MICROBIOLOGICAL QUALITY OF AND ANTIMICROBIAL RESISTANCE IN COMMERCIAL PROBIOTIC PRODUCTS FOR FOOD ANIMALS IN THAILAND

Miss Hoang My Tran

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2020

คุณภาพทางจุลชีววิทยาและการดื้อยาต้านจุลชีพในผลิตภัณฑ์โปรไบโอติกเชิงพาณิชย์สำหรับสัตว์ที่เลี้ยงเพื่อการบริโภคในประเทศไทย

น.ส.ฮวง มาย ทราน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563

Thesis Title	MICROBIOLOGICAL QUALITY OF AND ANTIMICROBIAL RESISTANCE IN COMMERCIAL PROBIOTIC PRODUCTS FOR FOOD ANIMALS IN THAILAND
By	Miss Hoang My Tran
Field of Study	Veterinary Science and technology
Thesis Advisor	Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor CHANNARONG RODKHUM, D.V.M., Ph.D.)

..... Thesis Advisor

(Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.)

..... Examiner

(Professor ALONGKORN AMONSIN, D.V.M., Ph.D.)

..... Examiner

(TARADON LUANGTONGKUM, D.V.M., Ph.D.)

..... External Examiner

(Associate Professor Sunpeth Angkittitrakul, D.V.M., M.Sc., Ph.D.)

ฮวง มาย ทราน :

คุณภาพทางจุลชีววิทยาและการดื้อยาต้านจุลชีพในผลิตภัณฑ์โปรไบโอติกเชิงพาณิชย์สำหรับสัตว์ที่เลี้ยงเพื่อการบริโภคในประเทศไทย. (MICROBIOLOGICAL QUALITY OF AND ANTIMICROBIAL RESISTANCE IN COMMERCIAL PROBIOTIC PRODUCTS FOR FOOD ANIMALS IN THAILAND) อ.ที่ปรึกษาหลัก : ศ. สพ.ญ.ดร.รุ่งทิพย์ ชวนชื่น

การศึกษาฉบับนี้ได้ทำการศึกษาเพื่อตรวจสอบปริมาณสายพันธุ์และยีนดื้อยาต้านจุลชีพของเชื้อแบคทีเรียในผลิตภัณฑ์โพรไบโอติกส์สำหรับปศุสัตว์ จำนวน 45 ตัวอย่าง โดยทำการนับจำนวนเชื้อแบคทีเรียสายพันธุ์ *Lactobacillus Bacillus* และ *Enterococcus* ในตัวอย่างทั้งหมด วิธีการทดสอบยืนยันสายพันธุ์ของเชื้อแบคทีเรีย *Lactobacillus* (n=20) และ *Enterococcus* (n=20) ใช้เทคนิค Multiplex polymerase Chain Reaction (PCR) เชื้อ *Bacillus* (n=190) ใช้เทคนิค Amplified Ribosomal DNA Restriction Analysis (ARDRA) และ เชื้อ *Clostridium* (n=4) ใช้เทคนิค PCR นอกจากนี้ได้ทำการทดสอบการปนเปื้อนเชื้อ *E. coli* และ *Salmonella* ในผลิตภัณฑ์ทั้งหมด จากนั้นทำการตรวจหาความไวรับของเชื้อต่อ ยาต้านจุลชีพด้วยการหาค่า Minimal Inhibitory Concentrations (MICs) ต่อยาต้านจุลชีพจำนวน 14 ชนิดในเชื้อแบคทีเรียที่แยกได้ (n=64) จากนั้นทำการทดสอบหายีนดื้อยาที่สำคัญทางคลินิกจำนวน 111 ยีน ในตัวอย่างโพรไบโอติกส์ 45 ตัวอย่าง รวมถึงทดสอบความสามารถในการส่งต่อยีน (gene transferability) ในเชื้อแบคทีเรียที่แสดงออกถึงการดื้อยาต้านจุลชีพ ผลการทดสอบพบว่าในผลิตภัณฑ์จำนวน 34 จาก 45 ตัวอย่าง (75.5%) แสดงจำนวนหรือชนิดของเชื้อของแบคทีเรียไม่ตรงกับฉลาก ไม่พบผลิตภัณฑ์ใดที่มีการปนเปื้อนเชื้อ *E. coli* และ *Salmonella* การดื้อยาของเชื้อโพรไบโอติส์พบว่า 33 จาก 64 isolates (51.6%) มีการดื้อยาต้านจุลชีพอย่างน้อย 1 ชนิด โดยการดื้อยาและเปอร์เซ็นต์ของการดื้อยาแต่ละชนิดดังนี้ chloramphenicol (21%), trimethoprim (17%), clindamycin (16%) sulfamethoxazole (15%), ampicillin (10%), erythromycin (9%), vancomycin (9%), tetracycline (8%), ciprofloxacin (6%), streptomycin (5%) และ kanamycin (5%) ผลิตภัณฑ์ 16 จาก 45 ตัวอย่างพบยีนดื้อยาอย่างน้อยหนึ่งชนิดดังต่อไปนี้ beta-lactamase ($bla_{OXA-1-like}$ และ bla_{SHV}), ciprofloxacin [*oqxAB, qnrD, aac(6')-Ib-cr, qrnB,* และ *qnrS*), streptomycin (*aadA1, aadA2, aadE* และ *strA-strB*), gentamicin [*aac(3)-II* และ *aac(6')-aph(2'')*], kanamycin [*ant(4')-Ia* และ *aph(3')-IIIa*], tetracycline *(tetA, tetB, tetL* และ *tetM*), choramphenicol (*catA* และ *cmlA),* macrolide (*mefA),* trimethoprim (*dfrA12* และ *dfrA14),* sulfonamide (*sul1*) และ vancomycin (*vanC*) โดยยีนดื้อยาที่พบทั้งหมดไม่สัมพันธ์กับคุณสมบัติการแสดงออกการดื้อยาต้านจุลชีพของเชื้อ นอกจากนี้เชื้อ *Lactobacillus* ที่ดื้อต่อยา streptomycin จำนวน 3 isolates สามารถถ่ายทอดพันธุกรรมการดื้อยาได้แบบ horizontal transfer จากการศึกษานี้แสดงให้เห็นว่าผลิตภัณฑ์โพรไบโอติกส์สามารถเป็นแหล่งแพร่ยีนดื้อยาต้านจุลชีพและอาจไม่ส่งผลประโยชน์ให้กับตัวสัตว์ ดังนั้นจึงจำเป็นอย่างยิ่งในการควบคุมคุณภาพผลิตภัณฑ์โพรไบโอติกส์ให้ดียิ่งขึ้น

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6175407031 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY
KEYWORD: antimicrobial resistance food animals probiotics Thailand

Hoang My Tran : MICROBIOLOGICAL QUALITY OF AND ANTIMICROBIAL RESISTANCE IN COMMERCIAL PROBIOTIC PRODUCTS FOR FOOD ANIMALS IN THAILAND. Advisor: Prof. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

A total of 45 commercial probiotic products for food animals were investigated for the number of viable cells, bacterial species and the presence of antimicrobial resistance (AMR) genes. All products were enumerated for viable bacterial cells of *Lactobacillus*, *Bacillus* and *Enterococcus*. Confirmation of species was carried out by multiplex Polymerase Chain Reaction (PCR) for *Lactobacillus* (n=20), *Enterococcus* (n=20), and Amplified Ribosomal DNA Restriction Analysis (ARDRA) for *Bacillus* (n=190). The presence of *Clostridium* species was examined by PCR. The contamination of *E. coli* and *Salmonella* was also determined. Minimal Inhibitory Concentrations (MICs) for 14 antimicrobials was examined in the bacterial isolates obtained (n=64). The presence of 111 genes encoding resistance to clinically important antibiotics was tested in probiotic products (n=45). Possible resistance gene transferability was investigated in the isolates with resistance phenotype. The results showed that 34 of 45 products (75.5%) were incorrectly labeled in either numbers of viable cells or bacterial species or both. None of the products tested were contaminated with *E. coli* and *Salmonella*. Thirty-three out of 64 isolates (51.6%) exhibited resistance to at least one antimicrobial agent. Resistance to chloramphenicol (21%) was highest among probiotic bacteria, followed by trimethoprim (17%), clindamycin (16%) sulfamethoxazole (15%), ampicillin (10%), erythromycin (9%), vancomycin (9%), tetracycline (8%), ciprofloxacin (6%), streptomycin (5%) and kanamycin (5%). Sixteen in 45 products (35.5%) were positive to at least one AMR genes, including genes encoding resistance to β-lactamase ($bla_{\text{OXA-1-like}}$ and bla_{SHV}), ciprofloxacin [*oqxAB*, *qnrD*, *aac(6')-Ib-cr*, *qrnB*, and *qnrS*), streptomycin (*aadA1*, *aadA2*, *aadE* and *strA-strB*), gentamicin [*aac(3)-II* and *aac(6')-aph(2'')*], kanamycin [*ant(4')-Ia* and *aph(3')-IIIa*], tetracycline (*tetA, tetB, tetL* and *tetM*), chloramphenicol (*catA* and *cmlA*), macrolide (*mefA*), trimethoprim (*dfrA12* and *dfrA14*), sulfonamide (*sul1*) and vancomycin (*vanC*). Almost AMR genes detected in probiotic products were not correlated to AMR phenotype of probiotic bacteria found in these products. Three streptomycin- resistant *Lactobacillus* isolates could horizontally transfer their resistance determinants. The findings demonstrated that the probiotic products could serve as reservoirs for spread of AMR genes and may not yield benefits to animals as claimed. The observations highlight the need for the adequate quality control of probiotic products.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my deep gratitude and appreciation to Prof. Dr. Rungtip Chuanchuen to be my advisors, for her patient guidance, advices, enthusiastic encouragement and useful critiques throughout my study.

I would like to sincerely thank my thesis committee members, Prof. Dr, Alongkorn Amosin, Assc. Prof. Dr. Channarong Rodkhum, Dr. Taradon Luangtomkum, and Assc. Prof. Dr. Sunpetch Angkititrakul for their valuable comments and advices.

I would like to express my sincere thanks to all staffs and friends in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for their warm and kind support during my research.

I would like to thank Huvepharma Company for their financial support of my research. In particular, I would like to appreciate Dr. Sumeth Sapchukun, Dr. Korntip Kanjavaikoon, and their team from Huvepharm Company for their kindly support during my research work.

Last but not at least, I would like to express my deepest gratitude to my family for giving me lots of encouragements and supporting me spiritually throughout my life and my study.

Hoang My Tran

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ARDRA	Ribosomal DNA restriction analysis
BAE	Bile Aesculin agar
BHI	Brain Heart Infusion
bp	base pair (s)
BPW	Buffered Peptone Water
CFU	colony-forming unit
CLSI	Clinical & Laboratory Standards Institute
CVM	Center for Veterinary Medicine
°C	degree Celsius
DNA	Deoxyribonucleic acid
EC	European Commission
ECOFFs	Epidemiological cut-off values
EMB	Eosin Methylene Blue
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
etc.	et cetara, so on
et al.	et alii, and others
DLD	Department of Livestock Development
FAO	Food and Organization
h	hour (s)
H_2S	hydrogen sulfide
ISR	Intergenic spacer regions
IST	Iso-Sensitest
g	gram
GIT	Gastrointestinal tract
GRAS	Generally Recognised as Safe
KF	Kenner Fecal

kg	kilogram
LAB	lactic acid bacteria
LB	Luria-Bertani
LSM	Lactic acid bacteria susceptibility test medium
M	molar
MCK	MacConkey
mg	milligram (s)
min	minute (s)
ml	millilitre (s)
MHA	Muller-Hinton agar
MIC	Minimum inhibitory concentration
MRS	De Man, Rogosa and Sharpe
MSRV	Modified Semi-Solid Rappaport-Vassiliadis
MYP	Mannitol Egg Yolk Polymyxin
NaCl	sodium chloride
NSS	normal saline solution
PCR	polymerase chain reaction
PSD	peptone saline diluting
pH	potential of hydrogen
QPS	Qualified Presumption of Safety
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
sec	second (s)
spp.	species
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron
TAE	Tris-Acetate-EDTA
US FDA	United State Food and Drug Administration
UV	Ultraviolet
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

%	percentage
µg	microgram (s)
µl	microliter (s)

CHAPTER I INTRODUCTION

Global population has increased with the prediction to reach more than 9 billions by 2050. Thus, the food demand is expected to drive up between 59% - 98% (Valin et al., 2014). Due to economic growth, the consumption of animal-sourced products has increased and generated pressure on the livestock sector. Livestock production is one of the fastest growing sectors in agriculture, contributing around 40% and 20% of the global value of agricultural production in developed and developing countries, respectively (FAO, 2018). However, the intensification of animal production has led to an increasing overall use of antimicrobials. The antimicrobial consumption was 93,309 tons in 2017 and is expected to rise by 11.5% to 104,079 tons in 2030 (Tiseo et al., 2020). Such increase in antimicrobial use has primarily considered a major cause of emergence and spread of antimicrobial resistance (AMR) in bacteria.

In recent decades, the increase of AMR in bacterial pathogens is considered one of the significant global public health concerns. The issue has affected human, animal, plant and environmental health and so referred to as One Health issue. According to World Health Organization (WHO), approximate 700,000 people die each year as a result of infections with AMR bacteria and 60% of all human diseases have originated from animals (WHO, 2018). Currently, the AMR issue has generated the implications to food safety, food security and economic system worldwide (FAO, 2016a). Food plays an important role in development and spread of AMR bacteria to humans. If the AMR bacteria are pathogens, they can cause human illnesses that may not be treated with antibiotics currently available. If the AMR bacteria are not themselves pathogenic, they can be a reservoir of resistance determinants that may be transmitted to other bacterial species.

Attempts to reduce antimicrobial use to minimize the emergence and spread of AMR have been conducted in almost all parts of the world. The strategic actions include the enforcement of law and regulation for antimicrobial usage in animals,

promotion of diseases prevention program, production and application of alternatives to antibiotics etc. In European countries, the use of antibiotic as growth promoters (AGPs) in food animals has been banned under Regulation (EC) No. 1831/2003 since 2006. In Thailand, the Department of Livestock Development (DLD) has launched Animal Feed Quality Control Act B.E. 2558 (2015) to prohibit the direct use of active pharmaceutical ingredients in animal feed (Lekagul et al., 2018). The Act has been enforced for all livestock sector throughout the country.

Alternatives to antibiotics have been researched, and their applications to replace antibiotics have gained popularity, especially in the food animal production sector. Probiotic product is one of the most popular antibiotic alternatives that are widely used in food animals for a long time. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). Several species belonging to the genera of *Lactobacillus*, *Bacillus,* and *Enterococcus* are commonly used as animal-feed probiotics. Currently, the commercially available probiotic products are formulated using a single to multi-strains or species (FAO, 2016b).

The basic properties of probiotic products for animals were defined (FAO, 2016b). The number of probiotic bacteria used in feed additives is usually about 10^{10} CFU/g, while pre-mixtures contain 10^{8} CFU/g (Coeuret et al., 2004). For the safety purposes, microorganisms used as probiotics in animal feed should be examined to ensure accurate identification of species/strains, not being a cause of infection, no toxin production, and lack of transferable AMR genes (EFSA, 2007). Recently, several studies have demonstrated that the actual quality of some commercial probiotic products deviated from the declared label (Weese, 2003; Wannaprasat et al., 2009). The common deviations included low levels of viable bacterial cells and misidentified species of microorganisms. The presence of bacterial species/strains not stated by manufacturers was frequently found. Health benefits of probiotics are dependent on the appropriate number of viable bacterial cells and loss of the probiotic-bacterial

cells will cause the loss of probiotic effects. The safety and functionality of probiotics depend on species and strains of the probiotic microorganisms. *Lactobacillus* spp. is a member of gut flora and is rarely associated with infections (Adams and Marteau, 1995). However, *Bacillus* spp., *Enterococcus* spp. and *Clostridium* spp. have been implicated in food poisoning and clinical symptoms (Cassir et al., 2016; Elshaghabee et al., 2017; Hanchi et al., 2018). Besides, the risk of contamination of pathogenic bacteria (e.g., *Salmonella* and *Escherichia coli*) is of particular concern and cannot be underestimated. The presence of *E. coli* and *Salmonella* in probiotic products of human was demonstrated in previous finding and this can be detrimental to consumer's health (Joosten et al., 2006; Makut et al., 2014). However, the studies of adverse bacteria contaminated in probiotic products used for animal feed are still limited. Therefore, the particular concerns have been raised for both beneficial effects and potential health risks of probiotic products for animal consumption.

Importantly, the presence of AMR bacteria and determinants in several probiotic products was previously reported (Wannaprasat et al., 2009). Previous studies demonstrated that many probiotic bacteria were resistant to various clinically important antibiotics and carried resistance determinants potentially transferred to commensal flora and pathogenic bacteria in gut through horizontal gene transfer (Imperial and Ibana, 2016). Due to horizontal gene transfer, concerns are still raised particularly in probiotic strains that carry mobile genetic elements such as plasmids, transposons and integrons. In a recent study, it was found that plasmid encoded genes such as *erm*(B) and multiple *tet* genes were successfully transferred between *Lactobacillus* spp and bacterial pathogens *in vivo*, *in vitro* and during food fermentation (Thumu and Halami, 2019). The transferable tetracycline resistance gene, *tet*(L) found in *Bacillus* spp was encoded by a plasmid (Phelan et al., 2011). Therefore, the use of such probiotics in animal feed may be a double-edged sword, leading to a wide distribution of AMR and failure in the implementations for combating AMR (Imperial and Ibana, 2016).

The United State Food and Drug Administration (US FDA) and European Food Safety Authority (EFSA) have developed the concept of Generally Recognised as Safe (GRAS) and Qualified Presumption of Safety (QPS), respectively, for safety evaluation of microorganisms intentionally introduced into the human food and animal feed (EFSA, 2007; FDA, 2019). However, in Thailand, the regulation of probiotic products, either imported or locally manufactured, has not been clearly stated and those existing for animal feed have not been effectively enforced. The mechanisms for quality control of the products remain largely unclear. At the product registration process, there is no specific requirement for the detection of microorganism number, species and AMR determinants. Most studies on probiotic products for food-producing animals in Thailand have focused on testing the effectiveness, but not the safety of probiotics in terms of the microbiological quality and the potential contribution to the spread of AMR determinants. This will open a chance for dispersing poor-quality products and introducing AMR bacteria and determinants into the farms. Therefore, research studies to examine the microbiology quality of and AMR in probiotic products commercially available for food animals in Thailand are required.

Objectives of Study

1. To isolate, enumerate and identify common probiotic bacteria (*Lactobacillus, Bacillus,* and *Enterococcus*) in probiotic products for food animals in Thailand.
2. To detect the presence of *Clostridium* species in probiotic products for food animals in Thailand.
3. To detect the contamination of *E. coli* and *Salmonella* in probiotic products used for food animals in Thailand.
4. To examine the AMR characteristics of bacterial strains formulated in probiotic products for food animals in Thailand.

Questions of Study

1. What are the number and species of common probiotic bacteria (*Lactobacillus*, *Bacillus* and *Enterococcus*) in probiotic products for food animals in Thailand?
2. What are species of *Clostridium* found in probiotic products for food animals in Thailand?
3. Is there any contamination of *E. coli* and *Salmonella* in probiotic products for food animals in Thailand?
4. What are characteristics of AMR of bacterial strains formulated in probiotic products for food animals in Thailand?

CHAPTER II LITERATURE REVIEW

1. Probiotic: definition and classification

As AMR continues to evolve and spread, it is crucial to minimize the use of antibiotics in food animals and develop alternatives to antibiotics. Alternative products, including bacteriophages, phytochemicals, antimicrobial peptides, organic acids, probiotics, prebiotics, immune modulators and vaccines, play a crucial role in allowing farmers and veterinarians to simultaneously prevent infections and improve animal performance (Papatsiros et al., 2013).

The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) defined that probiotics are "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). The microorganisms used as probiotics can be categorized as follows bacterial vs. non-bacterial probiotics, spore-forming vs. non-spore forming probiotics, single-strain probiotics (or single-species) probiotics vs. multi-strain (or multi-species) probiotics, allochothonous probiotics vs. autothonous probiotics. The source of microorganisms used for probiotic production can be originated from humans or animal origins like microflora and food sources like fermented milk. The strain and species should be accurately identified before use (Shewale et al., 2014). The non-pathogenic microorganisms chosen to produce probiotics should survive in gastrointestinal environment with low pH and high concentration of bile and enable to adhere to intestinal epithelium (Collado and Sanz, 2006; Collado et al., 2007). Moreover, the bacterial probiotics should have capacity to withstand and maintain their viability and desired functionalities during production, transportation, storage and handling (Collins et al., 1998). Probiotic microorganisms are generally manufactured by fermentation, followed by drying processes (FAO, 2016b).

2. Mode of action of probiotics

The mode of action of probiotics, which appears to be different among probiotic bacteria species/strains (Fioramonti et al., 2003), are generally rested on competitive exclusion, bacterial antagonism, and immunomodulation (Yirga, 2015). Competitive exclusion is indicated that beneficial bacteria supplemented to the animal feed compete with pathogenic bacteria concerning adhesion sides and nutrients in guts (Yirga, 2015). Certain strains of *Lactobacillus* can reduce the growth of pathogens like *Salmonella* and *E. coli* O157:H7 by competitively adhering to epithelial cells (Hudault et al., 1997; Johnson-Henry et al., 2007). In terms of bacterial antagonism or antimicrobial substance production, probiotics can secrete many antimicrobial substances, including bacteriocins, organic acids, biosurfactants, and hydrogen peroxide, that can inhibit the growth of pathogenic bacteria in GIT (Hossain et al., 2017). For example, lactic acid bacteria (LAB) release lactic acids and acetic acids that reduce the pH of GIT to a lethal level for harmful pathogens (Fayol-Messaoudi et al., 2005). In addition, LABs can produce bacteriocins which can bind the cell wall precursor to form pores in the cell wall of pathogenic microbes leading to fluid loss and bacterial death (Hassan et al., 2012). Probiotics can be able to affect both innate and adaptive immunity. On the one hand, probiotics can improve the function of an epithelial barrier, which is the first line of defence of GIT but is easily disrupted by stress factors and disease conditions (García-Lafuente et al., 2001). On the other hand, certain probiotics can stimulate the adaptive immune system in animals by either drifting through the intestinal wall as viable cells or multiply to a limited amount, and thereby the dead organisms can release the antigens which are absorbed and directly stimulate the immunity (Fuller, 1992). Several studies showed that probiotics can exert their immunostimulatory effects by producing cytokines and antibodies and enhancing phagocytic activity, thus preventing invasion of entero-pathogens (Dunne et al., 2001; Bai et al., 2013)

3. Global regulatory agencies of probiotics used for food animals

Although most microorganisms used as probiotics in animal feed are apparently considered safe, some microbial species and/or strains may theoretically pose risks described as follows gastrointestinal/systemic infection of animal consumed the probiotic, handlers of animal and animal feed, consumers who consume animal products produced by animals fed probiotics; transfer of antibiotic resistant determinants from probiotic bacteria to other pathogenic microbes; production of toxins by probiotic bacteria in the host; contamination of detrimental microorganisms or harmful compounds from the animal production systems to the environments; and hyper-stimulation of host's immunity (Marteau, 2001; Doron and Snydman, 2015). Therefore, the microorganisms intentionally added to food or feed additives should be assessed against the above-mentioned risks on a case-by-case basis.

3.1 Qualified Presumption of Safety (QPS)

In Europe, the European Food Safety Authority (EFSA) was established to provide scientific evidence and carry out risk assessments of food and feed and their effects on the environment. EFSA has used the QPS since 2007 as a generic safety pre-assessment tool to support the risk assessment of a microorganism intended to introduce into the food chain (EFSA, 2007). According to the QPS concept, a safety assessment of a biological taxonomic group, including genus or group of related species, can be made based on four pillars, such as establishing identity, body of knowledge, possible pathogenicity, and end use (EFSA, 2007). If the taxonomic group does not pose any risk or the risk can be unambiguously defined and excluded, it could be granted the QPS status (EFSA, 2007). Thenceforth, any microorganisms assigned a QPS group would be freed from a pre-market safety assessment other than satisfying any pre-determination of specific qualifications (EFSA, 2007). In contrast, microorganisms without QPS status would be subject to a full pre-market safety assessment (EFSA, 2007). The QPS status can only be used to prove the safety of

microorganisms but not the safety of products containing such microorganisms (EFSA, 2007). There are more than 100 species of microorganisms under QPS status, which are generally classified into four groups (i) Gram-positive non-sporulating bacteria, (ii) *Bacillus* species, (iii) yeasts and (iv) filamentous fungi (EFSA, 2007). Probiotic products can only be marketed following assessment and approval from EFSA and authorization under EU regulation (EC) No. 1831/2003 and (EC) No. 429/2008. The authorization of Europe Commission (EC) granted for new probiotics is valid for ten years and should be renewed thereafter.

3.2 Generally Recognized as Safe (GRAS)

The Center for Veterinary Medicine (CVM), which is a branch of the United States Food and Drug administration (US FDA), is responsible for regulations of animal feed products. CVM has a GRAS for microorganisms used for food processing and animal feed (FDA, 2018). The microorganisms are GRAS based either on a history of safe use in animal feed (before 1958) or on scientific justification (FAO, 2016b).

4. General characteristics of probiotic bacteria

4.1 *Lactobacillus*

The genus *Lactobacillus* is microaerophilic, rod-shaped, non-spore-forming, acid-tolerant, Gram-positive bacteria belonging to a group of LAB, which ferment sugar to produce lactic acid (Makarova et al., 2006). Due to acid-tolerant ability, *Lactobacillus* can adapt to grow in various environmental conditions, so they can be found in milk, dairy products, fermented food and intestinal tracts of humans and animals (Brashears et al., 2005). Regarding taxonomy, the studies about 16S rRNA genes of *Lactobacillus* have shown significant variety in this genus. More than 180 species have been identified until now, but many were later undergone genera reclassification thus, expanding the species and subspecies in taxonomic rank (Pot et al., 2014). *Lactobacillus* was considered one of the safest candidate microorganisms as probiotics due to, first it has been used to produce traditional fermented food such as yogurt, pickles, e.g.

