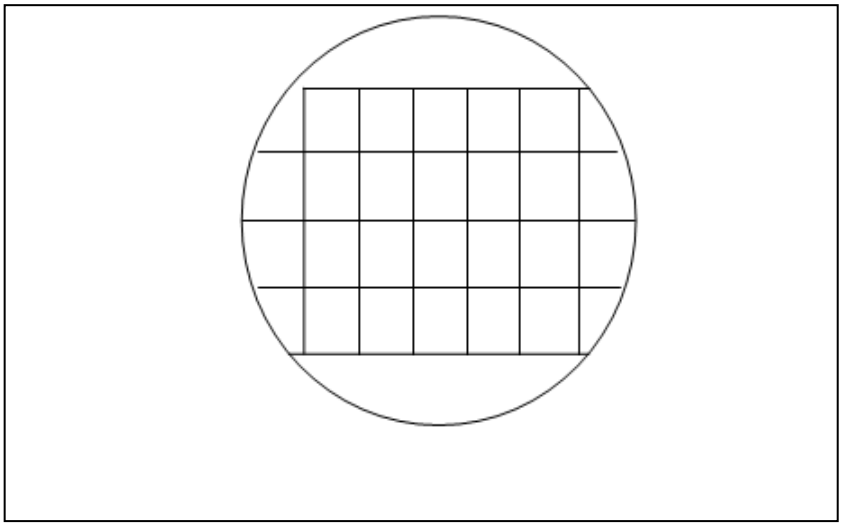


Figure 9 Table on the bottom of plate

Preparation of tetracycline solution

Tetracycline powder (57.7 mg.) was weighed and dissolved in 11 ml of sterile water. So, the concentration of tetracycline solution was 5120 µg/ml. The 1 ml of

tetracycline solution was diluted with 3 ml of sterile water to meet the final concentration at 1,280 µg/ml. Then the 7 ml of sterile water was added in the 1 ml of tetracycline solution (1,280 µg/ml). So, the final concentration of tetracycline solution was 160 µg/ml. And then the solution was sterilized by 0.2 micron of filter.

Plate count agar containing tetracycline 16 µg/ml

The 18 ml of plate count agar solution were prepared into the test tube. Then, the solution of media was sterilized by autoclaving at 121ºC for 15 minutes. Before mixing the media with the sterile tetracycline solution, the media was be cooled at 45-50°C in water bath. The sterile tetracycline solution (160 µg/ml) was mixed with PCA by the ratio of tetracycline solution and PCA was 1:9 (2 ml of tetracycline solution, 18 ml of PCA) that made the concentration be 16 µg/ml. Then it was poured onto the plate.

3.2 Starting culture (*Enterococcus* spp.)

The selected isolates were inoculated by steaking thinly on the surface of plate count agar and incubated at 37°C for 16 hours for a single colonies.

3.3 The instruments for replica plate

The instrument for replica the colony was the skewer that has blunt end. It was washed cleanly and dried, then covered by foil and sterilized by autoclaving at 121ºC for 15 minutes. And then it was dried in hot air oven at 60°C for 30 minutes.

4. Preparation of media, inoculum preparation, and antibiotic discs in disk diffusion method

4.1 Muller-Hinton Agar (MHA)

Muller-Hinton agar was prepared according to the manufacturer's instructions. The pH of each preparation of MHA has be checked and must lie between 7.2-7.4. After that, MHA was steriled by autoclaving at 121ºC for 15 minutes. Then, MHA was cooled at 45-50°C in water bath and were poured onto the sterile plates.

4.2 Inoculum preparation

The selected isolates were inoculated by steaking thinly on the surface of plate count agar and incubated at 37°C for 16 hours for a single colony. After that, the inoculum was prepared by emulsifying three to five colonies from the starting sulture in sterile 0.9% saline. The inoculums were standardized to have a concentration of 1 X 10^8-2 X 10^8 CFU/ml by standardized to have an optical density 0.08-0.13 (1 cm light path) which achieve a turbidity equivalent to 0.5 Mcfarland standard.

4.3 Antibiotic discs preparation

Antibiotic discs of tetracycline (Oxoid, UK) supplied by a reputable manufacturer have concentration per disc at 30 µg and has the diameter of discs approximate to 6 mm. All discs should be stored at 2-8°C.

5. Preparation of media, inoculum, and tetracycline stock solution in broth microdilution method

5.1 Cation supplemented Mueller-Hinton broth (CSMHB)

The 6 ml, 9 ml, and 100 ml of CSMHB broth were prepared according to the manufacturer instructions and adjusted pH of each volume at 7.2-7.4. After that, CSMHB broth was sterilized by autoclaving at 121ºC for 15 minutes.