(Bernardeau et al., 2006); second, the bacteria was a natural inhabitant of GIT in large quantities; and finally, there are some rare infection cases associated with these bacteria (Adams and Marteau, 1995). Until now, there are 37 *Lactobacillus* species included in the EFSA QPS list (EFSA, 2020) of which several species generally used in animal feed such as *L. acidophilus, L. casei, L. delbrueckii sub* sp. *bulgaricus, L. brevis, L. cellobiosus, L. curvatus, L. fermentum, L. plantarum, L. reuteri, L. salivarius sub* sp. *thermophiles* and *L. gasseri* (Dowarah et al., 2017). Due to a wide genetic diversity in this genus, it is necessary to find a reliable identification method to find candidate *Lactobacillus* strains used for probiotics (Nakagawa et al., 1994; Heilig et al., 2002). Because of limitations for conventional methods such as biochemical and physiological tests (Berthier and Ehrlich, 1999), many studies have been performed using molecular techniques for rapid discrimination of *Lactobacillus* species including DNA-DNA hybridization, sequencing, Restriction Fragment Length Polymorphism (RFLP) and analysis of 16S/23S rRNA sequences (Pot et al., 2014). Multiplex PCR using a combination of sequences 16S and 23S rRNA genes and intergenic spacer regions (ISR) was developed and has become one of optimal methods for rapid identification of many species at the same time (Kwon et al., 2004).

4.2 *Bacillus*

The genus *Bacillus,* a spore-forming, Gram-positive, obligate aerobic or facultative anaerobic bacteria, has been used as probiotics for more than 50 years (Cutting, 2011). *Bacillus* is ubiquitous, so it is commonly isolated from food, plants, soil, aquatic environment, and GIT of animals such as pigs, chickens, ruminants and aquatic animals (Mingmongkolchai and Panbangred, 2018). The presence of *Bacillus* in GIT and feces of animals is associated with the ingestion of contaminated food because *Bacillus* is generally considered as allochthonous microorganism (Hong et al., 2005). Endospores produced by *Bacillus* can survive without nutrients in harsh environmental conditions such as heat, UV radiation, solvents and enzymes (Nicholson et al., 2000). Based on spore-forming characteristics, *Bacillus* can be long-term storage at room

temperature without loss of viability and survive at acidic pH of gastric barrier that can eventually reach the small intestine to exert its effects (Barbosa et al., 2005). There are more than 100 species and subspecies of *Bacillus* genus, however, only several *Bacillus* species have been on the list of QPS status, including *B. subtilis, B. licheniformis, B. clausii, B. coagulans, B. amynoliquencies, B. atrophaeus, B. fusiformis, B. lentus, B. megaterium, B. mojavensis, B. pumilus, B. subtilis* and *B. vallismortis* (EFSA, 2007). *B. cereus* and *B. thuringiensis*, which have been implicated in foodborne diseases because of the production of enterotoxins, are not proposed for QPS (EFSA, 2007). Interestingly, the product Toyocerin® containing *B. cereus* var. *toyoi* was approved by the European Committee in 2001 due to this species was proven non-pathogenic and incapable of transferring antibiotic resistance genes to other bacteria. Analysis of the 16S rRNA sequence has also been one of the most reliable methods for rapid identification of *Bacillus* species (Wang et al., 2003), of which a group-specific PCR accomplished by amplified ribosomal DNA restriction analysis (ARDRA) has proved to be a suitable method for the classification of most important *Bacillus* species in the environment (Wu et al., 2006).

4.3 *Enterococcus*

Enterococcus is also a member of LAB group including both pathogenic and commensal microorganisms in intestine of humans and animals (Facklam et al., 2002). They are Gram-positive, ubiquitous, facultative anaerobic and non-spore forming organisms (Facklam et al., 2002). The most common *Enterococcus* species used as probiotics are *E. faecalis* and *E. faecium*, which were formerly classified as group D *Streptococcus* until the year 1984, when a distinct genetic characteristic was analysed, resulting in *Streptococcus faecalis* and *Streptococcus faecium* were renamed as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer and Kilpper-Bälz, 1984). Bacteriocins produced by several *Enterococcus* have become a promising trait of probiotic, which are considered as either food preservatives or alternatives to antibiotics (Cotter et al., 2013). Nevertheless, the *Enterococcus* species are also

opportunistic pathogens, which are associated to nosocomial infection and human disease such as endocarditis and bacteraemia (Morrison et al., 1997). Such pathogenic strains can confer virulence factors and antimicrobial resistance genes (Franz et al., 2011). There are different virulence factors implicated in pathogenesis of enterococci, including aggregation substance (Shankar et al., 2002), cytolysin (Coburn and Gilmore, 2003), gelatinase (Singh et al., 2005) and surface adhesion (Chandler et al., 2005). Besides, some foodborne species of *Enterococcus* were found to harbour resistance genes, and the transmission of resistance genes and virulence determinants due to mobile genetic elements such as conjugative plasmid and transposons was described in previous studies (Cocconcelli et al., 2003; Hummel et al., 2007a). Although some species of *Enterococcus* have been used as probiotics for a long time, due to lack of information on the safety, the *Enterococcus* species have not yet granted QPS status (EFSA, 2007). It is necessary to accurately identify the which species can cause disease to apply a correct treatment for each pathogenic strain. While classical methods including biochemicals tests have become less accurate and reliable, the multiplex PCR using genus- and species- specific primers to 16S rRNA genes was developed to provide a simple, efficient and reliable method to identify 23 species of *Enterococcus* (Jackson et al., 2004).

4.4 *Clostridium*

The genus *Clostridium* is Gram-positive, spore-forming, rod-shaped and obligate anaerobes bacteria (Cassir et al., 2016). They can be found in soils and intestinal tract of humans and animals. Whereas non-toxigenic strains, *C. butyricum,* are currently used as probiotic in Asia, other strains have been human pathogens, such as botulism and tetanus (Cassir et al., 2016). EFSA has issued some opinions on the safety and efficacy of Miya-Gold® formulated by *C. butyricum* as active ingredients for pigs and chickens (EFSA, 2011).

5. Antimicrobial resistance associated with probiotics

Besides many health benefits of probiotics, there are concerns regrading to the safety of probiotics, especially the potential transfer of AMR determinants in the gut bacterial population. Resistance to antibiotics and transferable ability of resistance genes have been observed in probiotic species (EFSA, 2007).

There are two major pathways of transfer of resistant bacteria and their AMR determinants consist of (i) clonal transfer or vertical transfer of resistant bacteria of food animal origin or (ii) horizontal transfer of AMR genes of food animal origin to humans (FAO, 2016a). Horizontal gene transfer occurs through three main mechanisms including (i) transformation is the uptake of free DNA from extracellular environment; (ii) conjugation is the transfer of DNA via cell-to-cell contact between donor and recipient bacteria; and (iii) transduction requires bacteriophages to transfer the genes between two bacteria (FAO, 2016a).

Acquired antibiotic resistance genes have been found in many *Lactobacillus* species, of which tetracycline resistance genes (*tet*) have been detected in high frequency. For example, *tet(M)* was found in *L. brevis, L. paracasei, L. plantarum, L. salivarius* (Devirgiliis et al., 2009; Nawaz et al., 2011; Thumu and Halami, 2019). The *erm(B)* genes coding for erythromycin resistance was found in several *Lactobacillus* species (Nawaz et al., 2011; Thumu and Halami, 2012). Some resistant genes found in *Lactobacillus* species were harboured by plasmids (Gfeller et al., 2003; Huys et al., 2006). However, the studies of resistant-gene transferability are still limited (Rossi et al., 2014). Regarding vancomycin, some *Lactobacillus* species intrinsically displayed resistance without the capability of horizontal gene transfer (Klein et al., 2000).

Resistance to antibiotics and transferable ability of resistance genes have been observed in several *Bacillus* species (EFSA, 2007). Tetracycline resistance genes (*tet*) have frequently been detected on mobile elements of *B. subtilis*, of which *tet*(M) was found on conjugative transposon Tn5397 (Roberts et al., 1999) and *tet*(L) was encoded

by a plasmid (Phelan et al., 2011). Regarding macrolides resistance in *Bacillus* species, the *erm*(C) was found on a plasmid of *B. subtilis* (Monod et al., 1986) while *erm*(D) is the most prevalence but transferability of this gene has not been determined (Gryczan et al., 1984; EFSA, 2007)

6. Probiotic products available for food animals in Thailand

The use of probiotics as feed additives has gained popularity in Thailand. This is a result of the ban of all antibiotics used for growth promoters in food animals by the Food and Drug Administration (FDA), Ministry of Public Health. FDA works in cooperation with the Department of Livestock Development (DLD), Ministry of Agriculture and Cooperatives in the regulation of veterinary drugs. With the development and expansion of animal feed business, a new government unit, the Division of Animal Feed and Veterinary Product was established within the DLD to control animal feed under Animal Feed Quality Control Act. Under the Animal Feed Quality Control Act B.E. 2558, animal feed must be registered prior to domestically manufacturing or importing into Thailand. At present, the data of probiotics used for food animals available in Thailand has not been completed, and it is impossible to determine an exact number of products available in Thailand. As stated in the Veterinary and Animal Health Product Directory published in 2012, only 24 probiotic products for food animals were sold in Thailand; however, some of them were discontinued. In addition, many imported or new products have been available for food animals in Thailand market until now.

CHAPTER III MATERIALS AND METHOD

The research project was divided into 3 phases, including Phase 1: Sample collection (n=45); Phase 2: Determination of microbiological quality of probiotic products (n=45); and Phase 3: Determination of AMR characteristics in probiotic products (n=45) (Figure 1).

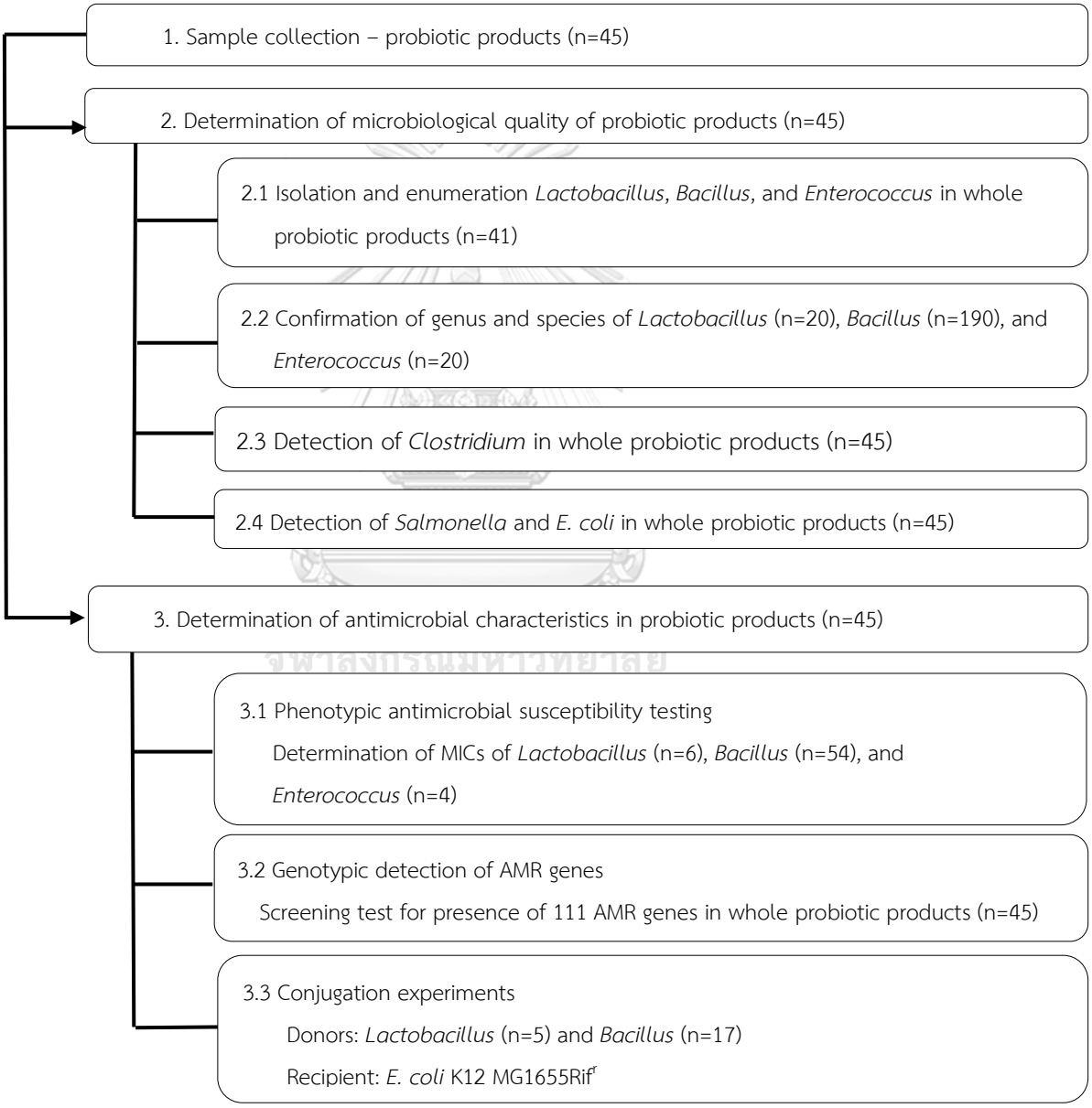

Figure 1. Flow of experiments performed in this study

1. Sample collection (n=45)

A total of 45 commercial probiotic products used for food animals including 2 liquid products and 43 powder products were collected during March 2019 - December 2020. The probiotic product distributors that agreed to participate in the project submitted the products to Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. At least 100 grams or milliliters of samples were aseptically obtained from the original products. If necessary, the whole package was purchased. Each sample was collected in bottles or bags with lightproof and submitted to the laboratory within 24 hours. All products were kept at room temperature and analyzed within 24 hours after arrival or within 7 days of being purchased. The samples from the same batch were avoided. All samples tested were at least 3 months before the expiration date. The information declared on the leaflet, including numbers of bacterial cells, bacterial species, and expiry date, was collected.

The information of probiotic products indicated by manufacturers including bacterial strains, number of viable cells and days left before expiration is described in Table 1.

Table 1. Information of probiotic products (n=45)

Product	Species/strains	Number[a]	Product type	Days left before expiration
P1	*B. licheniformis, B. subtilis*	$1.9x10^{11}$	Liquid product	6 months
P2	*B. subtilis*	$1.48x10^{11}$	Dried product	6 months
P3	*B. licheniformis*	$10.04x10^{10}$	Dried product	6 months
	B. subtilis	$4.76x10^{10}$		
P4	*B. subtilis*	$4x10^{11}$	Dried product	6 months
P5	*B. subtilis*	$4x10^{11}$	Dried product	6 months
P6	*B. subtilis*	$5x10^{9}$	Dried product	7 months
	S. faecium	$5x10^{9}$		

[a] Unit is cfu/kg for all products except for product P1 and P7 (cfu/l)

Table 1 (Continued)

Product	Species/strains	Number[a]	Product type	Days left before expiration
P7	*L. acidophilus*	$1x10^9$	Liquid product	6 months
	L. plantarum	$1x10^9$		
	B. subtilis	$1x10^9$		
	B. licheniformis	$1x10^9$		
P8	*E. faecium*	$8.4x10^{11}$	Dried product	6 months
P9	*B. amyloliquefaciens*	$1x10^{13}$	Dried product	6 months
P10	*B. subtilis*	$1x10^{13}$	Dried product	6 months
P11	*B. licheniformis*	$1.6x10^{12}$	Dried product	8 months
P12	*B. coagulans*	$1.5x10^{12}$	Dried product	22 months
	B. subtilis	$1x10^{12}$		
	L. acidophilus	$1.5x10^{12}$		
P13	*B. licheniformis, B. subtilis*	$2.56x10^{11}$	Dried product	6 months
P14	*E. faecium*	$5x10^{14}$	Dried product	6 months
P15	*Cl. butyricum*	$1.25x10^{12}$	Dried product	6 months
P16	*Cl. butyricum*	$5x10^8$	Dried product	6 months
P17	*Cl. butyricum*	$5x10^8$	Dried product	6 months
P18	*B. licheniformis*	$1.6x10^{13}$	Dried product	6 months
P19	*B. subtilis*	$1x10^{13}$	Dried product	6 months
P20	*B. subtilis*	$1x10^{13}$	Dried product	6 months
P21	*B. subtilis*	$1x10^{13}$	Dried product	6 months
P22	*B. subtilis*	$1x10^{12}$	Dried product	6 months
P23	*B. subtilis*	$1x10^{12}$	Dried product	6 months
P24	*B. cereus toyoi*	$1x10^{13}$	Dried product	6 months
P25	*B. cereus toyoi*	$1x10^{13}$	Dried product	6 months
P26	*B. licheniformis*	$3.2x10^{12}$	Dried product	6 months
P27	*B. subtilis*	$1.48x10^{11}$	Dried product	6 months
P28	*B. subtilis*	$7.5x10^{10}$	Dried product	6 months
P29	*B. subtilis*	$7.5x10^{10}$	Dried product	6 months
P30	*B. subtilis, B. licheniformis*	$1.48x10^{11}$	Dried product	6 months

[a] Unit is cfu/kg for all products except for product P1 and P7 (cfu/l)

Table 1 (Continued)

Product	Species/strains	Number[a]	Product type	Days left before expiration
P31	*B. subtilis*	$6.5x10^{10}$	Dried product	6 months
	B. licheniformis	$5.8x10^{10}$		
	L. acidophilus	$6x10^{9}$		
	L. casei	$1x10^{9}$		
	S. faecium	$1.5x10^{9}$		
P32	*B. subtilis*	$6.5x10^{10}$	Dried product	6 months
	B. licheniformis	$5.8x10^{10}$		
	L. acidophilus	$6x10^{9}$		
	L. casei	$1x10^{9}$		
	S. faecium	$1.5x10^{9}$		
P33	*B. subtilis*	$4.7x10^{8}$	Dried product	6 months
P34	*B. subtilis*	$4.7x10^{8}$	Dried product	6 months
P35	*B. subtilis*	$2x10^{11}$	Dried product	6 months
P36	*B. subtilis*	$2x10^{11}$	Dried product	6 months
P37	*B. subtilis*	$2x10^{11}$	Dried product	6 months
P38	*B. licheniformis*	$3.2x10^{12}$	Dried product	6 months
P39	*B. licheniformis*	$3.2x10^{12}$	Dried product	6 months
P40	*B. licheniformis*	$3.2x10^{12}$	Dried product	6 months
P41	Lactic acid bacteria	$1.34x10^{12}$	Dried product	6 months
P42	*Cl. butyricum*	$5x10^{8}$	Dried product	6 months
P43	*B. licheniformis, B. subtilis,*	$\geq 1x10^{12}$	Dried product	8 months
	B. pumilus			
	E. faecium, E. faecalis	$\geq 1x10^{11}$		
P44	Lactic acid bacteria	$\geq 7x10^{12}$	Dried product	12 months
	B. subtilis	$\geq 3x10^{12}$		
P45	Lactic acid bacteria	$\geq 7x10^{12}$	Dried product	12 months
	B. subtilis	$\geq 3x10^{12}$		

[a] Unit is cfu/kg for all products except for product P1 and P7 (cfu/l)

2. Determination of microbiological quality of probiotic products (n=45)

Of 45 samples, 41 probiotic products (n=41) including 2 liquid products and 39 dried products were examined for number of viable cells including *Lactobacillus*, *Bacillus* and *Enterococcus,* except 4 products (P15, P16, P17, and P44) that were formulated with *Clostridium* species. Then, genus and species of *Lactobacillus*, *Bacillus, Enterococcus* and *Clostridium* were confirmed. All products (n=45) were also examined for the presence of *E. coli* and *Salmonella*.

2.1 Isolation and enumeration *Lactobacillus, Bacillus and Enterococcus* in whole probiotic products (n=41)

Prior to isolation and enumeration of target bacteria, all samples (n = 41), either liquid or dried products, were prepared as follows. For dried products, 20 g of each sample was dissolved in 180 ml peptone saline diluting fluid (PSD; peptone 1.0 g and NaCl 8.5 g in 1,000 ml distilled water). For liquid products, one ml of each liquid product was diluted in 9 ml PSD. The samples were 10-fold serially diluted to reach the final concentration which was based concentration of probiotic bacteria claimed on labels (ISO, 2017). For example, if the label mentioned the number of bacteria was $1x10^9$ cfu/g, the sample would be diluted 9 times from 10^0 to 10^{-9}. The colonies were isolated and counted on duplicate plates of corresponded selective media. The number of colonies on plates showing between 30 and 300 colonies was counted. For each product, the numbers of bacteria were the means of duplicated counts. Three to five typical colonies of each target bacteria were selected for further identification of species. The bacterial species tested were according to the species declared on the label. The standard methods for isolation and enumeration of probiotic bacteria are described as follows.

Isolation and enumeration of *Lactobacillus* was performed by pour plate method using De Man, Rogosa and Sharpe (MRS) agar (Difco®, MD, USA) (ISO, 1998). One milliliter of diluted samples was spread on MRS agar and the inoculating plates were under microaerophilic condition at 37°C for 24 hours. After counting the number of isolates, 5 single colonies were picked up and sub-cultured onto MRS agar containing 0.3% calcium carbonate precipitated (QReC, Auckland, New Zealand) and then, incubated under microaerophilic condition at 37 °C for 24 hours. The single colonies surrounded by clear zone on MRS agar were pick and put into MRS broth and incubated at 37°C overnight. The *Lactobacillus* species appear as small, white and creamy colonies on MRS agar. From each positive sample, one isolate from each typical *Lactobacillus* colony was selected for further examination. A total of 20 *Lactobacillus* isolates were selected for confirmation of genus and species. All isolates were stored in 20% glycerol at -80°C.

Bacillus were isolated and counted by spread plate method using Mannitol Egg Yolk Polymyxin (MYP) agar (Difco®) (ISO, 2004). A hundred-µl diluted sample was spread on MYP agar and incubated at 37 °C for 24 hours. After bacterial enumeration, 5 typical colonies were streaked on MYP agar to get the single pure colonies and then incubated at 37°C for 24 hours. The colony morphology of *Bacillus* varies among species. For example, typical colonies of *B. cereus* are pink with precipitation halo, while colonies of *B. subtilis* are yellow without precipitation halo. The colonies were inoculated into Tryptic Soy Broth (TSB) (Difco®) at 37°C overnight. From each positive sample, each bacterial isolate from each colony with typical characteristics of *Bacillus* was collected for further examination. A total of 190 *Bacillus* isolates were selected for confirmation of genus and species. All *Bacillus* isolates were stored in 20% glycerol at -80°C.

Enterococcus were isolated and enumerated by spread plate method (Domig et al., 2003). The diluted samples were spread onto Bile Aesculin agar (BEA) (Oxoid®, Hampshire, UK) and incubated at 37°C for 24 hours. The colonies were counted, and 5 single colonies with typical characteristics of *Enterococcus* were chosen to sub-

cultured onto Kenner Fecal (KF) agar (HiMedia®, Mumbai, India). After 24-hour incubation, 5 red or pink single colonies from KF agar were isolated into Brain Heart Infusion (BHI) agar (Difco®) overnight. The overnight colonies on BHI agar were cultured in BHI broth (Difco®) at 37°C for 24 hours. One isolate from one typical *Enterococcus* colony collected from each positive sample was selected for further examination. A total of 20 *Enterococcus* isolates were selected for confirmation of genus and species. All *Enterococcus* isolates were stored in 20% glycerol at -80°C.

2.2 Confirmation of genus and species of *Lactobacillus* (n=20), *Bacillus* (n=190) and *Enterococcus* (n=20)

A total of 190 *Bacillus*, 20 *Lactobacillus* and 20 *Enterococcus* isolates were confirmed the genus and species by different types of PCR. All bacterial isolates were extracted template DNA using whole cell boiled lysate procedure (Lévesque et al., 1995). Those bacteria were grown on Luria-Bertani (LB) (Difco®) agar at 37°C overnight. A single colony was picked and put in 100 µl of sterile distilled water. Then, the suspension was heated in a boiled water for 10 minutes and immediately placed on ice. The suspension was centrifuged at 12,000 rpm for 5 minutes. The supernatant was placed into a new 1.5 ml Eppendorf tube and stored at -20°C. The PCR conditions for confirmation of genus and species were described in Table 2 and PCR primers are all listed in Appendix A.

The genus of *Lactobacillus, Bacillus* and *Enterococcus* was verified using simplex PCR (Nakagawa et al., 1994; Dubernet et al., 2002; Jackson et al., 2004; Wu et al., 2006). The PCR reactions consisted of 12.5 µl TopTaq Master Mix (Qiagen, Hilden, Germany), 1.25 µl of each primer at 0.5 µM, 5 µl DNA template, and RNase-free water to get 25 µl of final volume.

Table 2. PCR reaction conditions for detection of genus and species of probiotic bacteria

Probiotic bacteria	PCR type	PCR reaction conditions (Temperature – Time)						Gel concentration (%)	References
		Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles		
Lactobacillus									
Genus	Simplex	95°C – 5 min	95°C – 40 sec	55 °C – 30 sec	72 °C – 30 sec	72 °C – 7 min	30	1.5	(Nakagawa et al., 1994; Dubernet et al., 2002)
Species	Multiplex	95°C – 2 min	95°C – 40 sec	55 °C – 30 sec	72 °C – 30 sec	72 °C – 7 min	30	1.5	(Kwon et al., 2004)
Bacillus									
Genus	Simplex	94°C – 3 min	94°C – 30 sec	63 °C – 30 sec	72 °C – 2 min	72 °C – 10 min	25	2	(Wu et al., 2006)
Enterococcus									
Genus	Simplex	95°C – 3 min	95°C – 30 sec	55 °C – 30 sec	72 °C – 1 min	72 °C – 7 min	30	2	(Ke et al., 1999)
Species	Multiplex	95°C – 4 min	95°C – 30 sec	55°C – 1 min	72 °C – 1 min	72 °C – 7 min	30	2	(Jackson et al., 2004)
Clostridium									
Genus	Simplex	95°C – 3 min	95°C – 30 sec	60°C – 1 min	72 °C – 30 sec	72 °C – 5 min	30	2	(Dhalluin et al., 2003)
Species	Simplex	94°C – 2 min	94°C – 30 sec	60 °C – 30 sec	72 °C – 2 min	72 °C – 2 min	35	0.8	(Kikuchi et al., 2002)

2.2.1 *Lactobacillus* (n=20)

A total of 20 *Lactobacillus* isolates were confirmed for seven *Lactobacillus* species including *L. acidophilus, L. delbrueckii, L. casei, L. gasseri, L. plantarum, L. reuteri* and *L. rhamnosus*, were confirmed using multiplex PCR assay (Kwon et al., 2004). Each PCR reaction included 1 µl of each primer at 0.2 µM, 25 µl TopTaq Master Mix (Qiagen, Hilden, Germany), 5 µl DNA template, and RNase-free water to make final volume of 50 µl.

2.2.2. *Enterococcus* (n=20)

A total of 20 *Enterococcus* isolates were verified six species of *Enterococcus* consisting of *E. faecalis, E. faecium, E. durans, E. gallinarum, E. casseliflavus* and *E. hirae* using multiplex PCR assay (Jackson et al., 2004). Each PCR reaction consisted of 25 µl TopTaq Master Mix (Qiagen®), 1 µl of each primer at 0.2 µM, 5 µl DNA template and RNase-free water to get 50 µl of final volume.

2.2.3 *Bacillus* (n=190)

For confirmation of *Bacillus* species, a total of 190 *Bacillus* isolates were verified using Amplified Ribosomal DNA Restriction Analysis (ARDRA) to identify seven-teen species were identified including *B. subtilis, B. licheniformis, B. subtilis* cluster (*B. pumilus, B. amyloliquefaciens* and *B. atrophaeus*), *B. cereus* cluster (*B. cereus, B. thuringiencies* and *B. anthracis*), *B. laterosporus*, *B. coagulans, B. sphaericus, B. circulans, B. badius, B. clausii, P. polymyxa, P. larvae* and *P. lentimorbus* (Wu et al., 2006). The PCR products from genus detection were digested with restriction enzymes such as *Alu*I and *Taq*I (Thermo Scientific, Massachusetts, USA). For *Alu*I, a 32 µl reaction volume consisted of 10 µl PCR reaction mixture, 18 µl RNase-free water, 2 µl of 10X Buffer Tango and 2 µl of *Alu*I. Similarly, a 10 µl PCR reaction mixture was mixed with 18 µl RNase-free water, 2 µl of 10X Buffer *Taq*I and 2 µl of *Taq*I in a 32 µl reaction volume. The reaction mixtures were then incubated at 37°C and 65°C for *Alu*I and *Taq*I, respectively, in 1 – 16 hours as described by the manufacturer.

The *Alu*I and *Taq*I restriction profiles of each isolate were then compared to the ARDRA patterns, as shown in Figure 2, to identify the specific species of *Bacillus*.

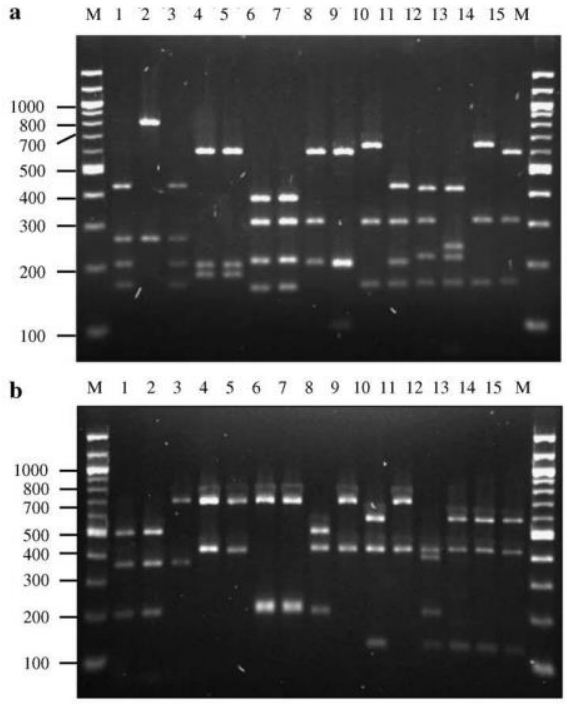

Figure 2. *Alu*I (a) and *Taq*I (a) restriction profiles of amplified regions of the 16S rRNA genes of *Bacillus* reference strains.

Lane M, 100 bp+ DNA ladder; lane 1, *B. subtilis* ATCC6633; lane 2, *B. licheniformis* ATCC25972; lane 3, *B. pumilus* ATCC21356; lane 4, *B. cereus* ATCC14579; lane 5, *B. thuringiensis* ATCC10792; lane 6, *B. laterosporus* ATCC64; lane 7, *B. laterosporus* ACM5117; lane 8, *B. coagulans* ATCC7050; lane 9, *B. sphaericus* ATCC14577; lane 10, *B. circulans* ATCC15518; lane 11, *B. badius* ATCC14574; lane 12, *B. clausii* ATCC700160; lane 13, *P. polymyxa* ATCC842; lane 14, *P. larvae* ATCC9545; lane 15, *P. lentimorbus* ATCC 14707 (Wu et al., 2006).

Electrophoresis was used to separate amplicons on agarose gel (concentrations as shown in Table 2) (Vivantis®, Subang Jaya, Malaysia) stained by RedSafe™ Nucleic Acid Staining Solution (iNtROn Biotechnology®, Seongnam, South Korea) in 1xTris-actate/EDTA (1X TAE) buffer. A 100 bp+ DNA ladder (Thermo Scientific, Massachusetts, USA) was used to estimate the sizes of DNA fragments. The PCR products were then

visualized using UV light by Bio-Rad Gel Documentation System (Bio-Rad Laboratories, California, USA). PCR product sizes were shown in Appendix A.

2.3 Detection of *Clostridium* in whole probiotic products (n=45)

All probiotic products (n=45), including 4 probiotic products (P15, P16, P17, and P44) that were claimed *Clostridium* species on the labels and 41 other products, were extracted DNA using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, Massachusetts, USA). Simplex PCR assays were performed to detect genus and thirteen species of *Clostridium* including *C. butyricum, C. perfringens, C. paraputrificum, C. bifermentans, C. difficile, C. sordellii, C. clostridiiforme, C. nexile, C. sphenoides, C. indolis, C. innocuum, C. ramosum* and *C. cocleatum* (Kikuchi et al., 2002; Dhalluin et al., 2003). PCR reactions contained 5 µl of DNA template, 12.5 µl TopTaq Master Mix (Qiagen®), 1.25 µl of each primer at 0.5 µM, and RNase-free water to make a final volume at 25 µl. PCR conditions were shown in Table 2 and all primers used were described in Appendix A.

Five-µl of each PCR product was electrophoresed using agarose gel (Vivantis®) stained with RedSafe™ Nucleic Acid Staining Solution (iNtROn Biotechnology®) in 1x TAE buffer. A 100 bp+ DNA ladder (Thermo®) was used to estimate the PCR product sizes. The PCR products were then visualized using UV light by Bio-Rad Gel Documentation System (Bio-Rad Laboratories, California, USA).

2.4 Determination of *Salmonella* and *E. coli* in whole probiotic products (n=45)

All samples (n=45) were prepared for detection of the presence of *Salmonella* and *E. coli* according to ISO 6887-1:2017(en) (ISO, 2017). Twenty-five grams of each dried product was dissolved in 225 ml Buffered Peptone Water (BPW) (Difco®). For liquid products, 25 ml of each liquid product was diluted in 225 ml BPW. The mixtures were then incubated at 37°C for 18 ± 2 hours and proceeded as follows.

All *Salmonella* strains were isolated using the standard methods described in ISO 6579:2002(en) (ISO, 2002). A hundred-µl of pre-enriched sample was placed on Modified Semi-Solid Rappaport-Vassiliadis (MSRV) agar (Difco®) and incubated at 41.5°C for 24 hours. A loopful of material from the edge of turbid growth zone was sub-cultured on Xylose Lysine Deoxycholate (XLD) agar (Difco®) and incubated at 37°C for 24 hours. Three red colonies with black centers was selected for biochemincal tests using Triple Sugar Iron (TSI) agar (Difco®). The colonies were inoculated by stabbing to the butt and streaking on the slant and incubated at 37°C for 24 hours. *Salmonella* can grow on TSI producing red slant, yellow butt, gas possitive and black precipitation. Single colonies were picked and inoculated at 37°C for 24 hours on LB agar. Finally, single colonies were grown in LB broth overnight at 37°C. The isolates were stored at 20% glycerol at -80°C for further analyses. All *Salmonella* isolates were subjected to serotyping by slide agglutination based on the Kauffmann-White schemes using commercially available antiserum (S&A Reagents Lab Ltd., Bangkok, Thailand) (Gueimonde et al., 2013).

E. coli was isolated and confirmed in all samples using standard protocols for *E. coli* isolation (BAM, 2017; ISO, 2017). One loop of incubated sample in BPW was streaked on Eosin Methylene Blue (EMB) agar (Difco®) and incubated at 37°C for 24 hours. The purple-coloured colonies with metallic sheen were sub-cultured on MacConkey (MCK) agar (Difco®) at 37°C overnight. The red colonies on MCK agar were biochemically confirmed by indole test. The colonies with *E. coli* typical characteristics were inoculated into 4 ml Tryptophan broth (Difco®) at 37°C overnight. A 0.5 ml of Kovac's reagents was added to the inoculum. *E. coli* can form a pink to red colour, called cherry-red ring, in the reagent layer on the top of medium within seconds. The *E. coli* isolates were purified on LB agar to get single colonies. One colony from each positive sample was grown in LB broth and stored in 20% glycerol at -80°C for further investigations.

3. Determination of AMR characteristics in probiotic products (n=45)

3.1 Phenotypic antimicrobial susceptibility testing

Since the same species found in the same product were expected to have the same antimicrobial susceptibility test (AST) pattern, one isolate of one probiotic species found in each positive sample was chosen for examining for their susceptibilities. The bacterial isolates examined for AMR phenotypes are described in Table 3.

Table 3. Bacterial isolates (n=64) selected for determination of AMR phenotypes

Product	Isolate code	Probiotic bacterial species		
		Bacillus (n=54)	*Lactobacillus* (n=6)	*Enterococcus* (n=4)
P1	B1.1	*B. subtilis*		
	B1.3	Members of *B. subtilis* cluster[a]		
	B1.5	*B. sphaericus*		
P2	B2.1	Other *Bacillus* spp.		
	B2.2	*B. subtilis*		
	B2.3	Members of *B. subtilis* cluster		
P3	B3.1	Members of *B. subtilis* cluster		
	B3.3	*B. subtilis*		
P4	B4.1	Members of *B. subtilis* cluster		
P5	B5.1	Members of *B. subtilis* cluster		
P6	B6.1	*B. licheniformis*		
	E6.1			*E. feacium*
P7	B7.1	Members of *B. subtilis* cluster		
	L7.1		*L. casei*-group[b]	
	L7.2		*L. plantarum*	
	L7.4		*L. rhamnosus*	
P8	E8.1			*E. feacium*
P9	B9.1	Members of *B. subtilis* cluster		
P10	B10.1	Members of *B. subtilis* cluster		
P11	B11.1	*B. licheniformis*		

[a] *B. pumilus, B. amynoliquencies* and *B. atrophaeus*

[b] *L. casei* and *L. paracasei*

Table 3 (Continued)

Product	Isolate code	Probiotic bacterial species		
		***Bacillus* (n=54)**	***Lactobacillus* (n=6)**	***Enterococcus* (n=4)**
P12	B12.1	Members of *B. subtilis* cluster[a]		
	B12.2	*B. sphaericus*		
P13	B13.1	*B. licheniformis*		
	B13.3	*B. subtilis*		
P14	E14.1			*E. feacium*
P18	B18.1	*B. licheniformis*		
P19	B19.1	Other *Bacillus* spp.		
P20	B20.1	*B. subtilis*		
P21	B21.1	*B. subtilis*		
P22	B22.1	Members of *B. subtilis* cluster		
P23	B23.1	Members of *B. subtilis* cluster		
P24	B24.1	Other *Bacillus* spp.		
P25	B25.1	Other *Bacillus* spp.		
P26	B26.1	*B. licheniformis*		
P27	B27.1	*B. subtilis*		
	B27.2	Members of *B. subtilis* cluster		
P28	B28.1	Other *Bacillus* spp.		
	B28.5	Members of *B. subtilis* cluster		
P29	B29.1	Other *Bacillus* spp.		
P30	B30.2	*B. subtilis*		
	B30.4	Other *Bacillus* spp.		
	B30.5	Members of *B. subtilis* cluster		
P31	B31.1	Other *Bacillus* spp.		
	B31.4	Members of *B. subtilis* cluster		
P32	B32.1	Other *Bacillus* spp.		
	B32.4	Members of *B. subtilis* cluster		
P33	B33.1	Members of *B. subtilis* cluster		
	B33.3	Other *Bacillus* spp.		
	B33.4	*B. sphaericus*		
P34	B34.1	*B. sphaericus*		

[a] *B. pumilus, B. amynoliquencies* and *B. atrophaeus*

Table 3 (Continued)

Product	Isolate code	Probiotic bacterial species		
		***Bacillus* (n=54)**	***Lactobacillus* (n=6)**	***Enterococcus* (n=4)**
P35	B35.1	Members of *B. subtilis* cluster[a]		
P36	B36.1	Members of *B. subtilis* cluster		
P37	B37.1	*B. licheniformis*		
	B37.3	Members of *B. subtilis* cluster		
P38	B38.1	*B. licheniformis*		
P39	B39.1	*B. licheniformis*		
P40	B40.1	*B. licheniformis*		
P41	L41.1		*L. delbrueckii*	
P43	B43.1	Members of *B. subtilis* cluster		
	E43.1			*E. feacium*
P44	B44.1	Members of *B. subtilis* cluster		
	L44.1		Other *Lactobacillus* spp.	
P45	B45.1	Members of *B. subtilis* cluster		
	L45.1		Other *Lactobacillus* spp.	

[a] *B. pumilus, B. amynoliquencies* and *B. atrophaeus*

A total of 64 probiotic bacterial isolates, including *Bacillus* (n=54), *Lactobacillus* (n=6) and *Enterococcus* (n=4), were examined for their susceptibilities to 14 antimicrobial agents such as ampicillin (AMP), meropenem (MER), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), chloramphenicol (CHL), tetracycline (TET), erythromycin (ERY), vancomycin (VAN), trimethoprim (TRI), sulfamethoxazole (SUL), ciprofloxacin (CIP), clindamycin (CLI), and rifampicin (RIF) by determining the minimum inhibitory concentrations (MICs). All antimicrobial agents were purchased from Sigma-Aldrich® (Steinheim, Germany). The antimicrobial agents were prepared in appropriate concentrations with diluents as shown in Appendix B. MICs of *Lactobacillus* were determined by broth microdilution method using LAB susceptibility test medium (Klare et al., 2005). For *Bacillus* and *Enterococcus*, the determination of MICs was performed in Muller Hinton agar (MHA) using a two-fold agar dilution method (CLSI, 2019).

For the interpretive criteria, the priority was given to clinical breakpoints according to Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (CLSI, 2015; EUCAST, 2020). When the breakpoints were not available, the epidemiology cut-off (ECOFF) values according to EUCAST and EFSA Panel were used (EFSA, 2012; EUCAST, 2020).

3.1.1 Broth microdilution method

MICs of *Lactobacillus* isolates (n=6) were determined by broth microdilution method using lactic acid bacteria susceptibility test medium (LSM) (Klare et al., 2005). The LSM broth was the mixture of 90% Iso-Sensitest (IST) broth (Oxoid®, Hampshire, UK) and 10% MRS broth. Each 50-µl volume of LSM broth was added into microtiter plate by multichannel pipette, except the first column. The first and the second column were filled with 50 µl of double strength antibiotic solution. Two-fold serial dilution was made by transferring 50 µl of suspension from the second column to next column and repeated until finish expect for the control at the last column. The *Lactobacillus* isolates were grown overnight at 37°C on MRS agar. Single colony was picked and resuspended in 0.9% normal saline solution (NSS) and the cell density was adjusted to 0.5 McFarland (~10^8 CFU/ml). Then, the ten-fold dilution of bacterial suspension was performed by adding 1 ml of bacterial suspension into 9 ml of LSM and repeated twice to obtain approximately 10^6 CFU/ml. Fifty-µl volume of suspensions were then transferred into the microtiter plates with two-fold serially diluted antibiotic solution. When 50 µl of bacterial suspension was transferred into microtiter plate with 50 µl diluted antibiotic solution, the final concentration of bacterial suspension was approximately 5 x 10^5 CFU/ml or 5 x 10^4 CFU/well. To prevent drying, each tray was sealed with paraffin before incubation. The microtiter plates were incubated at 37°C for 16 – 20 hours.

The MIC results were recorded as the lowest concentration of antimicrobial agents the inhibits visible growth of the organism in microdilution wells. Three organisms were used as quality control including *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212.

The MICs of certain *Lactobacillus* species were interpreted using clinical breakpoints and ECOFFs as shown in Table 4.

3.1.2 Agar dilution method

The MICs of *Bacillus* (n=54) and *Enterococcus* (n=4) were determined using agar dilution method (CLSI, 2019). The *Bacillus* and *Enterococcus* isolates were cultured overnight at 37°C on Muller-Hinton agar (MHA) (Difco®). The well-isolated colonies were picked and transferred to a tube containing 2 ml sterile NSS (0.9%). The turbidity of inoculum was adjusted to 0.5 McFarland (~ 1.5×10^8 CFU/ml). The suspension was ten-fold diluted to 10^7 CFU/ml by adding 1 ml of bacterial suspension to 9 ml NSS. Then, one hundred-µl suspension was transferred into microtiter plates and inoculated onto the MHA plates containing suitable concentrations of antibiotics using multipoint inoculator. After incubation for 16 – 20 hours, the MICs were recorded as the lowest concentration of antimicrobial agent the completely inhibits the visible growth of bacteria. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as quality control organisms.

The ECOFFs and clinical breakpoints were used to interpret the MICs of *Bacillus* and *Enterococcus* isolates (Table 5).

Table 4. Clinical breakpoints and ECOFFs (μg/ml) for interpretation of antimicrobial susceptibility of *Lactobacillus*

MIC interpretation	Antimicrobials	Concentration range (μg/ml)	*Lactobacillus* spp					References
			L. plantarum	*L. rhamnosus*	*L. casei*	*L. delbrueckii*	*Other Lactobacillus* species	
Clinical breakpoints	Meropenem	0, 0.0625 – 64	≥4	≥4	≥4	≥4	≥4	(CLSI, 2015)
	Sulfamethoxazole*	0, 2 – 2048	≥512	≥512	≥512	≥512	≥512	(CLSI, 2020)
	Trimethoprim	0, 0.5 – 512	≥32	≥32	≥32	≥32	≥32	(SCAN, 2003)
	Ciprofloxacin	0, 0.0625 – 64	≥4	≥4	≥4	≥4	≥4	(SCAN, 2003)
	Rifampicin	0, 0.125 – 128	≥32	≥32	≥32	≥32	≥32	(SCAN, 2003)
ECOFFs	Ampicillin	0, 0.125 – 128	>2	>4	>4	>1	>1	(EFSA, 2012)
	Streptomycin	0, 1 – 1024	>16	>32	>64	>16	>16	(EFSA, 2012)
	Kanamycin	0, 1 – 1024	>64	>64	>64	>16	>16	(EFSA, 2012)
	Gentamicin	0, 0.5 – 512	>16	>16	>32	>16	>16	(EFSA, 2012)
	Chloramphenicol	0, 0.25 – 256	>8	>4	>4	>4	>4	(EFSA, 2012)
	Tetracycline	0, 0.5 – 512	>32	>8	>4	>4	>4	(EFSA, 2012)
	Erythromycin	0, 0.125 – 128	>1	>1	>1	>1	>1	(EFSA, 2012)
	Vancomycin	0, 0.25 – 256	>2	>2	>2	>2	>2	(EFSA, 2012)
	Clindamycin	0, 0.0625 – 64	>2	>1	>1	>1	>1	(EFSA, 2012)

* Since the clinical breakpoint of sulfamethoxazole for *Lactobacillus* spp was not available, therefore, the resistant breakpoint of ≥ 512 μg/ml for *S. aureus* was applied (CLSI, 2020)

Table 5. The clinical breakpoints and ECOFFs (µg/ml) for interpretation of antimicrobial susceptibility of *Bacillus* (n=54) and *Enterococcus* (n=4)

Antimicrobials	Species	Concentration range (µg/ml)	Clinical breakpoints (µg/ml)	ECOFFs (µg/ml)	References
Ampicillin	*Bacillus*	0, 0.0625 – 32	>2	-	(EUCAST, 2020)
	Enterococcus	0, 0.125 – 256	-	>2	(EFSA, 2012)
Meropenem	*Bacillus*	0, 0.0625 – 256	≥16	-	(CLSI, 2015)
	Enterococcus	0, 0.625 – 128	-	>8	(EUCAST, 2020)
Streptomycin	*Bacillus*	0, 1 – 1024	-	>8	(EFSA, 2012)
	Enterococcus	0, 1 – 1024	-	>128	(EFSA, 2012)
Kanamycin	*Bacillus*	0, 1 – 1024	-	>8	(EFSA, 2012)
	Enterococcus	0, 1 – 1024	-	>1024	(EFSA, 2012)
Gentamicin	*Bacillus*	0, 0.125 – 256	-	>4	(EFSA, 2012)
	Enterococcus	0, 1 – 1024	-	>32	(EFSA, 2012)
Chloramphenicol	*Bacillus*	0, 0.5 – 512	-	>8	(EFSA, 2012)
	Enterococcus	0, 1 – 512	-	>16	(EFSA, 2012)
Tetracycline	*Bacillus*	0, 0.125 – 256	-	>8	(EFSA, 2012)
	Enterococcus	0, 0.125 – 256	-	>4	(EFSA, 2012)
Erythromycin	*Bacillus*	0, 0.125 – 128	-	>4	(EFSA, 2012)
	Enterococcus	0, 0.25 – 128	-	>4	(EFSA, 2012)
Vancomycin	*Bacillus*	0, 0.25 – 64	-	>4	(EFSA, 2012)
	Enterococcus	0, 0.25 – 256	-	>4	(EFSA, 2012)
Trimethoprim	*Bacillus*	0, 0.125 – 256	>8	-	(EUCAST, 2020)
	Enterococcus	0, 0.125 – 128	≥8	-	(SCAN, 2003)
Sulfamethoxazole*	*Bacillus*	0, 0.5 – 2048	≥512	-	(CLSI, 2020)
	Enterococcus	0, 1 - 2048	≥512	-	(CLSI, 2020)
Ciprofloxacin	*Bacillus*	0, 0.15625 – 64	>1	-	(EUCAST, 2020)
	Enterococcus	0, 0.15625 – 64	-	>8	(EUCAST, 2020)
Clindamycin	*Bacillus*	0, 0.0625 – 64	-	>4	(EFSA, 2012)
	Enterococcus	0, 0.0625 – 64	-	>4	(EFSA, 2012)
Rifampicin	*Bacillus*	0, 0.125 – 128	≥4	-	(SCAN, 2003)
	Enterococcus	0, 0.125 – 128	≥4	-	(SCAN, 2003)

* MIC breakpoints for *Bacillus* and *Enterococcus* for sulfamethoxazole were recommended using breakpoints of *S. aureus* from CLSI (CLSI, 2020).

3.2 Genotypic detection of AMR genes in whole probiotic products (n=45)

The presence of 111 genes encoding resistance to clinically important antibiotics was screened in whole probiotic products (n = 45). The PCR primers used are listed in Appendix C.

Template DNA were directly extracted from each probiotic product using a GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, Massachusetts, USA). All PCR reactions were prepared in a final volume of 25 µl using TopTaq Master Mix Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A PCR reaction mixture contained 12.5 µl of 2X TopTaq Master Mix, 1.25 µl of each primer (0.5 µM), 1.25 µl of CoralLoad, 5 µl of DNA template and 5 µl of RNase-free water to obtain final volume at 25 µl.

The amplification conditions for all genes were an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 45 seconds, primer annealing for 45 seconds with annealing temperature described in Appendix C an extension at 72°C for 45 seconds, and a final extension for 10 minutes. All primers, annealing temperature and PCR product size (bp) were shown in Appendix C. The PCR products were separated by electrophoresis on 1.5% agarose gel in 1X TAE buffer and visualized under UV light by Gel Documentation System.

The PCR products of all positive samples were purified using Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany) and then submitted for sequencing at First Base Laboratories (Selangor Darul Ehsan, Malaysia). The DNA sequencing results were analyzed by comparing with those published on GeneBank Database using BLAST available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

3.3 Conjugation experiment

The *Lactobacillus* (n=5) and *Bacillus* (n=17) isolates with resistant phenotypes were performed to test transferability of AMR genes by biparental mating method (Khemtong and Chuanchuen, 2008). All *Lactobacillus* (n=5) and *Bacillus* (n=16) isolates that were resistant to antibiotics tested served as donors. All *Bacillus* isolates (n=17) used as donors were resistant to chloramphenicol, tetracycline, trimethoprim and clindamycin, while *Lactobacillus* isolates (n=5) served as donors were resistant to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline and ciprofloxacin.

The spontaneous rifampicin-resistant *E. coli* K12 strain MG1655 (MG1655Rif^r, MIC = 256 µg/ml) was used as recipients. *E. coli* MG1655Rif^r is susceptible to all antimicrobials tested and does not carry either plasmid or class 1 integrons.