5.2 Inoculum preparation

The starting culture should be prepared from a culture of the test organism steaked for a single colony on a plate count agar and incubate at 37°C for 16 hours. After that, the inoculum should be prepared by emulsifying one colonies to CSMHB and was incubated at 37°C for 4 hours. After 4 hours, the culture was measured the optimal density by spectrophotometer. The optimal density of the culture was adjusted to achieve 0.08-0.13 (or 1 to 2 x 10^8 CFU/ml) by CSMHB. Then, the culture was diluted to achieve 1:100 dilution by pipeted 0.1 ml into CSMHB 9.9 ml. The final concentration was 1 x 10^6 cfu/ml.

5.3 Tetracycline stock solution

Tetracycline powder (0.01 g) was dissolved in 4.88 ml of sterile water. The concentration of tetracycline was 2,000 µg/ml. The 2.56 ml of tetracycline solution was diluted with 2.44 ml of CSMHB to meet the final concentration at 1,024 µg/ml. Then, the solution was sterilized by 0.2 micron of membrane filter.

6. Recipe of Ready-to-eat Thai traditional fermented product.

6.1Pla-ra (Steinkraus, 2005)

Ingredient

1. Freshwater fish : pla-chorn, pla ta-pian, pla kra-dee, pla-soi, pla ta-kok
2. Salt
3. Rice bran
4. Roasted rice or dried cooked rice

Process

Freshwater fish are descaled, deheaded, eviscerated, and washed with water and then rapidly dried. The prepares fish is mixed with salt in a fish to salt ratio of 3-5:1 by weight and then left at ambient temperature for 12-24 h before packing in an earthen water jar and letting it ferment for 1 month. Then, salted fish is added with roasted rice or rice bran in earthenware jars and held at least 6 months. The production process flow sheet for pla-ra is shown in Fig10.

6.2 pla-jom (วิลาวัณย์ เจริญจิระตระกูล, 2539)

Ingredient

1. Freshwater fish (pla-soi, pla-mhotes)
2. Salt
3. Steamed rice

Process

Freshwater fish are washed with water. The prepared fish is mixed with salt in a fish to salt ratio of 7:3 by weight and then left at ambient temperature for 48 h. After that, salted fish is added with streamed rice ratio of 5:1 by weight and packing in an earthenwater jar and letting it ferment for 3 days.

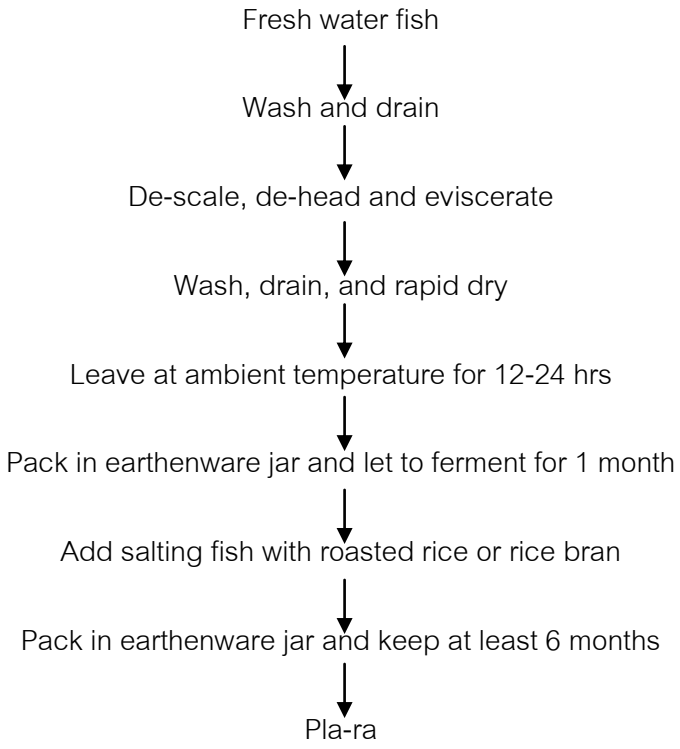


Figure 10 Process flow sheet for plara

6.3 som-fak (Steinkraus, 2005; วิลาวัณย์ เจริญจิระตระกูล, 2539)

Ingredient

1. Fresh water fish e.g. great snakehead
2. Salt
3. Cooked rice
4. Garlic

Process

Freshwater fish are cleaned, filleted, and washed to remove the skin and blood. The flesh is collected and the water is pressed out in a cloth bag; it is then minced and mixed with ingredients consisting of (ratio of fish to each ingredient by weight) cooked rice (6:1), garlic (20:1), and salt (20:1). The mixture is kneaded until the appearance becomes gel-like and forms a sticky, elastic paste. The paste is divided into small portions and then packed and tightly wrapped in banana leaves or plastic sheeting. The raw som-fak is elastic and firm and left to ferment at ambient temperature for 3-5 days. The production process flowsheet for som-fak is shown in Fig11.