Non-selective media used for filter mating were LB media and BHI media for *Bacillus* and *Lactobacillus*, respectively. Both donor and recipient were cultured on non-selective agar overnight at 37°C. The single colonies were the put into 4 ml non-selective broth and were incubated in shaking incubator at 37°C for 24 hours. The 80 µl overnight culture of donors and recipients was added to 4 ml fresh non-selective broth and grown at 37°C until the log phase for 3 – 4 hours in shaking incubator. The donor culture (700 µl) was mixed with recipient culture (700 µl) (ratio 1: 1) and the mixtures were centrifuged at 8,000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended by 30 µl fresh non-selective broth (warmed at 37°C). The bacterial mixtures were placed on a 0.45-µm-pore-size filter (Sartorius, Gottingen, Germany) on non-selective agar plates without antibiotics and incubated at 37°C. The bacteria grown on filter membrane were then scraped and washed wash with 1 ml 0.9% NSS in an Eppendorf. The filter membrane was removed and the mixture was centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the bacterial pellet was then re-suspended with 200 µl fresh non-selective broth. A hundred-µl of conjugation mixtures was spread on non-selective agar (duplicated

plate, 100 µl/plate) supplemented with 32 µg/ml of rifampicin and one of following antibiotic such as ampicillin (100 µg/ml), streptomycin (50 µg/ml), kanamycin (35 µg/ml), chloramphenicol (25 µg/ml), tetracycline (10 µg/ml), trimethoprim (100 µg/ml) and ciprofloxacin (0.064 µg/ml) and incubated at 37°C overnight. The colonies were picked up and grown on EMB agar with corresponding antibiotics at 37°C for 24 hours. The colonies appeared metallic green sheen color were streaked on non-selective agar supplemented with antibiotics and incubated at 37°C overnight. The colonies were then cultured in non-selective broth at 37°C for 24 hours and put in 20% glycerol at -80°C for keeping stock.

The transconjugants were examined MICs for 14 antibiotics mentioned above. The transconjugants with a 4-fold MIC increase compared to recipients were confirmed to receive AMR determinants from donors. DNA templates were extracted from each transconjugant using whole cell boiled lysate method and the presence of AMR genes encoding for resistance of corresponding antibiotics were detected using PCR as described above.

CHAPTER IV RESULTS

1. Numbers and species of probiotic bacteria

Overall, 11 out of 45 products (11/45, 24.4%) were accurately labeled in both numbers and bacterial species, while 34 remaining products (34/45, 75.6%) were not in agreement with their declared labels in different ways, for example, poor viable cell count and incorrect species, or both (Figure 3). The comparison between information given on labels and the analysis of each probiotic product is shown in Table 6.

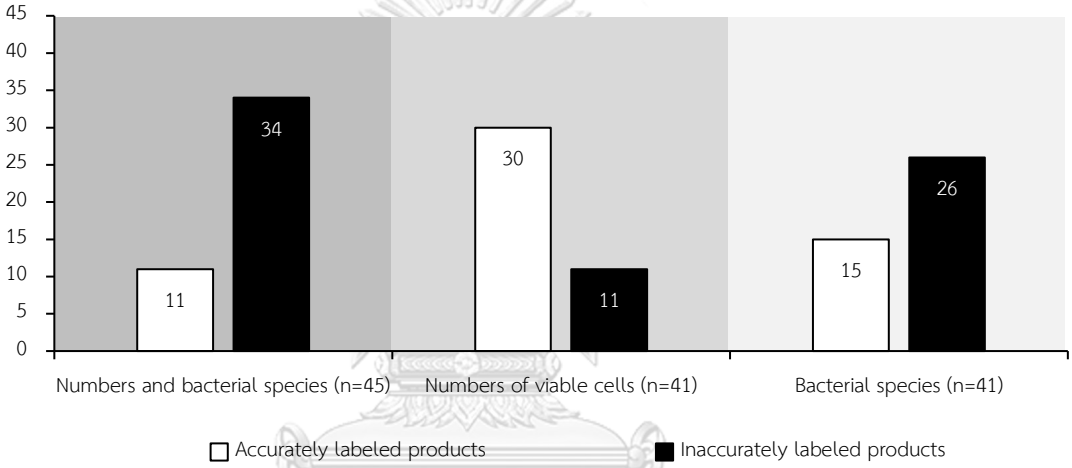

Figure 3. Number of probiotic products by microbiological properties

Of 41 products, the numbers of viable organisms in 11 products (11/41, 26.8%) were lower than their label claims (Figure 3). The numbers of viable cells ranged from 0 to 3.85 x 10^{15} cfu/g (Table 6). No viable *Lactobacillus* was found in products P12, P31 and P32, although high numbers of these bacteria were present on the label. Thirty out of 41 products (30/41, 73.2%) contained viable bacteria cells approximately equivalent to or exceeded the declared contents. The viable cells of *Bacillus* and/or *Enterococcus* counted in 3 products (P5, P6 and P8) were 10 to 10,000 times higher than that indicated on the labels.

Table 6. Comparison of information given on labels and analysis of probiotic products (n = 45)

Product	Labelling information		Results		
	Strains	Number[a]	Strains	Number[a]	Specific species
P1	*B. licheniformis*	$1.9x10^{11}$	*Bacillus* spp	$2.14x10^{9}$	*B. subtilis, B. sphaericus,* members of the *B. subtilis* cluster[b]
	B. subtilis				
P2	*B. subtilis*	$1.48x10^{11}$	*Bacillus* spp	$9.2x10^{10}$	*B. subtilis,* members of the *B. subtilis* cluster, other *Bacillus* species[c]
P3	*B. licheniformis*	$10.04x10^{10}$	*Bacillus* spp	$9.4x10^{10}$	*B. subtilis,* members of the *B. subtilis* cluster
	B. subtilis	$4.76x10^{10}$			
P4	*B. subtilis*	$4x10^{11}$	*Bacillus* spp	$7.65x10^{11}$	Members of the *B. subtilis* cluster
P5	*B. subtilis*	$4x10^{11}$	*Bacillus* spp	$7.2x10^{12}$	Members of the *B. subtilis* cluster
P6	*B. subtilis*	$5x10^{9}$	*Bacillus* spp	$7.2x10^{10}$	*B. licheniformis, E. faecium*
	S. faecium	$5x10^{9}$	*Enterococcus* spp	$9.2x10^{11}$	
P7	*L. acidophilus*	$1x10^{9}$	*Lactobacillus spp*	$1.88x10^{9}$	*L. plantarum, L. rhamnosus, L. casei*-group[d], members of the *B. subtilis* cluster
	L. plantarum	$1x10^{9}$	*Bacillus* spp	$8.4x10^{13}$	
	B. subtilis	$1x10^{9}$			
	B. licheniformis	$1x10^{9}$			
P8	*E. faecium*	$8.4x10^{11}$	*Enterococcus* spp	$3.85x10^{15}$	*E. faecium*
P9	*B. amyloliquefaciens*	$1x10^{13}$	*Bacillus* spp	$2.01x10^{13}$	Members of the *B. subtilis* cluster
P10	*B. subtilis*	$1x10^{13}$	*Bacillus* spp	$6x10^{13}$	Members of the *B. subtilis* cluster
P11	*B. licheniformis*	$1.6x10^{12}$	*Bacillus* spp	$5.6x10^{12}$	*B. licheniformis*
P12	*B. coagulans*	$1.5x10^{12}$	*Bacillus* spp	$1.31x10^{7}$	Members of the *B. subtilis* cluster, *B. sphaericus*
	B. subtilis	$1x10^{12}$			
	L. acidophilus	$1.5x10^{12}$			
P13	*B. licheniformis*	$2.56x10^{11}$	*Bacillus* spp	$2.3x10^{11}$	*B. licheniformis, B. subtilis*
	B. subtilis				
P14	*E. faecium*	$5x10^{14}$	*Enterococcus* spp	$1.85x10^{14}$	*E. faecium*
P15	*Cl. butyricum*	$1.25x10^{12}$	*Clostridium* spp	NT	*Cl. butyricum*
P16	*Cl. butyricum*	$5x10^{8}$	*Clostridium* spp	NT	*Cl. butyricum*
P17	*Cl. butyricum*	$5x10^{8}$	*Clostridium* spp	NT	*Cl. butyricum*
P18	*B. licheniformis*	$1.6x10^{13}$	*Bacillus* spp	$8.4x10^{13}$	*B. licheniformis*
P19	*B. subtilis*	$1x10^{13}$	*Bacillus* spp	$4.9x10^{13}$	Other *Bacillus* species
P20	*B. subtilis*	$1x10^{13}$	*Bacillus* spp	$2.15x10^{13}$	*B. subtilis*
P21	*B. subtilis*	$1x10^{13}$	*Bacillus* spp	$3.7x10^{13}$	*B. subtilis*
P22	*B. subtilis*	$1x10^{12}$	*Bacillus* spp	$3.2x10^{12}$	Members of the *B. subtilis* cluster
P23	*B. subtilis*	$1x10^{12}$	*Bacillus* spp	$5.05x10^{12}$	Members of the *B. subtilis* cluster

Table 6 (Continued)

Product	Labelling information		Results		
	Strains	Number[a]	Strains	Number[a]	Specific species
P24	*B. cereus toyoi*	$1x10^{13}$	*Bacillus* spp	$6.3x10^{12}$	Other *Bacillus* species
P25	*B. cereus toyoi*	$1x10^{13}$	*Bacillus* spp	$2.85x10^{12}$	Other *Bacillus* species
P26	*B. licheniformis*	$3.2x10^{12}$	*Bacillus* spp	$3.7x10^{12}$	*B. licheniformis*
P27	*B. subtilis*	$1.48x10^{11}$	*Bacillus* spp	$8.3x10^{10}$	*B. subtilis*, members of the *B. subtilis* cluster
P28	*B. subtilis*	$7.5x10^{10}$	*Bacillus* spp	$4.55x10^{10}$	Members of the *B. subtilis* cluster, other *Bacillus* species
P29	*B. subtilis*	$7.5x10^{10}$	*Bacillus* spp	$3.85x10^{10}$	Other *Bacillus* species
P30	*B. subtilis* *B. licheniformis*	$1.48x10^{11}$	*Bacillus* spp	$5.35x10^{10}$	*B. subtilis*, members of the *B. subtilis* cluster, other *Bacillus* species
P31	*B. subtilis* *B. licheniformis* *L. acidophilus* *L. casei* *S. faecium*	$6.5x10^{10}$ $5.8x10^{10}$ $6x10^{9}$ $1x10^{9}$ $1.5x10^{9}$	*Bacillus* spp	$1.93x10^{10}$	Members of the *B. subtilis* cluster, other *Bacillus* species
P32	*B. subtilis* *B. licheniformis* *L. acidophilus* *L. casei* *S. faecium*	$6.5x10^{10}$ $5.8x10^{10}$ $6x10^{9}$ $1x10^{9}$ $1.5x10^{9}$	*Bacillus* spp	$2.45x10^{10}$	Members of the *B. subtilis* cluster, other *Bacillus* species
P33	*B. subtilis*	$4.7x10^{8}$	*Bacillus* spp	$3.25x10^{10}$	*B. sphaericus*, members of *B. subtilis* cluster, other *Bacillus* species
P34	*B. subtilis*	$4.7x10^{8}$	*Bacillus* spp	$1.65x10^{8}$	*B. sphaericus*
P35	*B. subtilis*	$2x10^{11}$	*Bacillus* spp	$3.9x10^{11}$	Members of the *B. subtilis* cluster
P36	*B. subtilis*	$2x10^{11}$	*Bacillus* spp	$4.65x10^{11}$	Members of the *B. subtilis* cluster
P37	*B. subtilis*	$2x10^{11}$	*Bacillus* spp	$3.3x10^{11}$	*B. licheniformis,* members of the *B. subtilis* cluster
P38	*B. licheniformis*	$3.2x10^{12}$	*Bacillus* spp	$1.8x10^{12}$	*B. licheniformis*
P39	*B. licheniformis*	$3.2x10^{12}$	*Bacillus* spp	$1.7x10^{12}$	*B. licheniformis*
P40	*B. licheniformis*	$3.2x10^{12}$	*Bacillus* spp	$2.45x10^{12}$	*B. licheniformis*
P41	Lactic acid bacteria	$1.34x10^{12}$	*Lactobacillus* spp	$2.7x10^{11}$	*L. delbrueckii*, other lactic acid species[f]

Table 6 (Continued)

Product	Labelling information		Results		
	Strains	Number[a]	Strains	Number[a]	Specific species
P42	*Cl. butyricum*	$5x10^8$	*Clostridium* spp	NT	*Cl. butyricum*
P43	*B. licheniformis*	≥ $1x10^{12}$	*Bacillus* spp	$2.12x10^{12}$	Members of the *B. subtilis* cluster,
	B. subtilis		*Enterococcus* spp	$1.54x10^{11}$	*E. faecium*
	B. pumilus				
	E. faecium	≥ $1x10^{11}$			
	E. faecalis				
P44	Lactic acid bacteria	≥ $7x10^{12}$	*Lactobacillus* spp	$8.1x10^{11}$	Other *Lactobacillus* species[g],
	B. subtilis	≥ $3x10^{12}$	*Bacillus* spp	$7.35x10^{12}$	members of the *B. subtilis* cluster
P45	Lactic acid bacteria	≥ $7x10^{12}$	*Lactobacillus* spp	$1.85x10^{12}$	Other *Lactobacillus* species,
	B. subtilis	≥ $3x10^{12}$	*Bacillus* spp	$9.8x10^{12}$	members of the *B. subtilis* cluster

[a] Unit is cfu/kg for all products except for product P1 and P7 (cfu/l)

[b] *B. pumilus, B. amyloliquefaciens* and *B. atropheus*

[c] These *Bacillus* species could not confirmed by ARDRA.

[d] *L. casei* and *L. paracasei*

[f] These lactic acid bacteria were not species of genus *Lactobacillus.*

[g] These *Lactobacillus* species could not confirmed by multiplex PCR.

NT, not test

The genus and species of probiotic bacteria in 45 probiotic products are shown in Table 6. Twenty-six in 41 products (26/41, 63.4%) were inaccurately labelled in term of species (Figure 3). These products comprised other species than those claimed on the contents.

B. subtilis was stated on the labels of 29 products, of which only 8 products (P1, P2, P3, P13, P20, P21, P27 and P30) were found to contain the species. Twenty-two products claimed as containing *B. subtilis* consisted of members of *B. subtilis* cluster (*B. pumilus, B. amyloliquefaciens,* and *B. atrophaeus*). Thirteen products claimed to contain *B. licheniformis*, but only 7 of them (P11, P13, P18, P26, P38, P39 and P40) were found to carry this species. Two products (P6 and P37), which declared to harbor *B. subtilis* on the label were found to contain *B. licheniformis*. Other *Bacillus* species that could not be confirmed by ARDRA were detected in 9 products including P2, P19, P24, P25, P29, P30, P31, P32, and P33. In particular, *B. sphaericus*, that was

not listed on the label contents of all products, was present in 4 products including P1, P12, P33, and P34.

In this study, the ARDRA method can be used to differentiate most of *Bacillus* species, except *B. amyloquefaciens* declared on the label of product P9 and *B. cereus toyoi* claimed on that of products P24 and P25. In product P9, *B. amyloquefaciens* was claimed on the label and the findings obtained by using ARDRA was members of the *B. subtilis* cluster that included *B. amyloquefaciens*. In product P24 and P25, the *Bacillus* genus could be confirmed. Due to limitation of ARDRA method used, *B. cereus toyoi* could not be determined and thus defined as other *Bacillus* species.

In product P7, various *Lactobacillus* spp, such as *L. rhamnosus* and *L. casei*-group (*L. casei* and *L. paracasei*), were found but these bacteria were not listed on the label. *L. acidophilus* was stated on the contents of 4 products (P7, P12 P31 and P32), however none was found to carry this bacterial species. Lactic acid bacteria were listed on label of 3 products (P41, P44 and P45). Product P41 tested contained *L. delbruckii* and other lactic bacteria which were not species of genus *Lactobacillus.* Other *Lactobacillus* species that could not be identified by multiplex PCR were found in Product P44 and P45. Three products (P41, P44 and P45) were declared lactic acid bacteria on the labels but not specific species.

Three products (P6, P31 and P32) were labelled to contain *Streptococcus faecium*. *Enterococcus* spp*,* particularly *E. faecium,* was detected in 4 products (P6, P8, P14 and P43). *E. faecalis* was also labelled on product P43, but none were found.

Based on the PCR results, 4 products (P15, P16, P17, P42) consisting of only *Clostridium* spp were accurately labelled at both genus and species level. None was found to be positive to *Lactobacillus*, *Bacillus* and *Enterococcus*.

2. Contamination of *E. coli* and *Salmonella* in whole probiotic products (n=45)

None of the probiotic products tested (n=45) were positive to *E. coli* and *Salmonella.*

3. Phenotypic AMR in the bacterial isolates (n=64) from probiotic products

The MICs of 14 antimicrobials was analyzed in 64 isolates including *Bacillus* (n=54), *Enterococcus* (n=4) and *Lactobacillus* (n=6). Overall, resistance to chloramphenicol (21%) was highest among probiotic bacteria, followed by resistance to trimethoprim (17%), clindamycin (16%) sulfamethoxazole (15%), ampicillin (10%), erythromycin (9%), vancomycin (9%), tetracycline (8%), ciprofloxacin (6%), streptomycin (5%) and kanamycin (5%). Resistance to gentamycin, meropenem and rifampicin resistance were not observed in all isolates (Figure 4).

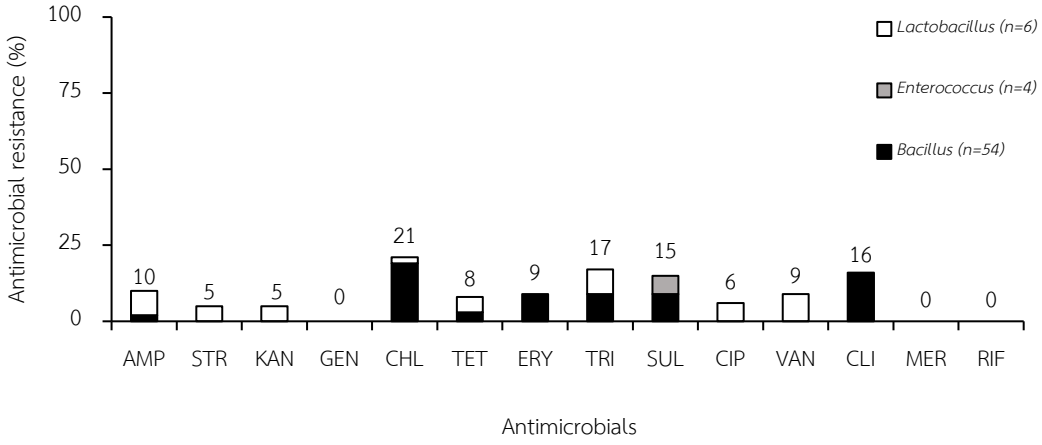

Figure 4. Antimicrobial resistance in bacterial species isolated from probiotic products (n=64).

Abbreviation: AMP, ampicillin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin.

Sixty-four isolates were classified into *B. subtilis* (n=8)*, B. licheniformis* (n=9), *B. sphaericus* (n=4), other *Bacillus* spp. (n=10), members of *B. subtilis* cluster (n=23), *E. faecium* (n=4), *L. casei*-group (n=1), *L. plantarum* (n=1), *L. rhamnosus* (n=1), *L. delbrueckii* (n=1) and other *Lactobacillus* spp. (n=2). Nine antimicrobial resistance patterns are defined (Table 7). The distribution of MICs for all antibiotics is shown in Table 8.

Table 7. Antimicrobial resistance patterns of bacterial isolates from probiotic products (n=64)

Resistant pattern	No. of isolates								
	B. licheniformis (n=9)	*B. sphaericus* (n=4)	Other *Bacillus* spp. (n=10)	*E. faecium* (n=4)	*L. casei*-group (n=1)	*L. plantarum* (n=1)	*L. rhamnosus* (n=1)	*L. delbrueckii* (n=1)	Other *Lactobacillus* spp. (n=2)
AMP-CIP-ERY-KAN-STR-TRI-VAN								1	2
AMP-CHL-TRI-VAN					1				
AMP-TRI-CIP-VAN						1			
CHL-TET-TRI-SUL			2						
CHL-CLI-ERY	5	1							
CHL-CLI	4								
TRI-SUL		3	1						
AMP			1						
SUL				4					
VAN							1		
Total	9	4	4	4	1	1	1	1	2

Abbreviation: AMP, ampicillin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin.

Table 8. Distribution of MICs of bacterial isolates from probiotic products (n=64)

Antibiotic	Strain (n)	Distribution of MICs (µg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
AMP	*B. subtilis* (8)		8																0
	B. licheniformis (9)		8		1														0
	B. sphaericus (4)		2	1				1											0
	Other *Bacillus* spp. (10)		8					1	1										1
	Members of *B. subtilis* cluster (23)		23																0
	E. faecium (4)						4												0
	L. casei-group (1)									1									1
	L. plantarum (1)									1									1
	L. rhamnosus (1)					1													0
	L. delbrueckii (1)							1											1
	Other *Lactobacillus* species (2)								2										2
	Subtotal	0	49	1	1	1	4	3	3	2	0	0	0	0	0	0	0	0	6
STR	*B. subtilis* (8)						8												0
	B. licheniformis (9)						9												0
	B. sphaericus (4)						3		1										0
	Other *Bacillus* spp. (10)						7	3											0
	Members of *B. subtilis* cluster (23)						22	1											0
	E. faecium (4)						4												0
	L. casei-group (1)										1								0
	L. plantarum (1)											1							0
	L. rhamnosus (1)											1							0
	L. delbrueckii (1)													1					1
	Other *Lactobacillus* species (2)											1	1						2
	Subtotal	0	0	0	0	0	53	4	1	0	1	3	1	1	0	0	0	0	3

Abbreviation: AMP, ampicillin; STR, streptomycin

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line.

Table 8 (Continued)

Antibiotic	Strain (n)	Distribution of MICs (μg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
KAN	*B. subtilis* (8)						8												0
	B. licheniformis (9)						9												0
	B. sphaericus (4)						4												0
	Other *Bacillus* spp. (10)						10												0
	Members of *B. subtilis* cluster (23)						23												0
	E. faecium (4)															4			0
	L. casei-group (1)												1						0
	L. plantarum (1)												1						0
	L. rhamnosus (1)												1						0
	L. delbrueckii (1)													1					1
	Other *Lactobacillus* species (2)											1		1					2
	Subtotal	0	0	0	0	0	54	0	0	0	0	1	4	1	0	0	0	0	3
GEN	*B. subtilis* (8)			8															0
	B. licheniformis (9)			5	4														0
	B. sphaericus (4)			3	1														0
	Other *Bacillus* spp. (10)			8	2														0
	Members of *B. subtilis* cluster (23)				23														0
	E. faecium (4)								4										0
	L. casei-group (1)							1											0
	L. plantarum (1)								1										0
	L. rhamnosus (1)							1											0
	L. delbrueckii (1)							1											0
	Other *Lactobacillus* species (2)							1	1										0
	Subtotal	0	0	24	30	0	0	4	6	0	0	0	0	0	0	0	0	0	0

Abbreviation: KAN, kanamycin; GEN, gentamicin.

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line.

Table 8 (Continued)

Antibiotic	Strain (n)	Distribution of MICs (μg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
CHL	*B. subtilis* (8)								7	1									0
	B. licheniformis (9)										8	1							9
	B. sphaericus (4)								2	1	1								1
	Other *Bacillus* spp. (10)							3	5				2						2
	Members of *B. subtilis* cluster (23)					1	5		17										0
	E. faecium (4)							4											0
	L. casei-group (1)									1									1
	L. plantarum (1)									1									0
	L. rhamnosus (1)								1										0
	L. delbrueckii (1)								1										0
	Other *Lactobacillus* species (2)								2										0
	Subtotal	0	0	0	0	1	5	7	35	4	9	1	2	0	0	0	0	0	13
TET	*B. subtilis* (8)			4					3	1									0
	B. licheniformis (9)			5				2	1	1									0
	B. sphaericus (4)			3	1														0
	Other *Bacillus* spp. (10)			2	1				3	2			2						2
	Members of *B. subtilis* cluster (23)			6				2	3	12									0
	E. faecium (4)			4															0
	L. casei-group (1)						1												0
	L. plantarum (1)										1								0
	L. rhamnosus (1)						1												0
	L. delbrueckii (1)														1				1
	Other *Lactobacillus* species (2)											2							2
	Subtotal	0	0	24	2	0	2	4	10	16	1	2	2	0	1	0	0	0	5

Abbreviation: CHL, chloramphenicol, TET, tetracycline

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line.

Table 8 (Continued)

Antibiotic	Strain (n)	Distribution of MICs (μg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
ERY	*B. subtilis* (8)				8														0
	B. licheniformis (9)				4									5					5
	B. sphaericus (4)				3									1					1
	Other *Bacillus* spp. (10)				7	1	2												0
	Members of *B. subtilis* cluster (23)				22	1													0
	E. faecium (4)			4															0
	L. casei-group (1)			1															0
	L. plantarum (1)			1															0
	L. rhamnosus (1)			1															0
	L. delbrueckii (1)			1															0
	Other *Lactobacillus* species (2)			1															0
	Subtotal	0	0	9	44	2	2	0	0	0	0	0	0	6	0	0	0	0	6
TRI	*B. subtilis* (8)			8															0
	B. licheniformis (9)			9															0
	B. sphaericus (4)			1										1	2				3
	Other *Bacillus* spp. (10)			3	2	2									3				3
	Members of *B. subtilis* cluster (23)			18	3	1	1												0
	E. faecium (4)			4															0
	L. casei-group (1)											1							1
	L. plantarum (1)															1			1
	L. rhamnosus (1)										1								0
	L. delbrueckii (1)											1							1
	Other *Lactobacillus* species (2)												1			1			2
	Subtotal	0	0	43	5	3	1	0	0	0	1	2	1	1	5	2	0	0	11

Abbreviation: ERY, erythromycin, TRI, trimethoprim

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line.

Table 8 (Continued)

Antibiotic	Strain (n)	Distribution of MICs (μg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
SUL	*B. subtilis* (8)					6	2												0
	B. licheniformis (9)					6	1	1	1										0
	B. sphaericus (4)						1											3	3
	Other *Bacillus* spp. (10)					2	1		3		1							3	3
	Members of *B. subtilis* cluster (23)					1	6	4	12										0
	E. faecium (4)																	4	4
	L. casei-group (1)													1					0
	L. plantarum (1)													1					0
	L. rhamnosus (1)													1					0
	L. delbrueckii (1)												1						0
	Other *Lactobacillus* species (2)													2					0
	Subtotal	0	0	0	0	15	11	5	16	0	1	0	1	5	0	0	0	10	10
CIP	*B. subtilis* (8)	8																	0
	B. licheniformis (9)	9																	0
	B. sphaericus (4)	3	1																0
	Other *Bacillus* spp. (10)	7	3																0
	Members of *B. subtilis* cluster (23)	22	1																0
	E. faecium (4)				4														0
	L. casei-group (1)							1											0
	L. plantarum (1)										1								1
	L. rhamnosus (1)						1												0
	L. delbrueckii (1)											1							1
	Other *Lactobacillus* species (2)												2						2
	Subtotal	49	5	0	4	0	1	1	0	0	1	1	2	0	0	0	0	0	4

Abbreviation: SUL, sulfamethoxazole, CIP, ciprofloxacin

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line.

Table 8 (Continued)

Antibiotic	Strain (n)	Distribution of MICs (μg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
VAN	*B. subtilis* (8)				8														0
	B. licheniformis (9)				9														0
	B. sphaericus (4)				3	1													0
	Other *Bacillus* spp. (10)				6	1	2		1										0
	Members of *B. subtilis* cluster (23)				22	1													0
	E. faecium (4)					4													0
	L. casei-group (1)														1				1
	L. plantarum (1)														1				1
	L. rhamnosus (1)														1				1
	L. delbrueckii (1)														1				1
	Other *Lactobacillus* species (2)														2				2
	Subtotal	0	0	0	48	7	2	0	1	0	0	0	0	0	6	0	0	0	6
CLI	*B. subtilis* (8)			2	1	3	2												0
	B. licheniformis (9)										3	5	1						9
	B. sphaericus (4)					2	1			1									1
	Other *Bacillus* spp. (10)			3	4	3													0
	Members of *B. subtilis* cluster (23)			2	13	7	0	1											0
	E. faecium (4)					1		2	1										0
	L. casei-group (1)	1																	0
	L. plantarum (1)				1														0
	L. rhamnosus (1)	1																	0
	L. delbrueckii (1)	1																	0
	Other *Lactobacillus* species (2)	2																	0
	Subtotal	5	0	7	19	16	3	3	1	1	3	5	1	0	0	0	0	0	10

Abbreviation: VAN, vancomycin; CLI, clindamycin

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line.

Table 8 (Continued)

Antibiotic	Strain (n)	Distribution of MICs (μg/ml)																	No. of resistance
		<0.0625	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
MER	*B. subtilis* (8)		8																0
	B. licheniformis (9)		9																0
	B. sphaericus (4)		4																0
	Other *Bacillus* spp. (10)		10																0
	Members of *B. subtilis* cluster (23)		23																0
	E. faecium (4)								3	1									0
	L. casei-group (1)		1																0
	L. plantarum (1)				1														0
	L. rhamnosus (1)							1											0
	L. delbrueckii (1)		1																0
	Other *Lactobacillus* species (2)					1													0
	Subtotal	0	56	0	1	1	0	1	3	1	0	0	0	0	0	0	0	0	0
RIF	*B. subtilis* (8)			8															0
	B. licheniformis (9)			9															0
	B. sphaericus (4)			4															0
	Other *Bacillus* spp. (10)			10															0
	Members of *B. subtilis* cluster (23)			23															0
	E. faecium (4)			4															0
	L. casei-group (1)									1									0
	L. plantarum (1)					1													0
	L. rhamnosus (1)					1													0
	L. delbrueckii (1)					1													0
	Other *Lactobacillus* species (2)					2													0
	Subtotal	0	0	58	0	5	0	0	0	1	0	0	0	0	0	0	0	0	0

The clinical breakpoints or ECOFFs for each antimicrobial are presented as a vertical line. Abbreviation: MER, meropenem; RIF, rifampicin.

The MIC range was 0.625 to 8 µg/ml for ampicillin, 1 to 128 µg/ml for streptomycin, 1 to 512 µg/ml for kanamycin, 0.125 to 4 µg/ml for gentamicin, 0.5 to 64 µg/ml for chloramphenicol, 0.125 to 256 µg/ml for tetracyclines, 0.125 to 128 µg/ml for erythromycin, 0.125 to 512 µg/ml for trimethoprim, 0.5 to >1024 µg/ml for sulfamethoxazole, <0.0625 to 64 µg/ml for ciprofloxacin, 0.25 to 256 µg/ml for vancomycin, <0.0625 to 64 µg/ml for clindamycin, 0.0625 to 8 µg/ml for meropenem, 0.0125 to 16 µg/ml for rifampicin (Table 8).

In general, antimicrobial susceptibilities appeared to vary according to bacterial species. Of 64 isolates tested, 33 isolates (51.6%) including *B. licheniformis* (n=9), *B. sphaericus* (n=4), other *Bacillus* spp. (n=10), *E. faecium* (n=4), *L. casei*-group (n=1), *L. plantarum* (n=1), *L. rhamnosus* (n=1), *L. delbrueckii* (n=1) and other *Lactobacillus* spp. (n=2) were resistant to at least one antimicrobial agent. However, 31 in 64 isolates (48.4%) consisting of *B. subtilis* (n=8) and members of *B. subtilis* cluster (n=23) were phenotypically susceptible to all antimicrobials.

In terms of *Bacillus* isolates, resistance to chloramphenicol (19%), followed clindamycin (16%), erythromycin (9%), trimethoprim (9%), sulfamethoxazole (9%), tetracycline (3%) and ampicillin (2%) were observed in *B. licheniformis*, *B. sphaericus* and other *Bacillus* spp. All *B. licheniformis* (n=9) were resistant to chloramphenicol (MIC, 16–32 µg/ml) and clindamycin (MIC, 16-64 µg/ml). The distribution of erythromycin MICs of *B. licheniformis* covered more than nine 2-fold dilutions, ranging from 0.25 to more than 128 µg/ml). The common AMR patterns found in *B. licheniformis* were CHL-CLI and CHL-CLI-ERY (Table 7). Of 4 *B. sphaericus* isolates, one was resistant to chloramphenicol (MIC=16 µg/ml), erythromycin (MIC>128 µg/ml) and clindamycin (MIC=8 µg/ml), followed by three isolates that were resistant to trimethoprim (MIC ranging from 128 to 256 µg/ml) and sulfamethoxazole (MIC=2048 µg/ml). The common AMR patterns observed in *B. sphaericus* were TRI-SUL, followed by CHL-CLI-ERY. Among 4 resistant isolates of other *Bacillus* spp., 2 isolates were resistant to chloramphenicol (MIC=64 µg/ml), tetracycline (MIC=64 µg/ml),

trimethoprim (MIC≥256 µg/ml) and sulfamethoxazole (MIC=2048 µg/ml), one isolate was resistant to trimethoprim (MIC=256 µg/ml) and sulfamethoxazole (MIC=2048 µg/ml), and one isolate was resistant to ampicillin (MIC=4 µg/ml). Several AMR patterns ware found including AMP, TRI-SUL and CHL-TET-TRI-SUL.

Most of *Enterococcus* isolates were sensitive to all antimicrobials test, except sulfamethoxazole. High level sulfamethoxazole resistance in all *E. faecium* (n=4) was determined showing MIC of ≥1024 µg/ml.

Among 6 *Lactobacillus* isolates tested, multidrug resistant (MDR) phenotypes were observed in 5 isolates. One in six *Lactobacillus* isolates was susceptibility to all antimicrobials, except vancomycin. The resistant *Lactobacillus* isolates showed high MIC values ranging from 2-8 µg/ml for ampicillin, 16-128 µg/ml for streptomycin, 32-128 µg/ml for kanamycin, 8 µg/ml for chloramphenicol, 32-256 µg/ml for tetracycline, 32-≥512 µg/ml for trimethoprim, 16-64 µg/ml for ciprofloxacin and ≥256 µg/ml for vancomycin. In particular, vancomycin resistance was detected in all *Lactobacillus* with MIC≥256 µg/ml. Different antibiotic resistance patterns were found in *Lactobacillus* of which the most common pattern was AMP-STR-KAN-TET-TRI-CIP-VAN, followed by AMP-CHL-TRI-VAN and AMP-TRI-CIP-VAN.

4. Presence of AMR genes in whole probiotic products (n=45)

Forty-five products (n=45) were performed screening test for the presence of 111 genes that encode resistance to clinically important antibiotics. Distribution of AMR genes is shown in Figure 5. The presence of AMR genes in each product is described in Table 9.

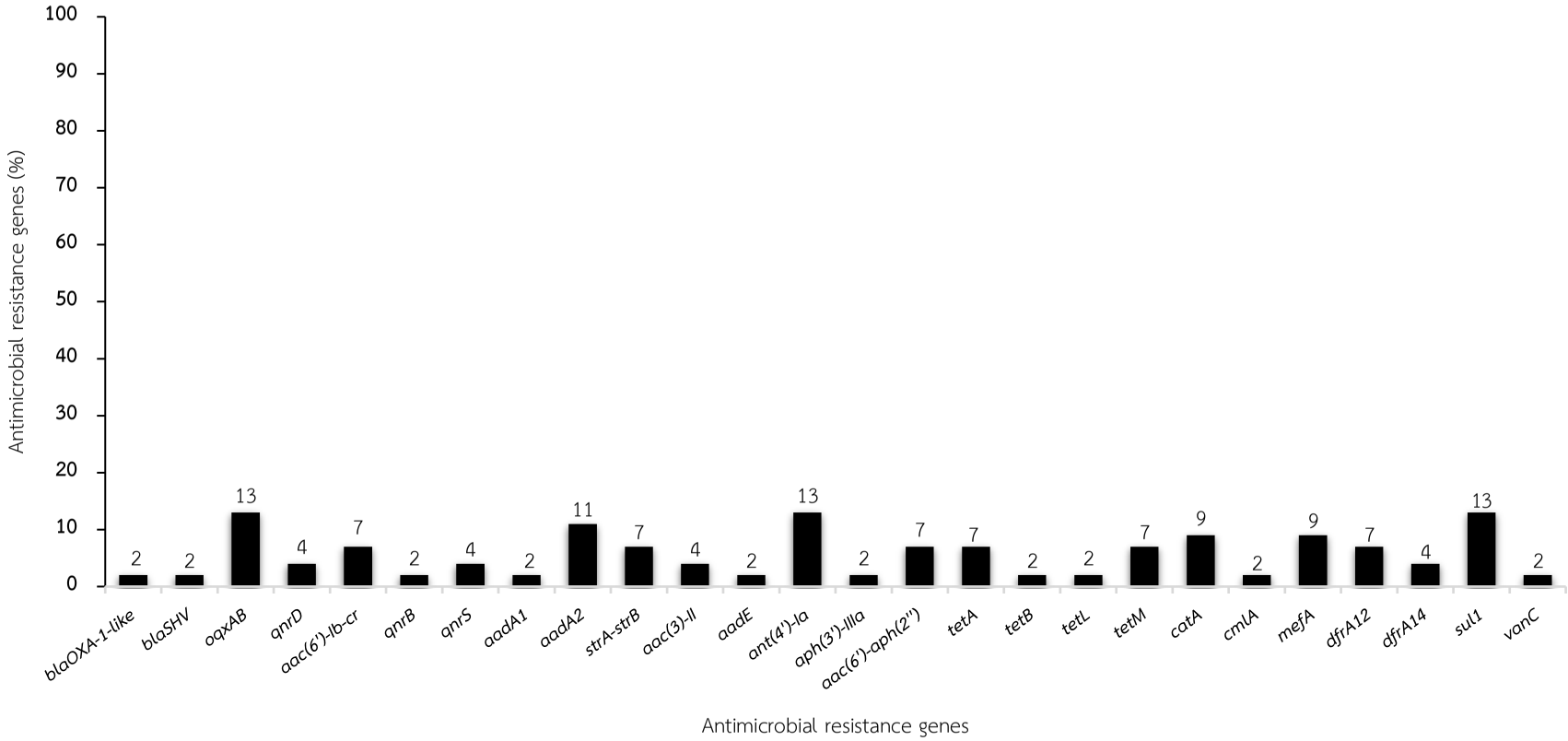

Figure 5. Antimicrobial resistance genes presented in probiotic products (n=45)

Table 9. Presence of AMR genes and their known encoding resistance phenotypes

Product	Known encoding resistance phenotypes								
	β-lactamase	Quinolone	Aminoglycosides	Tetracycline	Chloramphenicol	Macrolide	Trimethoprim	Sulfonamide	Vancomycin
P3		*oqxAB*[a]							
P4		*oqxAB*	*aadA2*[b]					*sul1*	
P5								*sul1*	
P6		*qnrD*[a]		*tetA*, *tetM*					
P12	$bla_{OXA-1-like}$	*aac(6')-Ib-cr*[a], *qnrB*[a]	*aadA1*[b], *aadA2*, *strA-strB*[b], *aac(3)-II*[c]	*tetA*	*catA*		*dfrA14*	*sul1*	
P13			*strA-strB*, *aadE*[b]	*tetM*					
P31			*ant(4')-Ia*[d]						
P32			*ant(4')-Ia*			*mefA*			
P38		*oqxAB*	*ant(4')-Ia*		*catA*	*mefA*			
P39			*ant(4')-Ia*, *aac(6')-aph(2'')*[c]		*catA*	*mefA*			
P40			*ant(4')-Ia*, *aph(3')-IIIa*[d]		*catA*				
P41				*tetM*, *tetL*		*mefA*			
P42		*qnrS*[a], *qnrD*	*ant(4')-Ia*						
P43	bla_{SHV}	*oqxAB*, *qnrS*	*aadA2*, *strA-strB*, *aac(3)-II*	*tetA*, *tetB*	*cmlA*		*dfrA12*, *dfrA14*	*sul1*	*vanC*
P44		*oqxAB*, *aac(6')-Ib-cr*	*aadA2*, *aac(6')-aph(2'')*				*dfrA12*	*sul1*	
P45		*oqxAB* , *aac(6')-Ib-cr*	*aadA2*, *aac(6')-aph(2'')*				*dfrA12*	*sul1*	

Genes encoding resistance to [a] ciprofloxacin, [b] streptomycin, [c] gentamicin and [d] kanamycin

Of 45 products, 16 products (35.5%) were positive to at least one resistance gene, whereas 13 products (28.9%) contained resistance genes encoding resistance to more than three antibiotic classes. In general, the most common AMR genes found in probiotic products were *oqxAB* (13%), *ant(4')-Ia* (13%) and *sul1* (13%) that confer resistance against ciprofloxacin, kanamycin and sulfonamide, respectively.

The genes encoding resistance to aminoglycosides were most commonly found among probiotic products (12/45, 26.6%). Twelve products were found to carry genes encoding resistance to various aminoglycoside antibiotics including streptomycin (*aadA1, aadA2, aadE* and *strA-strB*), gentamicin [*aac(3')-II* and *aac(6')-aph(2'')*], kanamycin [*ant(4')-Ia* and *aph(3')-IIIa*] (Table 9). The *ant(4')-Ia* gene observed in 6 products (P31, P32, P38, P39, P40 and P42) was the most common gene, followed by *aadA1* found in 5 products (P4, P12, P43, P44 and P45). Nine products contained genes encoding quinolone resistance, especially ciprofloxacin including *oqxAB, qnrB, qnrD*, *qnrS* and *aac(6')-Ib-cr* (Table 9). β-lactamase genes, $bla_{\text{OXA-1-like}}$ and bla_{SHV}, were detected in product P12 and P43, respectively. The *tet* genes, including *tetA, tetB, tetL* and *tetM,* which mediated tetracycline resistance, were found in 5 products (Table 9). The gene *catA* encoding chloramphenicol acetyltransferases was found in 4 products (P12, P38, P39 and P40), while *cmlA* encoding efflux pump was observed one product (P43). The *mefA* gene conferred macrolide efflux pump was observed in 4 products. Four products contained two trimethoprim resistance genes including *dfrA12* and *dfrA14*. Among three sulfonamide resistance genes tested (*sul1, sul2,* and *sul3*), only *sul1* gene was found in 6 products (P4, P5, P12, P43, P44 and P45). Only one (P43) carried *vanC* gene encoding resistance to vancomycin.

More than 10 AMR genes which mediated different antimicrobial classes were detected in two products (P12 and P43) (Table 9). Product P43 carried 14 AMR genes encoding 9 antimicrobial classes including β-lactams (bla_{SHV}), fluroquinolones (*oqxAB*), quinolones (*qnrS*), aminoglycosides [*aadA2, strA-strB* and *aac(3)-II*], tetracycline (*tetA* and *tetB*), chloramphenicol (*cmlA*), trimethoprim (*dfrA12* and *dfrA14*), sulfonamide

(*sul1*), and vancomycin (*vanC*). Product P44 and P45 were positive to identical AMR genes, which were *oqxAB, aac(6')-Ib-cr, aadA2, aac(6')-aph(2'')*, *dfrA12* and *sul1.*

Almost AMR phenotypes in bacterial isolates were not correlated with AMR genes found in probiotic products. The correlations between AMR phenotypes of bacterial isolates and AMR genes found 16 probiotic products are shown in Table 10.

Table 10. AMR phenotypes in bacterial isolates and AMR genes found in probiotic products (n=16)

Products	AMR phenotypes of bacterial isolates			AMR genes found in probiotic products
	Isolate	Species	Resistance patterns	
P3	-	-	-	*oqxAB*
P4	-	-	-	*oqxAB, aadA2, sul1*
P5	-	-	-	*sul1*
P6	B6.1	*B. licheniformis*	CHL-CLI-ERY	*qnrD, tetA, tetM*
	E6.1	*E. faecium*	SUL	
P12	B12.1	*B. sphaericus*	**TRI-SUL**	*bla*$_{OXA-1-like}$, *aac(6')-Ib-cr, qnrB, aadA1, aadA2, strA-strB, aac(3)-II, tetA, catA,* ***dfrA14***, ***sul1***
P13	B13.1	*B. licheniformis*	CHL-CLI-ERY	*strA-strB, aadE, tetM*
P31	-	-	-	*ant(4')-Ia*
P32	-	-	-	*ant(4')-Ia, mefA*
P38	B38.1	*B. licheniformis*	**CHL**-CLI	*oqxAB, ant(4')-Ia,* ***catA***, *mefA*
P39	B39.1	*B. licheniformis*	**CHL**-CLI	*ant(4')-Ia, aac(6')-aph(2'')*, ***catA***, *mefA*
P40	B40.1	*B. licheniformis*	**CHL**-CLI	*ant(4')-Ia, aph(3')-IIIa,* ***catA***
P41	L41.1	*L. delbrueckii*	AMP-CIP-ERY-KAN-STR-TRI-VAN	*tetM, tetL, mefA*
P42	-	-	-	*qnrS, qnrD, ant(4')-Ia,*
P43	E43.1	*E. faecium*	**SUL**	*bla*$_{SHV}$, *oqxAB, qnrS, aadA2, strA-strB, aac(3)-II, tetA, tetB, dfrA12, dfrA14,* ***sul1***, *vanC*
P44	L44.1	Other *Lactobacillus* spp.	AMP-**CIP-ERY-KAN-STR-TRI**-VAN	***oqxAB, aac(6')-Ib-cr, aadA2, aac(6')-aph(2''), dfrA12***, *sul1*
P45	L45.1	Other *Lactobacillus* spp.	AMP-**CIP-ERY-KAN-STR-TRI**-VAN	***oqxAB, aac(6')-Ib-cr, aadA2, aac(6')-aph(2''), dfrA12***, *sul1*

-, isolates were susceptible with all antimicrobials tested.

Bold letters indicate resistance genes that may correspond to the AMR genotypes of bacterial isolates from probiotic products.

Abbreviation: AMP, ampicillin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin.

The bacterial isolates from six products (P3, P4, P5, P31, P32, and P42) were susceptible to all antimicrobials, however, several AMR genes were found. Products

P6, P13 and P41 had AMR phenotypes that were not related to AMR genes found in those products. In product P12, resistance to trimethoprim and sulfamethoxazole of *B. sphaericus* might be correlated with *dfrA14* and *sul1*, respectively, however none of AMR phenotypes were found to be associated with other AMR genes including $bla_{OXA\text{-}1\text{-}like}$, *aac(6')-Ib-cr, qnrB, aadA1, aadA2, strA-strB, aac(3)-II, tetA* and *catA.* Three products (P38, P39 and P40) contained chloramphenicol-resistant *B. licheniformis* isolates that might carry *catA* gene found in those products. These *B. licheniformis* isolates were also resistant to clindamycin, however, none of clindamycin-resistant genes was found. Product P43 contained *E. faecium* that was only resistant to sulfamethoxazole correlated with *sul1* gene. This product was found to carry other AMR genes, including $bla_{SHV,}$ *oqxAB, qnrS, aadA2, strA-strB, aac(3)-II, tetA, tetB, dfrA12, dfrA14,* and *vanC,* however, the AMR phenotypes corresponding to these genes were not detected in bacterial isolates. *Lactobacillus* isolates from products P44 and P45 were resistance ciprofloxacin that might correspond to *oqxAB* and *aac(6')-Ib-cr*. In addition, resistance to streptomycin and trimethoprim might be correlated with *aadA2* and *dfrA12.* Although, these isolates exhibited resistance to ampicillin, erythromycin, kanamycin and vancomycin, none of corresponding AMR genes were detected in those products. Products P44 and P45 were positive to *aac(6')-aph(2'')* and *sul1,* however, corresponding AMR phenotypes were not found in bacterial isolates.

5. Transfer of AMR genes

All the *Bacillus* (n=17) and *Lactobacillus* (n=5) isolates that had resistance phenotypes were examined for transferability of AMR genes. The donors including *Bacillus* and *Lactobacillus* with their MICs for 14 antimicrobials are shown in Table 11. Antimicrobial susceptibilities of donors, recipients and transconjugants are described in Table 12.

Table 11. MICs of 14 antibiotics for donors in conjugation experiment (n=22)

No.	Donors		MIC (μg/ml)													
	Isolate	Species	AMP	STR	KAN	GEN	CHL	TET	ERY	TRI	SUL	CIP	VAN	CLI	MER	RIF
1	B1.5	*B. sphaericus*	0.125	1	1	0.125	16	0.125	>128	0.125	1	0.015625	0.25	8	0.0625	0.125
2	B2.1	Other *Bacillus* spp.	4	1	1	0.125	4	0.25	0.5	0.5	16	0.0625	4	0.5	0.0625	0.125
3	B6.1	*B. licheniformis*	0.125	1	1	0.125	16	0.125	>128	0.125	0.5	0.03125	0.25	64	0.0625	0.125
4	B11.1	*B. licheniformis*	0.125	1	1	0.125	16	2	>128	0.125	0.5	0.03125	0.25	16	0.0625	0.125
5	B12.2	*B. sphaericus*	2	4	1	0.125	4	0.25	0.25	256	2048	0.0625	0.5	1	0.0625	0.125
6	B13.1	*B. licheniformis*	0.125	1	1	0.125	16	4	>128	0.125	1	0.03125	0.25	32	0.0625	0.125
7	B18.1	*B. licheniformis*	0.25	1	1	0.125	16	0.125	0.25	0.125	0.5	0.03125	0.25	32	0.0625	0.125
8	B24.1	Other *Bacillus* spp.	0.0625	2	1	0.125	64	64	1	>256	2048	0.0625	1	0.25	0.0625	0.125
9	B25.1	Other *Bacillus* spp.	0.0625	2	1	0.25	64	64	1	>256	2048	0.0625	1	0.25	0.0625	0.125
10	B26.1	*B. licheniformis*	0.125	1	1	0.25	16	2	>128	0.125	0.5	0.015625	0.25	16	0.0625	0.125
11	B33.3	Other *Bacillus* spp.	0.0625	1	1	0.125	2	0.125	0.25	256	2048	0.03125	0.5	0.5	0.0625	0.125
12	B33.4	*B. sphaericus*	0.0625	1	1	0.125	4	0.125	0.25	256	2048	0.03125	0.5	0.5	0.0625	0.125
13	B34.1	*B. sphaericus*	0.0625	1	1	0.25	8	0.125	0.25	128	2048	0.03125	0.5	0.5	0.0625	0.125
14	B37.1	*B. licheniformis*	0.125	1	1	0.125	16	8	>128	0.125	2	0.015625	0.25	16	0.0625	0.125
15	B38.1	*B. licheniformis*	0.125	1	1	0.25	16	0.125	0.25	0.125	4	0.03125	0.25	32	0.0625	0.125
16	B39.1	*B. licheniformis*	0.125	1	1	0.25	16	0.125	0.25	0.125	0.5	0.015625	0.25	32	0.0625	0.125
17	B40.1	*B. licheniformis*	0.125	1	1	0.25	32	0.125	0.25	0.125	0.5	0.015625	0.25	32	0.0625	0.125
18	L7.1	*L. casei*-group	8	16	64	2	8	1	0.125	32	128	2	>256	0.03125	4	8
19	L7.2	*L. plantarum*	8	32	64	4	8	16	0.125	>512	128	16	>256	0.25	0.25	0.5
20	L41.1	*L. delbrueckii*	2	128	64	2	4	256	0.125	256	64	32	>256	0.03125	1	0.125
21	L44.1	Other *Lactobacillus* spp.	4	32	32	4	4	32	0.125	>512	128	64	>256	0.03125	0.25	0.5
22	L45.1	Other *Lactobacillus* spp.	4	64	128	2	4	32	0.125	64	128	64	>256	0.03125	0.5	0.5

MICs of antibiotics used for selection of transconjugants are highlighted in grey. AMP, ampicillin; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin; MER, meropenem; RIF, rifampicin.

Table 12. Comparison of MICs of 14 antibiotics for donors, recipients and transconjugants.

Type	Strains	MIC (μg/ml)													
		AMP	STR	KAN	GEN	CHL	TET	ERY	TRI	SUL	CIP	VAN	CLI	MER	RIF
Donor	*L. delbrueckii* L41.1	2	128	64	2	4	256	0.125	256	64	32	>256	0.03125	1	0.125
Recipient	*E. coli* K12 MG1655	16	4	4	2	2	0.25	128	0.25	16	0.015625	>256	>64	8	256
Transconjugant	TC-L41.1	16	**512**	8	2	4	2	128	0.25	16	0.015625	>256	>64	8	256
Donor	Other *Lactobacillus* spp. L44.1	4	32	32	4	4	32	0.125	>512	128	64	>256	0.03125	0.25	0.5
Recipient	*E. coli* K12 MG1655	16	4	4	2	2	0.25	128	0.25	16	0.015625	>256	>64	8	256
Transconjugant	TC-L44.1	16	**512**	8	2	4	1	128	0.25	16	0.015625	>256	>64	8	256
Donor	Other *Lactobacillus* spp. L45.1	4	64	128	2	4	32	0.125	64	128	64	>256	0.03125	0.5	0.5
Recipient	*E. coli* K12 MG1655	16	4	4	2	2	0.25	128	0.25	16	0.015625	>256	>64	8	256
Transconjugant	TC-L45.1	16	**512**	8	2	2	0.25	128	0.25	16	0.015625	>256	>64	8	256

Bold letters indicate MICs of transconjugant increased at least 4-fold from recipients.