Figure 11 Process flow sheet for som-fak

6.4 nham-moo (Steinkraus, 2005; วิลาวัณย์ เจริญจิระตระกูล, 2539)

Ingredient

1. fresh pork, pork rind
2. salt
3. garlic
4. cooked rice and sticky rice
5. potassium nitrate
6. seasonings

Process

All fibrous parts of pork are removed and then the meat is minced. It will provide the proper texture to the end product. In the preparation of pork rind, the hair and excess fat are removed. Then, the skin is boiled and shredded into thin slices. Washing the fresh meat before mincing is avoided because the water can cause product spoilage. The general formulation of nham is as follows: cooked rice and glutinous rice [7–10% (w/w) of mince meat], peeled garlic (3–10%), salt (2–3%), pepper (0–0.5%), and potassium nitrite (0–200 ppm). The quality of nham depends significantly on the quality of ingredients. Rice or sticky rice is cooked separately prior to mixing with other ingredients. Garlic is peeled and minced. Garlic serves as a seasoning agent and also contaminating a microorganism inhibitor. Minced red meat and sliced pork rind are mixed well, then the cooked rice and other ingredients are added and mixed. It was

noted that fermentation of nham was rapid during the first 4 days. The pH was decreased dramatically from 5.65 to 4.45. The production process flowsheet for nham is shown in Fig12.

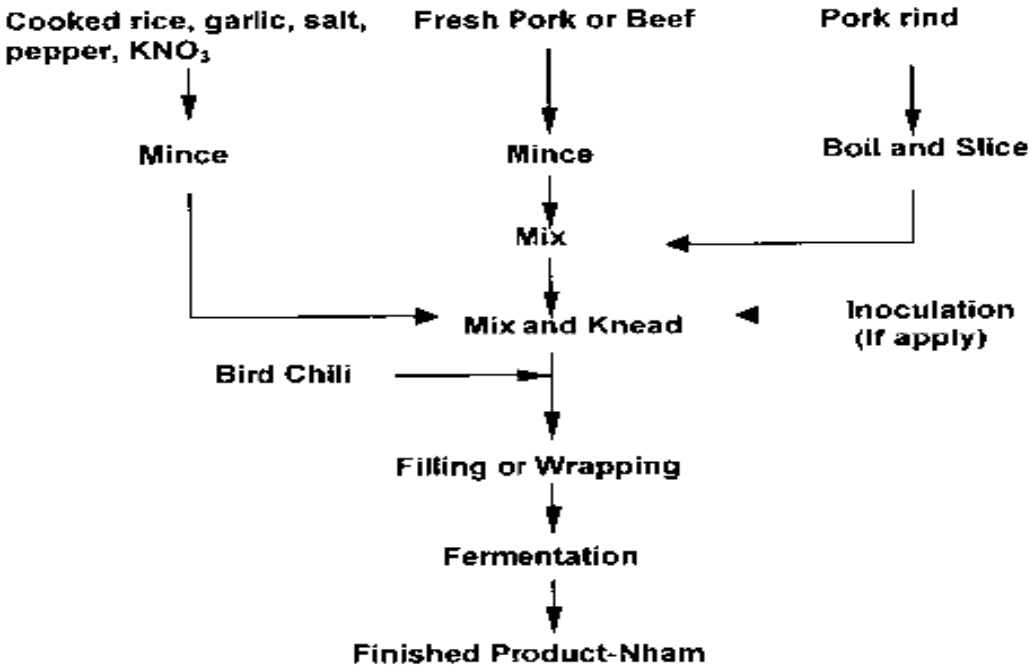


Figure 12 Process flowsheet of the nham

6.5 koung-jom (สุภาพ อัจฉริยศรีพงศ์, 2522)

Ingredient

1. Tiny freshwater shrimp
2. Salt
3. Roasted ground rice
4. Garlic

Process

Tiny freshwater shrimp are washed with tap water and dry to loss water. The tiny freshwater shrimp is mixed with salt, garlic and then letting it ferment for 2 days. After that, salted fish is added with roasted ground rice and packing in an earthenwater jar and letting it ferment for 7-8 days.

6.6 poo-dong (ศูนย์ทางด่วนข้อมูลเกษตร, 2552)

Ingredient

1. Field crab
2. Salt
3. Red line

Process

Dissolve salt to water until it can not soluable. Then, field crab is mixed with salt water and red line and then letting it ferment for 2 days.

6.7 hoi-dong (สุภาพ อัจฉริยศรีพงศ์, 2522)

Ingredient

1. Sea mussel
2. salt
3. sugar
4. fish sauce

Process

Sea mussel are washed with tap water and dry to loss water. The sea mussel is mixed is mixed with salt in a fish to salt ratio of 6-7:1 by weight and then mixed with sugar, fish sauce left in tight condition for 4-5 days.

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