Abbreviation: AMP, ampicillin; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin; MER, meropenem; RIF, rifampicin.

The conjugation experiments showed that only *Lactobacillus* isolates including L41.1, L44.1 and L45.1 could horizontally transfer streptomycin resistance determinants to *E. coli* recipients (*E. coli* K12 strain MG1655rif^r). MICs of streptomycin for transconjugants (TC-L41.1, TC-L44.1 and TC-L45.1) increased more than 4-fold, from 4 µg/ml to 512 µg/ml, were observed. The presence of genes encoding resistance to streptomycin including *aadA1*, *aadA2, strA-strB*, and *aadE* were tested in transconjugants by PCR, but none was found.

CHAPTER V DISCUSSION

Nowadays, the use of alternatives to antibiotics has become popular, particularly probiotic products that have been widely used in food animal production. In general, probiotic products have been produced from various microorganisms, for example, bacteria, fungi, and yeast, of which use of bacterial strains is most popular. The bacterial strains commonly formulated in probiotic products are Gram-positive bacteria including *Lactobacillus, Bacillus, Enterococcus* and *Clostridium.* In evaluating the potential probiotic strains, both QPS and GRAS status are considered as fundamental requirements of safety including taxonomy, pathogenicity, toxin production, antibiotic resistance, safe history of use, and other safety assessment information. However, Thailand has only the Animal Feed Quality Control Act B.E. 2558 without any guideline for the selection of probiotic strains intended for using in animal feed. Currently, the exact number of probiotic products commercially available in markets for animals in Thailand (population size) is not available. The DLD does not reveal the list of registered products due to its data protection policy. Based on the latest Veterinary and Animal Health Product Directory of feed-animal products in 2012, 24 probiotic products for food animals were sold in Thailand. Some were discontinued, while some new products have been launched in the markets. Due to the limited data available, convenience sampling method was performed. There were 45 probiotic products used in food animals tested in this study. However, most of them were problematic in terms of the amount of viable bacteria, species identification, and AMR determinants.

1. Number and strains of probiotic products sold for food animals in Thailand

Thirty-four in 45 probiotic products were unsatisfactory qualitatively or quantitatively as claimed on the label. As many previous studies, the amount of ingested viable bacteria and the specific species could affect the effectiveness of probiotics (Temmerman et al., 2003; Coeuret et al., 2004). Therefore, it is important

that each product must be guaranteed in number of viable cells and identification of the specific species.

The low number of live bacteria in 11 products, even no viable cells, was found in some products. This may negatively implicate the probiotic's health benefits. None of *Lactobacillus* spp was found in products P12, P31 and P32, although high numbers of these species were present on the label. This was in agreement with the results of previous studies revealing that no viable lactobacilli were found in food supplement (Temmerman et al., 2003) and some feed additives (Wannaprasat et al., 2009). This fault could be due to poor quality control at some stages of production, including drying process, packaging and storage conditions. It has been known that the viability of bacterial cells is strongly influenced during drying process (Morgan et al., 2006). Although freeze-drying has been preferred to preserve microorganisms, the losses in cell viability is inevitable due to the process of freezing and rate of freezing (Donev, 2002). Different bacterial species can have different tolerant levels of stress. This may explain why the multi-species probiotic products tested contained a very low number of some bacterial species, even none. Therefore, it is crucial to thoroughly choose appropriate bacterial species and the suitable manufacturing process, in order to reduce loss of viable cells. Moreover, the packaging and storage conditions, including pH, oxygen, moisture, light, temperature, that have effects on shelf-life of products, should be carefully evaluated (Morgan et al., 2006).

Of 45 products, 30 products had high numbers of *Bacillus* spp and *Enterococcus* spp that were equivalent to or exceeded declared labels. It is not surprising that high numbers of viable *Bacillus* spp could be found in these products because bacilli are spore-forming bacteria and bacterial spores can resist harsh conditions such as heat, dessication, chemicals and radition, enabling them to maintain their viability during drying, storage and handling (Cutting, 2011). The numbers of viable enterococci found in product P8 was 10,000 times higher than those mentioned on the label. The enterococci cannot produce endospores as bacilli, but they are also

able to survive in adverse environments better than many vegetative bacteria (Giraffa, 1999). This means that these bacteria can withstand the harshness during probiotic processing and storage.

The health benefits of probiotics can differ among bacterial strains and species. Different strains of the same species can produce different beneficial effects, so the label should accurately specify strains of probiotic species (Wannaprasat et al., 2009). However, none of products were labeled at the strain level and more than half of products were misidentified at species level. Previous studies have indicated that misidentification of microorganisms was common, mostly at the species level (Hoa et al., 2000; Wannaprasat et al., 2009). The present study showed that many products formulated by other species in *B. subtilis* group mislabelled as *B. subtilis*. Notably, the members of *B. subtilis* cluster were commonly misidentified as *B. subtilis*. It was similar with the results in previous studies (Hoa et al., 2000; Wannaprasat et al., 2009). The species *B. subtilis* itself was also frequently misidentified. The similar findings were reported from probiotic products used for food animals in Thailand, where *B. licheniformis*, *B. sphaericus* and members of *B. subtilis* cluster were frequently mislabelled as *B. subtilis* (Wannaprasat et al., 2009).

Due to limitation of differentiation ability of ARDRA, *B. amyloliquefaciens* and *B. cereus var toyoi* could not be confirmed. Therefore, the products containing *B. amyloliquefaciens* (Product P9) and *B. cereus var toyoi* (Product P24 and P25) on their label could not be defined as mislabelling.

According to EFSA, *B. subtilis*, *B. licheniformis* and members of *B. subtilis* cluster (*B. pumilus, B. amynoliquencies* and *B. atrophaeus*) have been listed of QPS status (EFSA, 2007), while *B. sphaericus* has not been included. *B. sphaericus* has been widely used in larvicides for mosquito control due to a specific protein in their spores (Ferreira et al., 2015). A previous study showed that *B. sphaericus* had potential properties to be formulated in probiotic for shrimp aquaculture (Puri et al., 2005) but further investigations are needed.

Three products were claimed to contain *Streptococcus faecium,* which was reclassified as *E. faecium* in 1984 (Schleifer and Kilpper-Bälz, 1984). *E. faecium* was commonly mislabeled as *S. faecium* in previous studies (Weese, 2003). All labeled *S. faecium* were actually identified as *E. faecium.* Although *Enterococcus* spp. have good probiotic properties, none of *Enterococcus* spp. is considered GRAS or QPS status due to their association with human illnesses, processing virulence factors and the transferability of AMR genes (Hanchi et al., 2018). Thus, the use of *Enteroccus* spp in feed additives should be scrutiny and the manufacturers should submit evidence of safety to relevant authorities.

The species *Cl. butyricum* were detected in 4 products corresponding to the label claim. Whereas some non-toxigenic strains of *Cl. butyricum* are currently used as probiotics in Asia, other strains have been reported to be pathogenic (Cassir et al., 2016). However, the specific strain of this species was not present on labels of 4 products tested.

The main reasons for mislabeling of probiotic products at species level were possibly the use of unreliable methods for identification and selection of bacterial species. The genus *Bacillus*, *Lactobacillus* and *Enterococcus* were in diverse groups including many species with a large variety of phenotypic, biochemical and physiological properties. Many manufacturers seem to use only conventional biochemical and physiological tests to identify the probiotic species. However, many species of the same genus show the similar biochemical and physiological characteristics thus misidentification is inevitable (Berthier and Ehrlich, 1999). To date, the application of phylogenetic molecular taxonomy and 16S rRNA gene sequence analysis have been developed for identification of probiotic species. This study was performed using the reliable identification methods based on 16S rRNA gene sequence analysis to test the accuracy of species mentioned on label.

2. Contamination of *Salmonella* and *E. coli*

None of the probiotic products in this study contaminated with neither *Salmonella* nor *E. coli*. In general, the likelihood of *Salmonella* and *E. coli* contamination in probiotic products are very low due to unsuitable conditions for growth. However, if the contamination of pathogens occurs, it indicates that a failure occurs during production process. This highlights that the manufacturing process needs to be carefully controlled. *Salmonella* and *E. coli* are foodborne pathogens and resistant to a wide range of antibiotics. Importantly, they can carry and transfer AMR determinants (Sinwat et al., 2016; Trongjit et al., 2016). Therefore, they can be the main source of AMR genes that are potentially transferred to probiotic bacteria and other pathogenic bacteria.

3. Phenotypic characterization of AMR in probiotic bacteria

The *Lactobacillus* and *Bacillus* isolates showed resistance to broad range of antibiotics as previously observed (Klare et al., 2007; Wannaprasat et al., 2009).

The different *Bacillus* species showed different resistance patterns with resistance commonly seen to chloramphenicol (19%), followed clindamycin (16%), erythromycin (9%), trimethoprim (9%), sulfamethoxazole (9%), tetracycline (3%) and ampicillin (2%). The *B. licheniformis* strains were mainly resistant to high levels of erythromycin, clindamycin and chloramphenicol compared to other antibiotics. High resistance to chloramphenicol and clindamycin was observed in all *B. licheniformis* strains in this study. Generally, high chloramphenicol and clindamycin MIC values were obtained for the *B. licheniformis* strains. This could be due to intrinsic resistance characteristics of this species since the uniform distributions of the MIC values were observed. This finding was similar to chloramphenicol and clindamycin resistance profiles among *B. licheniformis* strains from different geographical areas in previous studies (Adimpong et al., 2012; Jeong et al., 2017). In contrast, *B. subtilis* and members of *B. subtilis* cluster were fully sensitive to all antimicrobials tested. The antimicrobial

susceptibility results of *B. subtilis* strains in this present study was consistent with previous studies in the US reporting that *B. subtilis* MB40 used in food were susceptible to most antimicrobials tested (Spears et al., 2021).

Although the number of *Lactobacillus* isolates examined in this study was limited (n=6), the antimicrobial resistance among these isolates appeared to vary among species. Apart from *L. rhamnosus*, that was susceptible to all antimicrobials (except vancomycin), all other *Lactobacillus* isolates were resistant to at least 3 antimicrobial classes. It was observed in this study observed all *Lactobacillus* isolates were susceptible to gentamicin, erythromycin, clindamycin, rifampicin, and meropenem. Only one in 6 *Lactobacillus* was resistant to chloramphenicol. It is in agreement with a previous study, where 33 *Lactobacillus* strains isolated from dairy products were sensitive to gentamicin, erythromycin and clindamycin (Guo et al., 2017). Another study also described probiotic *Lactobacillus* strains that were sensitive to rifampicin and chloramphenicol (Zhou et al., 2005)

Conversely, high resistance to ampicillin, aminoglycosides (streptomycin and kanamycin), tetracycline, trimethoprim, ciprofloxacin and vancomycin was observed in all *Lactobacillus* strains in the present study. Most *Lactobacillus* isolates were low-level resistant to ampicillin with MICs ranging from 2 to 8 µg/ml. This is in line with the results in a previous study reporting that MICs of *Lactobacillus* strains were equal or close to MIC breakpoints (Hummel et al., 2007b). However, the mechanisms of resistance to ampicillin for *Lactobacillus* still remained largely unclear. All *Lactobacillus* strains in this study showed higher resistance to kanamycin and streptomycin than gentamicin. The high MIC values were observed for streptomycin (16 to 128 µg/ml) and kanamycin (32 to 128 µg/ml), but rather low MICs for gentamicin (2 to 8 µg/ml). It has been reported that *Lactobacillus* is intrinsically resistant to aminoglycosides (i.e. kanamycin and streptomycin) due to lack of cytochrome-mediated electron transport (Guo et al., 2017). Conversely, susceptibility to gentamicin is associated to its ability to cross the cell membrane better than other

aminoglycosides (Elkins and Mullis, 2004). For trimethoprim, the MIC values of *Lactobacillus* ranged from 16 to >512 µg/ml. In another study, *Lactobacillus* has a wide MIC range between 0.125 and 64 µg/ml (Guo et al., 2017). All *Lactobacillus* isolates were resistant to vancomycin with high MIC values of >256 µg/ml. This may not be surprising since several *Lactobacillus* species are intrinsically resistant to vancomycin due to the presence of D-Alanine-D-Lactate was rather than the D-Ala-D-Ala dipeptide in their peptidoglycan, which prevents vancomycin binding (Gueimonde et al., 2013).

4. Genotypic characteristics of AMR in probiotic product

Evaluation of safety of bacterial strains intended for use in food or feed is of particular concern. According to EFSA, AMR determinants and their potential mobility are one of the most selection criteria for safety assessment of a candidate microorganism prior to approval for QPS status.

Broad-spectrum β-lactamase genes, $bla_{\text{OXA-1-like}}$ and bla_{SHV}, were found in two probiotic products. Those genes were commonly found among Enterobacteriaceae isolated from food-producing animals and humans. Broad-spectrum β-lactamase genes are usually located on mobile genetic elements including plasmids, transposons and integrons (Smet et al., 2010). The presence of β-lactamase genes in probiotic strains remains obscure. A previous study demonstrated that bla_{OXA} and bla_{SHV} were less frequent among *Lactobacillus* strains (Anisimova and Yarullina, 2019).

Plasmid-mediated quinolone resistance (PMQR) genes including *oqxAB, qnrB*, *qnrS*, *qnrD* and *aac(6')-Ib-cr* were frequently detected among probiotic products in this study. The emergency of PMQR has indicated that quinolone resistance can be acquired through horizontal gene transfer (Strahilevitz et al., 2009). Three quinolone resistance mechanisms have been described such as (1) *qnr* genes encoding proteins to protect DNA gyrase and topoisomerase IV from quinolone inhibition, (2) *aac(6')-Ib-cr* gene encoding AAC(6')-Ib-cr that is able to acetylate quinolones including

norfloxacin and ciprofloxacin, and (3) *qepA* and *oqxAB* encoding efflux pump that can extrude fluroquinolones from the bacterial cell (Strahilevitz et al., 2009). Until now, numerous studies have indicated that the dissemination of PMQR genes among clinically Enterobacteriaceae isolates (Robicsek et al., 2006; Kim et al., 2009). Nevertheless, PMQR have not been detected in Gram-positive bacteria including probiotic strains.

Aminoglycosides play an important role in treatment of serious infections in humans and have broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Therefore, its use in animal husbandry has been strictly regulated in Europe and USA to avoid resistantace development in microbiota. However, various aminoglycoside resistance genes encoding resistance to streptomycin, gentamicin and kanamycin were present in probiotic products. High frequency of streptomycin resistance mediated by *aadA2*, *aadA1* and *strA-strB* was previously detected in *P. aeruginosa* clinical isolates from non-cystic fibrosis patients in Thailand (Poonsuk et al., 2013). In Gram-positive bacteria, it was reported that aminoglycoside-resistant LAB (*Enterococcus* and *Lactobacillus*) and their horizontal transfer were observed (Jaimee and Halami, 2016). *Enterococcus* was commonly positive *aadE* and *aac(6')-aph(2'')* that confer high level streptomycin resistance (HLSR) and high-level gentamicin resistance (HLGR), respectively (Thu et al., 2019). The presence of *aac(6')-aph(2'')*, *aph(3')-III*, *aadA*, and *aadE* was reported in *Lactobacillus* spp. (Jaimee and Halami, 2016). The *ant(4')-Ia* and aph(3')-IIIa gene found among clinically methicillin-resistant *Staphylococcus aureus* (MRSA) are of great concern (Khosravi et al., 2017).

Based on the comparison of MICs among transconjugants, recipients and donors, the streptomycin MIC of transconjugants was increased to 512 µg/ml. This could imply that *Lactobacillus* strains could transfer streptomycin resistance determinants to the recipients. The target genes encoding streptomycin including *aadA1, aadA2, strA-strB,* and *aadE* were not detected by PCR despite the MIC values

of streptomycin for transconjugants was high. It is possible that they may carry streptomycin resistance encoding genes that were not examined in this study.

Tetracycline resistance is also widespread among Gram-positive and Gram-negative bacteria. Different tetracycline resistance genes, including *tetA, tetB* and *tetL* encoding for efflux pumps and *tetM* encoding for ribosomal protection proteins, were detected in probiotic products. The *tet* genes are widely distributed in *Enterococcus* and *Lactobacillus* (Gueimonde et al., 2013). The *tetA* and *tetB* genes were commonly detected in *E. coli* isolated from humans, animals, foods of animal origin and the environment (Olowe et al., 2013; Jamali et al., 2018). The *tetM* gene is widely distributed among Gram-positive bacteria, but it has rarely been reported in Gram-negative bacteria. Studies on *L. salivarius* have shown that *tetM* and *tetL* commonly located on plasmid and linked with determinants for resistance to erythromycin (*ermB*). These genes could be transferred from *L. salivarius* to pathogenic strains under in *vivo*, *in vitro* and during food fermentation (Thumu and Halami, 2019).

Although the use of chloramphenicol is prohibited in food-producing animals, chloramphenicol resistance genes, *catA* and *cmlA,* were still detected in 5 probiotic products. The *catA* and *cmlA* genes encode chloramphenicol acetyltransferases and specific exporters, respectively. The *cmlA* gene was located on transferable plasmids and confer multi-drug resistance in *Salmonella* (Chuanchuen et al., 2008b). The *cat* genes have been identified in several *Lactobacillus* species including *L. acidophilus*, *L. delbrueckii*, and *L. johnsonii* (Gueimonde et al., 2013).

The presence of *mef*(A) gene coding for macrolide efflux pumps, was detected in 4 products. This gene was found to be widespread in *Streptococcus* spp. including *S. suis* and *S. pneumonia* (Chen et al., 2013). The *mef(A)* gene was found less frequently in lactobacilli (Cauwerts et al., 2006).

Trimethoprim is most commonly used in combination with sulfamethoxazole for treatment of urinary tract infections in humans. The *dfrA12* and *dfrA14* gene cassette array conferring resistance to trimethoprim and *sul1* encoding resistance to

sulfamethoxazole were detected in probiotic products in this study. The *sul1* gene was mainly associated with class 1 integrons that contributes to MDR phenotype in Gram-negative bacteria (Chuanchuen et al., 2007). The integrons consist of 2 conversed segments 5' CS and 3'CS, separated by a variable region that comprises none or at least one gene cassette. The 5′-CS includes an integrase genes (*intI1)*, a integration site (*att1*) and a promoter (P_{ant}). The 3′-CS region contains several open reading frames (ORFs) of unknow function, *qacEΔ1* conferring resistance to quaternary ammonium compounds, *sul1* conferring resistance to sulfonamides. Most of *dfr* genes have been found to locate on genes cassettes within class 1 integrons that is a potential source of horizontal spread of *dfr* among bacteria (Yu et al., 2004).

Vancomycin resistance gene, *vanC*, was detected in only one product in the present study. Vancomycin resistant enterococci (VRE) are emerging as a global threat to public health. There are 5 recognized genes *vanA, vanB, vanC*, *vanD* and *vanE* contributing to vancomycin resistance in enterococci. Among those genes, *vanA* confers inducible, high-level resistance to vancomycin and is transferable. Conversely, *vanC* is not transferable and demonstrates intrinsic, low-level resistance to vancomycin.

Six probiotic products (P3, P4, P5, P12, P13 and P43) carrying AMR genes in this study were imported products. It indicates that there is a circulation of AMR genes around the world. This is one of great concerns. In general, almost genes detected in this study encoded resistance to clinically important antimicrobials and are widespread among Gram-positive and Gram-negative bacteria. The origins of these genes could be from probiotic bacteria formulated in the products or contaminated into products during manufacturing. Microorganisms used to produce probiotics can be originated from many sources including common members of human or animal guts, soils, and food, so there is high possibility to uptake AMR genes from environment and transfer these genes to other bacteria. Therefore, the presence of AMR determinants in probiotic bacteria must be systematically screened before formulation. Previous

studies were conducted to test the presence of AMR genes in each probiotic strain isolated from probiotic products. From our knowledge, this is the first report for screening AMR genes in probiotic products using DNA templates extracted from whole product, instead of using DNA templates extracted from individual bacterial isolates as previous studies. Together with the results from correlations between AMR genotypes and AMR phenotypes, most of AMR phenotypes of bacterial were not correlated with AMR genes detected in probiotic products. This may imply that the AMR genes may not be originated from bacterial species used to produce those products. These genes may be derived from other sources including bacterial strains contaminated probiotic products that were not detected in this study. Therefore, screening AMR genes in probiotic products before launching to the market can provide an overview of sources of AMR genes to have a timely intervention.

CHAPTER VI CONCLUSION AND SUGGESTIONS

Forty-five probiotic products for food animals were examined the number and species probiotic bacteria including *Bacillus, Lactobacillus, Enterococcus* and *Clostridium*. Contamination of *E. coli* and *Salmonella* in probiotic products was also investigated. Moreover, the AMR characteristics including AMR phenotypes and AMR genotypes as well as transferability of AMR determinants were carried out. From the findings of this study, probiotic products used for food animals were incorrectly mislabeled in either number or species or both. In addition, misidentification at species level was the most common. All products only labelled bacteria at species level not strain level. The different phenotypic characteristics were found among different bacterial species and genes encoding resistance to clinically-important antimicrobial classes were detected in probiotic products. Therefore, the safety of microorganisms used in the formulation of probiotic products should be assessed with following criteria:

i. The microorganisms should be identified to strain level.
ii. The particular strain of microorganisms should not have been associated with any infection in humans or animals.
iii. The microorganisms should not harbor transferable AMR genes.
iv. The microorganisms should be nontoxic and nonpathogenic strains.

The results from this finding can be used to support the improvement of regulation of probiotic products by the relevant authority. The manufacturers and producers should have developed policies to control quality of their probiotic products. The farmers and other food-animal producers should wisely choose the probiotic products that are approved to be sold on the market by relevant authority. The data obtained can be also used as part of risk assessment of AMR in probiotic products.

Further studies are also suggested as follows:

1. Number of viable cells in probiotics can be assessed at the time of purchase and after 3 months, 6 months, and 1 year to evaluate the product stability.
2. Strain specific DNA fingerprints by randomly amplified polymorphic DNA (RAPD) should be carried out to discriminate the probiotic strains.
3. Characterization of genotypes corresponding to phenotypes in each isolate should be performed to find the association between AMR phenotypes and genotypes.
4. The transferability of AMR genes from probiotic bacteria to other pathogens such as *Salmonella*, *E. faecalis*, e.g., can be performed *in vitro* and *in vivo*.
5. Presence of mobile genetic elements including plasmids, transposons and integrons should be investigated in bacterial isolates for deep understanding of gene transfer mechanisms.
6. Whole-genome sequencing (WGS) on the probiotic isolates resistant to antimicrobials should be performed to understand the nature of their resistance and facilitate determination of their suitability for using in probiotic products.

REFERENCES

REFERENCES

Adams MR and Marteau P 1995. On the safety of lactic acid bacteria from food. Int J Food Microbiol. 27(2-3): 263-264.

Adimpong DB, Sørensen KI, Thorsen L, Stuer-Lauridsen B, Abdelgadir WS, Nielsen DS, Derkx PM and Jespersen L 2012. Antimicrobial susceptibility of *Bacillus* strains isolated from primary starters for African traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. Appl Environ Microbiol. 78(22): 7903-7914.

Aminov RI, Garrigues-Jeanjean N and Mackie RI 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol. 67(1): 22-32.

Angot P, Vergnaud M, Auzou M, Leclercq R and Observatoire de Normandie du P 2000. Macrolide resistance phenotypes and genotypes in French clinical isolates of *Streptococcus pneumoniae*. Eur J Clin Microbiol Infect Dis. 19(10): 755-758.

Anisimova EA and Yarullina DR 2019. Antibiotic resistance of *Lactobacillus* strains. Curr Microbiol. 76(12): 1407-1416.

Bai SP, Wu AM, Ding XM, Lei Y, Bai J, Zhang KY and Chio JS 2013. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult Sci. 92(3): 663-670.

BAM 2017. Bacteriological analytical manual (BAM). Chapter 4: Enumeration of *Escherichia coli* and the coliform bacteria. [Online]. Available: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-4-enumeration-escherichia-coli-and-coliform-bacteria.

Barbosa TM, Serra CR, La Ragione RM, Woodward MJ and Henriques AO 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Appl Environ Microbiol. 71(2): 968-978.

Batchelor M, Hopkins K, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH and Liebana E 2005. *bla*CTX-M genes in clinical *Salmonella* isolates recovered from

humans in England and Wales from 1992 to 2003. Antimicrob Agents Chemother. 49(4): 1319-1322.

Bernardeau M, Guguen M and Vernoux JP 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol Rev. 30(4): 487-513.

Berthier F and Ehrlich SD 1999. Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. Int J Syst Evol. 49(3): 997-1007.

Brashears MM, Amezquita A and Jaroni D 2005. Lactic acid bacteria and their uses in animal feeding to improve food safety. Adv Food Nutr Res. 50: 1-31.

Call DR, Bakko MK, Krug MJ and Roberts MC 2003. Identifying antimicrobial resistance genes with DNA microarrays. Antimicrob Agents Chemother. 47(10): 3290-3295.

Cassir N, Benamar S and La Scola B 2016. *Clostridium butyricum*: from beneficial to a new emerging pathogen. Clin Microbiol Infect. 22(1): 37-45.

Cauwerts K, Pasmans F, Devriese LA, Martel A, Haesebrouck F and Decostere A 2006. Cloacal *Lactobacillus* isolates from broilers show high prevalence of resistance towards macrolide and lincosamide antibiotics. Avian Pathol. 35(2): 160-164.

Cavaco LM, Hasman H, Xia S and Aarestrup FM 2009. *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and *Bovismorbificans* strains of human origin. Antimicrob Agent Chemother. 53(2): 603-608.

Chandler JR, Hirt H and Dunny GM 2005. A paracrine peptide sex pheromone also acts as an autocrine signal to induce plasmid transfer and virulence factor expression in vivo. Proc Natl Acad Sci U S A. 102(43): 15617-15622.

Chen L, Song Y, Wei Z, He H, Zhang A and Jin M 2013. Antimicrobial susceptibility, tetracycline and erythromycin resistance genes, and multilocus sequence typing of *Streptococcus suis* isolates from diseased pigs in China. J Vet Med Sci. 75(5): 583-587.

Chuanchuen R, Khemtong S and Padungtod P 2007. Occurrence of *qacE/qacEDelta1* genes and their correlation with class 1 integrons in *Salmonella enterica*

isolates from poultry and swine. Southeast Asian J Trop Med Public Health. 38(5): 855-862.

Chuanchuen R, Koowatananukul C and Khemtong S 2008a. Characterization of class 1 integrons with unusual 3' conserved region from *Salmonella enterica* isolates. Southeast Asian J Trop Med Public Health. 39(3): 419-424.

Chuanchuen R and Padungtod P 2009. Antimicrobial resistance genes in *Salmonella enterica* isolates from poultry and swine in Thailand. J Vet Med Sci. 71(10): 1349-1355.

Chuanchuen R, Pathanasophon P, Khemtong S, Wannaprasat W and Padungtod P 2008b. Susceptibilities to antimicrobials and disinfectants in *Salmonella* isolates obtained from poultry and swine in Thailand. J Vet Med Sci. 70(6): 595-601.

Chung WO, Werckenthin C, Schwarz S and Roberts MC 1999. Host range of the *ermF* rRNA methylase gene in bacteria of human and animal origin. J Antimicrob Chemother. 43(1): 5-14.

CLSI 2015. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacterial. Approval standard – Third edition M45.

CLSI 2019. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. Approval Edition - Fifth Edition VET01.

CLSI 2020. Performance Standards for Antimicrobial Susceptibility Testing. Approval standard – 30th edition M100.

Coburn PS and Gilmore MS 2003. The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. Cell Microbiol. 5(10): 661-669.

Cocconcelli PS, Cattivelli D and Gazzola S 2003. Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. Int J Food Microbiol. 88(2-3): 315-323.

Coeuret V, Gueguen M and Vernoux JP 2004. Numbers and strains of lactobacilli in some probiotic products. Int J Food Microbiol. 97(2): 147-156.

Collado MC, Grze**Ś**kowiak Ł and Salminen S 2007. Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa. Curr Microbiol. 55(3): 260-265.

Collado MC and Sanz Y 2006. Method for direct selection of potentially probiotic *Bifidobacterium* strains from human feces based on their acid-adaptation ability. J Microbiol Methods. 66(3): 560-563.

Collins JK, Thornton G and Sullivan GO 1998. Selection of probiotic strains for human applications. Int Dairy J. 8(5): 487-490.

Cotter PD, Ross RP and Hill C 2013. Bacteriocins - a viable alternative to antibiotics? Nat Rev Microbiol. 11(2): 95-105.

Cutting SM 2011. *Bacillus* probiotics. Food Microbiol. 28(2): 214-220.

Dallenne C, Da Costa A, Decre D, Favier C and Arlet G 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. J Antimicrob Chemother. 65(3): 490-495.

Devirgiliis C, Coppola D, Barile S, Colonna B and Perozzi G 2009. Characterization of the Tn916 conjugative transposon in a food-borne strain of *Lactobacillus paracasei*. Appl Environ Microbiol. 75(12): 3866-3871.

Dhalluin A, Lemée L, Pestel-Caron M, Mory F, Leluan G, Lemeland J-F and Pons J-L 2003. Genotypic differentiation of twelve *Clostridium* species by polymorphism analysis of the triosephosphate isomerase (tpi) gene. Syst Appl Microbiol. 26(1): 90-96.

Domig KJ, Mayer HK and Kneifel W 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp.: 2. Pheno- and genotypic criteria. Int J Food Microbiol. 88(2): 165-188.

Donev T 2002. Influence of the freezing rate on the survival of strains *Saccharomyces cerevisiae* after cryogenic preservation. J Cult Collect. 3. 78-83.

Doron S and Snydman DR 2015. Risk and safety of probiotics. Clin Infect Dis. 60 (Suppl 2): S129-134.

Dowarah R, Verma AK and Agarwal N 2017. The use of *Lactobacillus* as an alternative of antibiotic growth promoters in pigs: A review. Anim Nutr. 3(1): 1-6.

Dubernet S, Desmasures N and Guéguen M 2002. A PCR-based method for identification of lactobacilli at the genus level. FEMS Microbiol Lett. 214(2): 271-275.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F and Collins JK 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr. 73(2 Suppl): 386s-392s.

Dutka-Malen S, Evers S and Courvalin P 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 33(1): 24-27.

EFSA 2007. European Food Safety Authority. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA-opinion of the scientific committee. The EFSA Journal. 5(12): 587.

EFSA 2011. Scientific Opinion on Miya-Gold® (*Clostridium butyricum*) as a feed additive for weaned piglets, minor weaned porcine species and minor avian species. EFSA Journal. 9(1): 1951.

EFSA 2012. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (FEEDAP). EFSA Journal. 10(6): 2740.

EFSA 2020. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 12: suitability of taxonomic units notified to EFSA until March 2020. EFSA Journal. 18(7): 6174.

Elkins CA and Mullis LB 2004. Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. Appl Environ Microbiol. 70(12): 7200-7209.

Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C and Panwar H 2017. *Bacillus* As Potential Probiotics: Status, Concerns, and Future Perspectives. Front Microbiol. 8(1490).

EUCAST 2020. European Committee on Antimicrobial Susceptibility Testing. [Online]. Available: https://www.eucast.org/.

Facklam RR, Carvalho MdGS and Teixeira LM 2002. History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. In: The Enterococci. 1-54.

FAO 2016a. Drivers, dynamics and epidemiology of anitimicrobial resistance in animal production. [Online]. Available: http://www.fao.org/3/a-i6209e.pdf.

FAO 2016b. Probiotics in animal nutrition - Production, impact and regulation. In: FAO animal production and health paper No. 179. Food and Agriculture Organization of the United Nations. [Online]. Available: http://www.fao.org/3/a-i5933e.pdf. .

FAO 2018. Animal production. Available at: http://www.fao.org/animal-production/en/

FAO/WHO 2002. Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. [Online]. Available: https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Fayol-Messaoudi D, Berger CN, Coconnier-Polter MH, Liévin-Le Moal V and Servin AL 2005. pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic Lactobacilli against *Salmonella enterica* Serovar Typhimurium. Appl Environ Microbiol. 71(10): 6008-6013.

FDA 2018. Microorganisms & microbial-derived ingredients used in food (Partial list). Available at: https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list.

FDA 2019. Generally Recognized as Safe (GRAS). Available at: https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras.

Ferreira F, Arcos A, Sampaio R, Rodrigues I and Tadei W 2015. Effect of *Bacillus sphaericus* Neide on *Anopheles* (Diptera: Culicidae) and associated insect fauna in fish ponds in the Amazon. Rev Bras Entomol. 59. 234-239.

Fioramonti J, Theodorou V and Bueno L 2003. Probiotics: what are they? What are their effects on gut physiology? Best Pract Res Clin Gastroenterol. 17(5): 711-724.

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

Fuller R 1992. History and development of probiotics. In: Probiotics: The scientific basis. Roy Fuller (ed). Dordrecht: Springer Netherlands. 1-8.

García-Lafuente A, Antolín M, Guarner F, Crespo E and Malagelada JR 2001. Modulation of colonic barrier function by the composition of the commensal flora in the rat. Gut. 48(4): 503-507.

Gevers D, Danielsen M, Huys G and Swings J 2003. Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl Environ Microbiol. 69(2): 1270-1275.

Gfeller KY, Roth M, Meile L and Teuber M 2003. Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. Plasmid. 50(3): 190-201.

Giraffa G 1999. *Enterococcus*. In: Encyclopedia of Food Microbiology. Richard K. Robinson (ed). Oxford: Elsevier. 617-624.

Grape M, Motakefi A, Pavuluri S and Kahlmeter G 2007. Standard and real-time multiplex PCR methods for detection of trimethoprim resistance *dfr* genes in large collections of bacteria. Clin Microbiol Infect. 13(11): 1112-1118.

Gryczan T, Israeli-Reches M, Del Bue M and Dubnau D 1984. DNA sequence and regulation of *ermD*, a macrolide-lincosamide-streptogramin B resistance element from *Bacillus licheniformis*. Mol Gen Genet. 194(3): 349-356.

Gueimonde M, Sánchez B, G de Los Reyes-Gavilán C and Margolles A 2013. Antibiotic resistance in probiotic bacteria. Front Microbiol. 4: 202.

Guo H, Pan L, Li L, Lu J, Kwok L, Menghe B, Zhang H and Zhang W 2017. Characterization of antibiotic resistance genes from *Lactobacillus* isolated from traditional dairy products. J Food Sci. 82(3): 724-730.

Hanchi H, Mottawea W, Sebei K and Hammami R 2018. The genus *Enterococcus*: between probiotic potential and safety concerns-an update. Front Microbiol. 9: 1791-1791.

Hasman H, Mevius D, Veldman K, Olesen I and Aarestrup FM 2005. β-Lactamases among extended-spectrum beta-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. J Antimicrob Chemother. 56(1): 115-121.

Hassan M, Kjos M, Nes IF, Diep DB and Lotfipour F 2012. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol. 113(4): 723-736.

Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD and de Vos WM 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol. 68(1): 114-123.

Hoa NT, Baccigalupi L, Huxham A, Smertenko A, Van PH, Ammendola S, Ricca E and Cutting AS 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl Environ Microbiol. 66(12): 5241-5247.

Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R and Waldor MK 2001. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob Agents Chemother. 45(11): 2991-3000.

Hong HA, Duc le H and Cutting SM 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev. 29(4): 813-835.

Hossain MI, Sadekuzzaman M and Ha SD 2017. Probiotics as potential alternative biocontrol agents in the agriculture and food industries: A review. Food Res Int. 100(Pt 1): 63-73.

Hudault S, Liévin V, Bernet-Camard MF and Servin AL 1997. Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium C5* infection. Appl Environ Microbiol. 63(2): 513-518.

Hummel A, Holzapfel WH and Franz CM 2007a. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Syst Appl Microbiol. 30(1): 1-7.

Hummel AS, Hertel C, Holzapfel WH and Franz CM 2007b. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl Environ Microbiol. 73(3): 730-739.

Huys G, D'Haene K and Swings J 2006. Genetic basis of tetracycline and minocycline resistance in potentially probiotic *Lactobacillus plantarum* strain CCUG 43738. Antimicrob Agents Chemother. 50(4): 1550-1551.

Imperial ICVJ and Ibana JA 2016. Addressing the antibiotic resistance problem with probiotics: reducing the risk of its double-edged sword effect. Front Microbiol. 7: 1983.

ISO 1998. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of mesophilic lactic acid bacteria - Colony-count technique at 30°C. Reference number: ISO 15214:1998.

ISO 2002. Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp. Reference number: ISO 6579:2002(en).

ISO 2004. Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive *Bacillus cereus* — Colony-count technique at 30°C. Reference number: ISO 7932:2004.

ISO 2017. Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions. Reference number: ISO 6887-1:2017(en).

Jackson CR, Fedorka-Cray PJ and Barrett JB 2004. Use of a genus- and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol. 42(8): 3558-3565.

Jaimee G and Halami PM 2016. Emerging resistance to aminoglycosides in lactic acid bacteria of food origin—an impending menace. Appl Microbiol Biotechnol. 100(3): 1137-1151.

Jakobsen L, Sandvang D, Hansen L, Skjøt-Rasmussen L, Westh H, Jørgensen C, Hansen D, Pedersen B, Monnet D, Frimodt-Møller N, Sørensen S and Hammerum A 2008. Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the waste water environment. Environ Int. 34: 108-115.

Jamali H, Krylova K and Aïder M 2018. Identification and frequency of the associated genes with virulence and antibiotic resistance of *Escherichia coli* isolated from cow's milk presenting mastitis pathology. Anim Sci J. 89(12): 1701-1706.

Jeong D-W, Jeong M and Lee J-H 2017. Antibiotic susceptibilities and characteristics of *Bacillus licheniformis* isolates from traditional Korean fermented soybean foods. LWT. 75: 565-568.

Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA and Sherman PM 2007. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. Cell Microbiol. 9(2): 356-367.

Joosten H, Bidlas E and Garofalo N 2006. *Salmonella* detection in probiotic products. Int J Food Microbiol. 110(1): 104-107.

Kaase M, Lenga S, Friedrich S, Szabados F, Sakinc T, Kleine B and Gatermann SG 2008. Comparison of phenotypic methods for penicillinase detection in *Staphylococcus aureus*. Clin Microbiol Infect. 14(6): 614-616.

Ke D, Picard FJ, Martineau F, Ménard C, Roy PH, Ouellette M and Bergeron MG 1999. Development of a PCR assay for rapid detection of enterococci. J Clin Microbiol. 37(11): 3497-3503.

Khemtong S and Chuanchuen R 2008. Class 1 integrons and *Salmonella* genomic island 1 among *Salmonella enterica* isolated from poultry and swine. Microb Drug Resist. 14(1): 65-70.

Khosravi AD, Jenabi A and Montazeri EA 2017. Distribution of genes encoding resistance to aminoglycoside modifying enzymes in methicillin-resistant *Staphylococus aureus* (MRSA) strains. Kaohsiung J Med Sci. 33(12): 587-593.

Kikuchi E, Miyamoto Y, Narushima S and Itoh K 2002. Design of species-specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. Microbiol Immunol. 46(5): 353-358.

Kim HB, Wang M, Park CH, Kim EC, Jacoby GA and Hooper DC 2009. *oqxAB* encoding a multidrug efflux pump in human clinical isolates of Enterobacteriaceae. Antimicrob Agents Chemother. 53(8): 3582-3584.

Klare I, Konstabel C, Müller-Bertling S, Reissbrodt R, Huys G, Vancanneyt M, Swings J, Goossens H and Witte W 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of Lactobacilli, Pediococci, Lactococci, and Bifidobacteria. Appl Environ Microbiol. 71(12): 8982-8986.

Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Muller-Bertling S, Witte W and Goossens H 2007. Antimicrobial susceptibilities of *Lactobacillus, Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. J Antimicrob Chemother. 59(5): 900-912.

Klein G, Hallmann C, Casas IA, Abad J, Louwers J and Reuter G 2000. Exclusion of *vanA*, *vanB* and *vanC* type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. J Appl Microbiol. 89(5): 815-824.

Kwon HS, Yang EH, Yeon SW, Kang BH and Kim TY 2004. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol Lett. 239(2): 267-275.

Lekagul A, Chanvatik S, Sermsinsiri V, Sivilaikul S, Patcharanarumol W, Yeung S and Tangcharoensathien V 2018. Antibiotic distribution channels in Thailand: Results of key-informant interviews, reviews of drug regulations and database searches. Bull World Health Organ. 96: 101-109.

Lévesque C, Piché L, Larose C and Roy PH 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother. 39(1): 185-191.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B and Mills D 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A. 103(42): 15611-15616.

Makut M, Ogbonna A and Dalami H 2014. An assessment of the bacteriological quality of different brands of yoghurt sold in Keffi, Nasarawa State, Nigeria. J Nat Sci Res. 4: 19-22.

Marteau P 2001. Safety aspects of probiotic products. Food Nutr Res. 45.

Melville CM, Scott KP, Mercer DK and Flint HJ 2001. Novel tetracycline resistance gene, *tet(32)*, in the *Clostridium*-related human colonic anaerobe K10 and its transmission in vitro to the rumen anaerobe *Butyrivibrio fibrisolvens*. Antimicrob Agents Chemother. 45(11): 3246-3249.

Mingmongkolchai S and Panbangred W 2018. *Bacillus* probiotics: an alternative to antibiotics for livestock production. J Appl Microbiol. 124(6): 1334-1346.

Miranda A, Ávila B, Díaz P, Rivas L, Bravo K, Astudillo J, Bueno C, Ulloa MT, Hermosilla G, Del Canto F, Salazar JC and Toro CS 2016. Emergence of plasmid-borne *dfrA14* trimethoprim resistance gene in *Shigella sonnei*. Front Cell Infect Microbiol. 6: 77-77.

Mišić M, Čukić J, Vidanović D, Šekler M, Matić S, Vukašinović M and Baskić D 2017. Prevalence of genotypes that determine resistance of staphylococci to macrolides and lincosamides in Serbia. Front Public Health. 5: 200.

Monod M, Denoya C and Dubnau D 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. J Bacteriol. 167(1): 138-147.

Morgan CA, Herman N, White PA and Vesey G 2006. Preservation of micro-organisms by drying; A review. J Micro Methods. 66(2): 183-193.

Morrison D, Woodford N and Cookson B 1997. Enterococci as emerging pathogens of humans. Soc Appl Bacteriol Symp Ser. 26: 89s-99s.

Muzaheed, Doi Y, Adams-Haduch JM, Endimiani A, Sidjabat HE, Gaddad SM and Paterson DL 2008. High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* among inpatients and outpatients with urinary tract infection in Southern India. J Antimicrob Chemother. 61(6): 1393-1394.

Nakagawa S, Taneike I, Mimura D, Iwakura N, Nakayama T, Emura T, Kitatsuji M, Fujimoto A and Yamamoto T 2005. Gene sequences and specific detection for Panton-Valentine leukocidin. Biochem Biophys Res Commun. 328(4): 995-1002.

Nakagawa T, Shimada M, Mukai H, Asada K, Kato I, Fujino K and Sato T 1994. Detection of alcohol-tolerant hiochi bacteria by PCR. Appl Environ Microbiol. 60(2): 637-640.

Nawaz M, Wang J, Zhou A, Ma C, Wu X, Moore JE, Millar BC and Xu J 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol. 62(3): 1081-1089.

Ng LK, Martin I, Alfa M and Mulvey M 2001. Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes. 15(4): 209-215.

Nicholson WL, Munakata N, Horneck G, Melosh HJ and Setlow P 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev. 64(3): 548-572.

Olowe OA, Idris OJ and Taiwo SS 2013. Prevalence of tet genes mediating tetracycline resistance in *Escherichia coli* clinical isolates in Osun State, Nigeria. Eur J Microbiol Immunol. 3(2): 135-140.

Papatsiros V, Katsoulos P, Koutoulis K, Karatzia M, Dedousi A and Christodoulopoulos G 2013. Alternatives to antibiotics for farm animals. CAB Rev. 8.

Park CH, Robicsek A, Jacoby GA, Sahm D and Hooper DC 2006. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob Agents Chemother. 50(11): 3953-3955.

Patterson AJ, Rincon MT, Flint HJ and Scott KP 2007. Mosaic tetracycline resistance genes are widespread in human and animal fecal samples. Antimicrob Agents Chemother. 51(3): 1115-1118.

Phelan RW, Clarke C, Morrissey JP, Dobson AD, O'Gara F and Barbosa TM 2011. Tetracycline resistance-encoding plasmid from *Bacillus* sp. strain #24, isolated from the marine sponge Haliclona simulans. Appl Environ Microbiol. 77(1): 327-329.

Phuc Nguyen MC, Woerther P-L, Bouvet M, Andremont A, Leclercq R and Canu A 2009. *Escherichia coli* as reservoir for macrolide resistance genes. Emerg Infect Dis. 15(10): 1648-1650.

Poirel L, Walsh TR, Cuvillier V and Nordmann P 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis. 70(1): 119-123.

Poonsuk K, Tribuddharat C and Chuanchuen R 2013. Aminoglycoside resistance mechanisms in *Pseudomonas aeruginosa* isolates from non-cystic fibrosis patients in Thailand. Can J Microbiol. 59(1): 51-56.

Pot B, Felis GE, Bruyne KD, Tsakalidou E, Papadimitriou K, Leisner J and Vandamme P 2014. The genus *Lactobacillus*. In: Lactic Acid Bacteria: Biodiversity and Taxonomy. W.H. Holzapfel and B.J. Wood (eds). West Sussex: John Wiley & Sons, Ltd. 249-353.

Puri W, Maketon M and Areechon N 2005. Probiotic properties of *Bacillus pumilus*, *Bacillus sphaericus* and *Bacillus subtilis* in Black Tiger Shrimp (Penaeus monodon Fabricius) Culture. Kasetsart J Nat Sci. 39.

Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, Guerra B, Malorny B, Borowiak M, Hammerl JA, Battisti A, Franco A, Alba P, Perrin-Guyomard A, Granier SA, De Frutos Escobar C, Malhotra-Kumar S, Villa L, Carattoli A and Hendriksen RS 2018. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. Euro Surveill. 23(6).

Roberts AP, Pratten J, Wilson M and Mullany P 1999. Transfer of a conjugative transposon, Tn*5397* in a model oral biofilm. FEMS Microbiol Lett. 177(1): 63-66.

Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA and Hooper DC 2006. *qnr* prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. Antimicrob Agents Chemother. 50(8): 2872-2874.

Rojo-Bezares B, Saenz Y, Poeta P, Zarazaga M, Ruiz-Larrea F and Torres C 2006. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. Int J Food Microbiol. 111(3): 234-240.

Rossi F, Rizzotti L, Felis GE and Torriani S 2014. Horizontal gene transfer among microorganisms in food: current knowledge and future perspectives. Food Microbiol. 42: 232-243.

Sandvang D and Aarestrup FM 2000. Characterization of aminoglycoside resistance genes and class 1 integrons in porcine and bovine gentamicin-resistant *Escherichia coli*. Microb Drug Resist. 6(1): 19-27.

SCAN 2003. Opinion of the Scientific Committee on Animal Nutritrion on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance.

Schleifer KH and Kilpper-Bälz R 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Syst Evol Microbiol. 34(1): 31-34.

Schnellmann C, Gerber V, Rossano A, Jaquier V, Panchaud Y, Doherr MG, Thomann A, Straub R and Perreten V 2006. Presence of new *mecA* and *mph(C)* variants conferring antibiotic resistance in *Staphylococcus* spp. isolated from the skin of horses before and after clinic admission. J Clin Microbiol. 44(12): 4444-4454.

Shankar N, Baghdayan AS and Gilmore MS 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. Nature. 417(6890): 746-750.

Shewale RN, Sawale P, Khedkar C and Singh A 2014. Selection criteria for probiotics: A review. Int J Probiotics Prebiotics. 9: 17-22.

Singh KV, Nallapareddy SR, Nannini EC and Murray BE 2005. Fsr-independent production of protease(s) may explain the lack of attenuation of an *Enterococcus faecalis fsr* mutant versus a *gelE-sprE* mutant in induction of endocarditis. Infect Immun. 73(8): 4888-4894.

Sinwat N, Angkittitrakul S, Coulson KF, Pilapil F, Meunsene D and Chuanchuen R 2016. High prevalence and molecular characteristics of multidrug-resistant *Salmonella* in pigs, pork and humans in Thailand and Laos provinces. J Med Microbiol. 65(10): 1182-1193.

Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, Haesebrouck F and Butaye P 2010. Broad-spectrum β-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. FEMS Microbiology Reviews. 34(3): 295-316.

Spears JL, Kramer R, Nikiforov AI, Rihner MO and Lambert EA 2021. Safety assessment of *Bacillus subtilis* MB40 for use in foods and dietary supplements. Nutrients. 13(3): 733.

Strahilevitz J, Jacoby GA, Hooper DC and Robicsek A 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiolo Rev. 22(4): 664-689.

Sutcliffe J, Grebe T, Tait-Kamradt A and Wondrack L 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother. 40(11): 2562-2566.

Temmerman R, Pot B, Huys G and Swings J 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int J Food Microbiol. 81(1): 1-10.

Thu WP, Sinwat N, Bitrus AA, Angkittitrakul S, Prathan R and Chuanchuen R 2019. Prevalence, antimicrobial resistance, virulence gene, and class 1 integrons of *Enterococcus faecium* and *Enterococcus faecalis* from pigs, pork and humans in Thai-Laos border provinces. J Glob Antimicrob Resist. 18: 130-138.

Thumu SC and Halami PM 2012. Presence of erythromycin and tetracycline resistance genes in lactic acid bacteria from fermented foods of Indian origin. Antonie Van Leeuwenhoek. 102(4): 541-551.

Thumu SCR and Halami PM 2019. Conjugal transfer of *erm(B)* and multiple *tet* genes from *Lactobacillu*s spp. to bacterial pathogens in animal gut, *in vitro* and during food fermentation. Food Res Int. 116: 1066-1075.

Tiseo K, Huber L, Gilbert M, Robinson TP and Van Boeckel TP 2020. Global trends in antimicrobial use in food animals from 2017 to 2030. Antibiotics (Basel). 9(12): 918.

Toro CS, Farfán M, Contreras I, Flores O, Navarro N, Mora GC and Prado V 2005. Genetic analysis of antibiotic-resistance determinants in multidrug-resistant *Shigella* strains isolated from Chilean children. Epidemiol Infect. 133(1): 81-86.

Trongjit S, Angkittitrakul S and Chuanchuen R 2016. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces. Microbiol Immunol. 60(9): 575-585.

Vakulenko SB, Donabedian SM, Voskresenskiy AM, Zervos MJ, Lerner SA and Chow JW 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. Antimicrob Agents Chemother. 47(4): 1423-1426.

Valin H, Sands R, Van der Mensbrugghe D, Nelson G, Ahammad H, Blanc E, Bodirsky B, Fujimori S, Hasegawa T, Havlík P, Heyhoe E, Kyle P, Mason-D'Croz D, Paltsev S, Rolinski S, Tabeau A, Meijl H, von Lampe M and Willenbockel D 2014. The future of food demand: understanding differences in global economic models. Agric Econ. 45.

Wang M, Guo Q, Xu X, Wang X, Ye X, Wu S, Hooper DC and Wang M 2009. New plasmid-mediated quinolone resistance gene, *qnrC*, found in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother. 53(5): 1892-1897.

Wang X, Heazlewood SP, Krause DO and Florin TH 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J Appl Microbiol. 95(3): 508-520.

Wang X, Wang Y, Zhou Y, Li J, Yin W, Wang S, Zhang S, Shen J, Shen Z and Wang Y 2018. Emergence of a novel mobile colistin resistance gene, *mcr-8*, in NDM-producing *Klebsiella pneumoniae*. Emerg Microbes Infect 7(1): 122-122.

Wannaprasat W, Koowatananukul C, Ekkapobyotin C and Chuanchuen R 2009. Quality analysis of commercial probiotic products for food animals. Southeast Asian J Trop Med Public Health. 40(5): 1103-1112.

Weese JS 2003. Evaluation of deficiencies in labeling of commercial probiotics. Can Vet J. 44(12): 982-983.

Werner G, Willems RJ, Hildebrandt B, Klare I and Witte W 2003. Influence of transferable genetic determinants on the outcome of typing methods commonly used for *Enterococcus faecium*. J Clin Microbiol. 41(4): 1499-1506.

WHO 2018. Of all humans diseases, 60% originate in animals - "One Health" is the only way to keep antibiotics working. Available at: https://www.euro.who.int/en/health-topics/disease-prevention/food-safety/news/news/2018/11/of-all-human-diseases,-60-originate-in-animals-one-health-is-the-only-way-to-keep-antibiotics-working.

Wu XY, Walker MJ, Hornitzky M and Chin J 2006. Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. J Microbiol Methods. 64(1): 107-119.

Yamane K, Wachino J, Suzuki S and Arakawa Y 2008. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. Antimicrob Agents Chemother. 52(4): 1564-1566.

Yan JJ, Wu JJ, Ko WC, Tsai SH, Chuang CL, Wu HM, Lu YJ and Li JD 2004. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. J Antimicrob Chemother. 54(6): 1007-1012.

Ying Y, Wu F, Wu C, Jiang Y, Yin M, Zhou W, Zhu X, Cheng C, Zhu L, Li K, Lu J, Xu T and Bao Q 2019. Florfenicol resistance in Enterobacteriaceae and whole-genome sequence analysis of florfenicol-resistant *Leclercia adecarboxylata* strain R25. Int J Genomics. 2019: 1-10.

Yirga H 2015. The use of probiotics in animal nutrition. J Prob Health. 03.

Yu HS, Lee JC, Kang HY, Jeong YS, Lee EY, Choi CH, Tae SH, Lee YC, Seol SY and Cho DT 2004. Prevalence of *dfr* genes associated with integrons and dissemination of *dfrA17* among urinary isolates of *Escherichia coli* in Korea. J Antimicrob Chemother. 53(3): 445-450.

Zhou JS, Pillidge CJ, Gopal PK and Gill HS 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. Int J Food Microbiol. 98(2): 211-217.

APPENDICES

APPENDIX A

Primers used for determination of genus and species of probiotic bacteria

Primers	Sequence (5'-3')	PCR type	PCR product (bp)	References
Bacillus				
B-K1/F	TCACCAAGGCRACGATGCG	All *Bacillus*	~1,114	(Wu et al., 2006)
B-K1/R1	CGTATTCACCGCGGCATG			
Lactobacillus				
R16-1	CTTGTACACACCGCCCGTCA	Genus-specificity	Variable	(Nakagawa et al., 1994)
LbLMA1-rev	CTCAAAACTAAACAAAGTTTC	Genus-specificity		(Dubernet et al., 2002)
IDL03R	CCACCTTCCTCCGGTTTGTCA	All *Lactobacillus*	-	(Kwon et al., 2004)
IDL04F	AGGGTGAAGTCGTAACAAGTAGCC	All *Lactobacillus*	-	(Kwon et al., 2004)
IDL11F	TGGTCGGCAGAGTAACTGTTGTCG	*L. casei*-group	727	(Kwon et al., 2004)
IDL22R	AACTATCGCTTACGCTACCACTTTGC	*L. acidophilus*	606	(Kwon et al., 2004)
IDL31F	CTGTGCTACACCTAGAGATAGGTGG	*L. delbrueckii*	184	(Kwon et al., 2004)
IDL42R	ATTTCAAGTTGAGTCTCTCTCTC	*L. gasseri*	272	(Kwon et al., 2004)
IDL52F	ACCTGATTGACGATGGATCACCAGT	*L. reuteri*	1,105	(Kwon et al., 2004)
DL62R	CTAGTGGTAACAGTTGATTAAAACTGC	*L. plantarum*	428	(Kwon et al., 2004)
IDL73R	GCCAACAAGCTATGTGTTCGCTTGC	*L. rhamnosus*	448	(Kwon et al., 2004)
Enterococcus				
Ent1	TACTGACAAACCATTCATGATG	Genus-specificity	112	(Ke et al., 1999)
Ent2	AACTTCGTCACCAACGCGAAC			
FL1	ACTTATGTGACTAACTTAACC	*E. faecalis*	360	(Jackson et al., 2004)
FL2	TAATGGTGAAATCTTGGTTTGG			
FM1	GAAAAAACAATAGAAGAATTAT	*E. faecium*	215	(Jackson et al., 2004)
FM2	TGCTTTTTTGAATTCTTCTTTA			
GA1	TTACTTGCTGATTTTGATTCG	*E. gallinarum*	173	(Jackson et al., 2004)
GA2	TGAATTCTTCTTTGAAATCAG			
CA1	TCCTGAATTAGGTGAAAAAAC	*E. casseliflavus*	288	(Jackson et al., 2004)
CA2	GCTAGTTTACCGTCTTTAACG			
HI1	CTTTCTGATATGGATGCTGTC	*E. hirae*	187	(Jackson et al., 2004)
HI2	TAAATTCTTCCTTAAATGTTG			
DU1	CCTACTGATATTAAGACAGCG	*E. durans*	295	(Jackson et al., 2004)
DU2	TAATCCTAAGATAGGTGTTTG			
Clostridium				
16SA	GAGAGTTTGATCCTGGCTCAG	Genus-specificity	800	(Dhalluin et al., 2003)
16SB	GTGGACTACCAGGGTATCTAATCC			
ClPER-F	AGATGGCATCATCATTCAAC	*C. perfringens*	793	(Kikuchi et al., 2002)
ClPER-R	GCAAGGGATGTCAAGTGT			

Primers used for determination of genus and species of probiotic bacteria (Continued)

Primers	Sequence (5'-3')	PCR type	PCR product (bp)	References
ClBUT-F	TACCGCATGGTACAGCAATT	*C. butyricum*	1,056	(Kikuchi et al., 2002)
ClBUT-R	TCGCGAGGTTGCATCTCAT			
ClPAR-F	CCTGAATTACCATGTAATGTGG	*C. paraputrificum*	268	(Kikuchi et al., 2002)
ClPAR-R	TCACGGTATTGCATCTCGT			
ClBIF-F	CAAGTCGAGCGATCTCT	*C. bifermentans*	564	(Kikuchi et al., 2002)
ClBIF-R	CCTGCACTCAAGTTCTCT			
ClDIF-F	CTTGAATATCAAAGGTGAGCCA	*C. difficile*	1,085	(Kikuchi et al., 2002)
ClDIF-R	CTACAATCCGAACTGAGAGTA			
ClSOR-F	TCGAGCGACCTTCGG	*C. sordellii*	944	(Kikuchi et al., 2002)
ClSOR-R	CACCACCTGTCACCAT			
ClCLO-F	GAAGTTTTCGGATGGAATCTTGA	*C. clostridiiforme*	762	(Kikuchi et al., 2002)
ClCLO-R	CACCGAAGGCTTTGCC			
ClNEX-F	ATGGCACAGTGTAAAAACTCCG	*C. nexile*	1,054	(Kikuchi et al., 2002)
ClNEX-R	TTGCTTCCCCTCACAGGT			
ClSPH-F	GAAGTTTTCGGACGGATTTTGA	*C. sphenoides*	1,058	(Kikuchi et al., 2002)
ClSPH-R	AGAGTGCCCAACTTGACC			
ClIND-F	GACTGCTTTGGAAACTGTGT	*C. indolis*	369	(Kikuchi et al., 2002)
ClIND-R	AGGCCCCGTTACGGA			
ClINN-F	GGGGGATAATTATGGATCAC	*C. innocuum*	241	(Kikuchi et al., 2002)
ClINN-R	GTCGCTGCTCTTTGTGG			
ClRAM-F	GTGACCGTATTAAAAGTGCCT	*C. ramosum*	298	(Kikuchi et al., 2002)
ClRAM-R	TACCGTCACTCGGCTAC			
ClCOC-F	GTAATACATAAGTAACCTGGCCTTT	*C. cocleatum*	373	(Kikuchi et al., 2002)
ClCOC-R	CTCGGATGTCATTTCCTCC			

APPENDIX B

Solvents and diluents for antimicrobials

Antimicrobial	Solvent	Diluent
Ampicillin sodium salt	SDW	SDW
Streptomycin sulfate salt	SDW	SDW
Kanamycin	SDW	SDW
Gentamicin	SDW	SDW
Chloramphenicol	95% ethanol	SDW
Tetracycline	70% ethanol	SDW
Erythromycin	95% ethanol	SDW
Vancomycin	SDW	SDW
Trimethoprim	Dimethylacetamide	SDW
Sulfamethoxazole	0.1M NaOH, SDW	SDW
Ciprofloxacin	0.1M NaOH, SDW	SDW
Clindamycin	SDW	SDW
Meropenem	SDW	SDW
Rifampicin	SDW	SDW

SDW. Sterile distilled water

APPENDIX C

Primers used for detection of AMR genes (n=111) in this study

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
Beta-lactams					
bla$_{PSE-1}$	bla$_{PSE1}$-F	GCAAGTAGGGCAGGCAATCA	55	422	(Chuanchuen et al., 2008b)
	bla$_{PSE1}$-R	GAGCTAGATAGATGCTCACAA			
bla$_{TEM}$	bla$_{TEM}$-F	ATCAGTTGGGTGCACGAGTG	55	608	(Chuanchuen et al., 2008b)
	bla$_{TEM}$-R	ATCAGTTGGGTGCACGAGTG			
bla$_{SHV}$	blaSHV-F	TTCGCCTGTGTATTATCTCCCTG	50	854	(Hasman et al., 2005)
	blaSHV-R	TTAGCGTTGCCAGTGYTG			
bla$_{CMY-1}$	blaCMY-1F	GTGGTGGATGCCAGCATCC	58	915	(Hasman et al., 2005)
	blaCMY-1R	GGTCGAGCCGGTCTTGTTGAA			
bla$_{CMY-2}$	blaCMY-2F	GCACTTAGCCACCTATACGGCAG	58	758	(Hasman et al., 2005)
	blaCMY-2F	GCTTTTCAAGAATGCGCCAGG			
bla$_{CTX-M}$ universal	blaCTX-MF	CGATGTGCAGTACCAGTAA	60	585	(Batchelor et al., 2005)
	blaCTX-MR	AGTGACCAGAATCAGCGG			
bla$_{CTX-M}$ group 1	blaCTX-M group1-IF	TTAGGAARTGTGCCGCTGYA	60	688	(Dallenne et al., 2010)
	blaCTX-M group1-IR	CGATATCGTTGGTGGTRCCAT			
bla$_{CTX-M}$ group 2	blaCTX-M group2-IF	CGTTAACGGCACGATGAC	60	404	(Dallenne et al., 2010)
	blaCTX-M group2-IR	CGATATCGTTGGTGGTRCCAT			
bla$_{CTX-M}$ group 9	blaCTX-M group9-IF	TCAAGCCTGCCGATCTGGT	60	561	(Dallenne et al., 2010)
	blaCTX-M group9-IR	TGATTCTCGCCGCTGAAG			
bla$_{CTX-M}$ group 8/25	blaCTX-M group8-IF	AACRCRCAGACGCTCTAC	60	326	(Dallenne et al., 2010)
	blaCTX-M group8-IR	TCGAGCCGGAASGTGTYAT			

Primers used for detection of AMR genes (n=111) in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
$bla_{CTX-M-15}$	blaCTX-M 15-IF	CACACGTGGAATTTAGGGACT	56	995	(Muzaheed et al., 2008)
	blaCTX-M 15-IR	GCCGTCTAAGGCGATAAACA			
bla_{VEB}	MultiVEB_for	CATTTCCCGATGCAAAGCGT	60	648	(Dallenne et al., 2010)
	MultiVEB_rev	CGAAGTTTCTTTGGACTCTG			
bla_{GES}	MultiGES_for	AGTCGGCTAGACCGGAAAG	60	399	(Dallenne et al., 2010)
	MultiGES_rev	TTTGTCCGTGCTCAGGAT			
bla_{PER}	MultiPER_for	GCTCCGATAATGAAAGCGT	60	520	(Dallenne et al., 2010)
	MultiPER_rev	TTCGGCTTGACTCGGCTGA			
bla_{ACC}	MultiCaseACC_for	CACCTCCAGCGACTTGTTAC	60	346	(Dallenne et al., 2010)
	MultiCaseACC_rev	GTTAGCCAGCATCACGATCC			
bla_{FOX}	MultiCaseFOX_for	CTACAGTGCGGGTGGTTT	60	126	(Dallenne et al., 2010)
	MultiCaseFOX_rev	CTATTTGCGGCCAGGTGA			
bla_{MOX}	MultiCaseMOX_for	GCAACAACGACAATCCATCCT	60	895	(Dallenne et al., 2010)
	MultiCaseMOX_rev	GGGATAGGCGTAACTCTCCCAA			
bla_{DHA}	MultiCaseDHA_for	TGATGGCACAGCAGGATATTC	60	997	(Dallenne et al., 2010)
	MultiCaseDHA_rev	GCTTTGACTCTTTCGGTATTCG			
bla_{CIT}	MultiCaseCIT_for	CGAAGAGGCAATGACCAGAC	60	538	(Dallenne et al., 2010)
	MultiCaseCIT_rev	ACGGACAGGGTTAGGATAGY			
bla_{EBC}	MultiCaseEBC_for	GGCACCAGATTCAACTTTCAAG	60	683	(Dallenne et al., 2010)
	MultiCaseEBC_rev	GACCCCAAGTTTCCTGTAAGTG			
$bla_{OXA-1-like}$	MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	60	564	(Dallenne et al., 2010)
	MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG			
blaZ	stau-*blaZ*-F	CAAAGATGATATAGTTGCTTATTCTCC	50	421	(Kaase et al., 2008)
	stau-*blaZ*-R	TGCTTGACCACTTTTATCAGC			
mecA	mecA-F1	TGGTATGTGGAAGTTAGATTGGGAT	60	155	(Nakagawa et al., 2005)
	mecA-R1	CTAATCTCATATGTGTTCCTGTATTGGC			
bla_{KPC}	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	52	798	(Poirel et al., 2011)
	KPC-Rm	CTTGTCATCCTTGTTAGGCG			
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	52	621	(Poirel et al., 2011)
	NDM-R	CGGAATGGCTCATCACGATC			
bla_{OXA-48}	OXA-F	GCGTGGTTAAGGATGAACAC	52	438	(Poirel et al., 2011)
	OXA-R	CATCAAGTTCAACCCAACCG			
bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAAYTCTC	52	232	(Poirel et al., 2011)
	IMP-R	GGTTTAAYAAAACAACCACC			

Primers used for detection of AMR genes (n=111) in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	52	390	(Poirel et al., 2011)
	VIM-R	CGAATGCGCAGCACCAG			
Quinolones					
qnrA	qnrA-F	ATTTCTCACGCCAGGATTTG	53	516	(Robicsek et al., 2006)
	qnrA-R	GATCGGCAAAGGTTAGGTCA			
qnrB	qnrB-F	GATCGTGAAAGCCAGAAAGG	53	469	(Robicsek et al., 2006)
	qnrB-R	ACGATGCCTGGTAGTTGTCC			
qnrS	qnrS-F	ACGACATTCGTCAACTGCAA	53	417	(Robicsek et al., 2006)
	qnrS-R	TAAATTGGCACCCTGTAGGC			
qepA	QepA-F	GCAGGTCCAGCAGCGGGTAG	60	199	(Yamane et al., 2008)
	QepA-R	CTTCCTGCCCGAGTATCGTG			
aac(6')-Ib-cr	AAC(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	55	482	(Park et al., 2006)
	AAC(6')-Ib-R	CTCGAATGCCTGGCGTGTTT			
qnrC	qnrC-F	GGGTTGTACATTTATTGAATC	50	447	(Wang et al., 2009)
	qnrC-R	TCCACTTTACGAGGTTCT			
qnrD	qnrD fw	CGAGATCAATTTACGGGGAATA	50	582	(Cavaco et al., 2009)
	qnrD rev	AACAAGCTGAAGCGCCTG			
oqxA	oqxAF	CTCGGCGCGATGATGCT	55	392	(Kim et al., 2009)
	oqxAR	CCACTCTTCACGGGAGACGA			
oqxB	oqxBs	TTCTCCCCCGGCGGGAAGTAC	55	512	(Kim et al., 2009)
	oqxBa2	CTCGGCCATTTTGGCGCGTA			
Aminoglycosides					
aadA1	aadA1-F	CTCCGCAGTGGATGGCGG	55	631	(Chuanchuen et al., 2008b)
	aadA1-R	GATCTGCGCGCGAGGCCA			
aadA2	aadA2-F	CATTGAGCGCCATCTGGAAT	55	500	(Chuanchuen et al., 2008b)
	aadA2-R	ACATTTCGCTCATCGCCGGC			
aadB	aadB-F	CTAGCTGCGGCAGATGAGC	57	300	(Chuanchuen et al., 2008b)
	aadB-R	CTCAGCCGCCTCTGGGCA			
aad(E)	aadEI	GCAGAACAGGATGAACGTATTCG	55	369	(Klare et al., 2007)
	aadEII	ATCAGTCGGAACTATGTCCC			

Primers used for detection of AMR genes in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
aac(6')-aph(2")	aac(6')aph(2")F	CCAAGAGCAATAAGGGCATA	60	222	(Rojo-Bezares et al., 2006)
	aac(6')aph(2")R	CACTATCATAACCACTACCG			
strA	strA-F	TGGCAGGAGGAACAGGAGG	57	405	(Chuanchuen and Padungtod, 2009)
	strA-R	AGGTCGATCAGACCCGTGC			
strB	strB-F	GCGGACACCTTTTCCAGCCT	57	621	(Chuanchuen and Padungtod, 2009)
	strB-R	TCCGCCATCTGTGCAATGCG			
armA	armA-F	CCGAAATGACAGTTCCTATC	55	846	(Yan et al., 2004)
	armA-R	GAAAATGAGTGCCTTGGAGG			
rmtB	rmtB-F	ATGAACATCAACGATGCCCT	55	769	(Yan et al., 2004)
	rmtB-R	CCTTCTGATTGGCTTATCCA			
aac(3)-I	aac(3)-I F	GGGCATCATTCGCACATGTAGGC	64	429	(Jakobsen et al., 2008)
	aac(3)-I R	CATCACTTCTTCCCGTATGCCC			
aac(3)-II	aac(3)-II F	TGAAACGCTGACGGAGCCTC	58	369	(Sandvang and Aarestrup, 2000)
	aac(3)-II R	GTCGAACAGGTAGCACTGAG			
aac(3)-III	aac(3)-III F	GTGCATCGCAGCGCAAACCCC	64	436	(Jakobsen et al., 2008)
	aac(3)-III R	CAAGCCACTGCACCGCAAACCG			
aac(3)-IV	aac(3)-IV F	GTGTGCTGCTGGTCCACAGC	58	628	(Sandvang and Aarestrup, 2000)
	aac(3)-IV R	AGTTGACCCAGGGCTGTCGC			
aph(2'')-Ib	aph2-Ib-F	CTTGGACGCTGAGATATATGAGCAC	55	867	(Vakulenko et al., 2003)
	aph2-Ib-R	GTTTGTAGCAATTCAGAAACACCCTT			
aph(2'')-Ic	aph2-Ic-F	CCACAATGATAATGACTCAGTTCCC	55	444	(Vakulenko et al., 2003)
	aph2-Ic-R	CCACAGCTTCCGATAGCAAGAG			
aph(2'')-Id	aph2-Id-F	GTGGTTTTTACAGGAATGCCATC	55	641	(Vakulenko et al., 2003)
	aph2-Id-R	CCCTCTTCATACCAATCCATATAACC			
aph(3')-IIIa	aph3-IIIa-F	GGCTAAAATGAGAATATCACCGG	55	523	(Vakulenko et al., 2003)
	aph3-IIIa-R	CTTTAAAAAATCATACAGCTCGCG			
ant(4')-Ia (aadD)	ant4-Ia-F	CAAACTGCTAAATCGGTAGAAGCC	55	294	(Vakulenko et al., 2003)
	ant4-Ia-R	GGAAAGTTGACCAGACATTACGAACT			

Primers used for detection of AMR genes in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
Chloramphenicol					
catA	catA-F	CCAGACCGTTCAGCTGGATA	55	454	(Chuanchuen et al., 2008b)
	catA-R	CATCAGCACCTTGTCGCCT			
catB	catB-F	CGGATTCAGCCTGACCACC	55	461	(Chuanchuen et al., 2008b)
	catB-R	ATACGCGGTCACCTTCCTG			
cmlA	cmlA-F	TGGACCGCTATCGGACCG	57	641	(Chuanchuen et al., 2008a)
	cmlA-R	CGCAAGACACTTGGGCTGC			
florR	florR-F	ATGGTGATGCTCGGCGTGGGCCA	58	800	(Ying et al., 2019)
	florR-R	GCGCCGTTGGCGGTAACAGACACCGTGA			
Macrolides					
ermA	ermAI	TCTAAAAAGCATGTAAAAGAA	52	645	(Sutcliffe et al., 1996)
	ermAII	CTTCGATAGTTTATTAATATTAGT			
ermB	ermBI	GAAAAGGTACTCAACCAAATA	52	638	(Sutcliffe et al., 1996)
	ermBII	AGTAACGGTACTTAAATTGTTTAC			
ermC	ermCI	TCAAAACATAATATAGATAAA	52	643	(Sutcliffe et al., 1996)
	ermCII	GCTAATATTGTTTAAATCGTCAAT			
mefA	mef(A)-FW	CAATATGGGCAGGGCAAG	62	317	(Chen et al., 2013)
	mef(A)-RW	AAGCTGTTCCAATGCTACGG			
mph(A)	mphAF	GTGAGGAGGAGCTTCGCGAG	60	403	(Phuc Nguyen et al., 2009)
	mphAR	TGCCGCAGGACTCGGAGGTC			
mph(B)	mphBF	GATATTAAACAAGTAATCAGAATAG	58	494	(Phuc Nguyen et al., 2009)
	mphBR	GCTCTTACTGCATCCATACG			
mph(C)	mphCF	ATGACTCGACATAATGAAAT	45	900	(Schnellmann et al., 2006)
	mphCR	CTACTCTTTCATACCTAACTC			
ere(A)	ereAF	GCCGGTGCTCATGAACTTGAG	60	420	(Phuc Nguyen et al., 2009)
	ereAR	CGACTCTATTCGATCAGAGGC			

Primers used for detection of AMR genes in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
ere(B)	ereBF	TTGGAGATACCCAGATTGTAG	55	537	(Phuc Nguyen et al., 2009)
	ereBR	GAGCCATAGCTTCAACGC			
ermF	F1	CGGGTCAGCACTTTACTATTG	50	466	(Chung et al., 1999)
	F2	GGACCTACCTCATAGACAAG			
msrA	msrA F	GGCACAATAAGAGTGTTTAAAGG	60	940	(Mišić et al., 2017)
	msrA R	AAGTTATATCATGAATAGATTGTCCTGTT			
msrB	msrB F	TATGATATCCATAATAATTATCCAATC	60	595	(Mišić et al., 2017)
	msrB R	AAGTTATATCATGAATAGATTGTCCTGTT			
ermTR	TR3	CAATAAACAAGATAAAATAATAG	47	531	(Angot et al., 2000)
	TR4	CTTTTTGTAGTCCTTCTTTAA			
Trimethoprim					
dfrA1	dfrA1-F	CAATGGCTGTTGGTTGGAC	55	254	(Chuanchuen et al., 2008b)
	dfrA1-R	CCGGCTCGATGTCTATTGT			
dfrA10	dfrA10-F	TCAAGGCAAATTACCTTGGC	57	432	(Chuanchuen and Padungtod, 2009)
	dfrA10-R	ATCTATTGGATCACCTACCC			
dfrA12	dfrA12-F	TTCGCAGACTCACTGAGGG	55	330	(Chuanchuen et al., 2008b)
	dfrA12-R	CGGTTGAGACAAGCTCGAAT			
dfrA5	dfr5-f	AGCTACTCTTTAAAGCCTTGACGTA	55	341	(Grape et al., 2007)
	dfr5-r	GTGTTGCTCAAAAACAACTTCG			
dfrA7	dfr7&17-f	ACATTTGACTCTATGGGTGTTCTTC	55	227	(Grape et al., 2007)
	dfr7-r	ACCTCAACGTGAACAGTAGACAAAT			
dfrA17	dfr7&17-f	ACATTTGACTCTATGGGTGTTCTTC	55	171	(Grape et al., 2007)
	dfr17-r	TCTCTGGCGGGGGTCAAATCTAT			
dfrA14	dfrA14-F	TTAACCCAGGATGAGAACCT	52	510	(Miranda et al., 2016)
	dfrA14-R	CGATTGCATAGCTTTGTTAA			
dfr18	dfr18-F	TGGGTAAGACACTCGTCATGGG	43	389	(Hochhut et al., 2001)
	dfr18-R	ACTGCCGTTTTCGATAATGTGG			
dfrA8	dfrA8-F	GAGCTTCCGGGTGTTCGTGAC	55	247	(Toro et al., 2005)
	dfrA8-R	CTTCCATGCCATTCTGCTCGTAGT			

Primers used for detection of AMR genes in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
Sulfonamides					
sul1	Sul1-F	CGGACGCGAGGCCTGTATC	57	591	(Chuanchuen et al., 2007)
	Sul1-R	GGGTGCGGACGTAGTCAGC			
sul2	sul2-F	GCGCAGGCGCGTAAGCTGAT	57	514	(Chuanchuen and Padungtod, 2009)
	sul2-R	CGAAGCGCAGCCGCAATTC			
sul3	sul3-F	GGGAGCCGCTTCCAGTAAT	57	500	(Chuanchuen et al., 2008a)
	sul3-R	TCCGTGACACTGCAATCATTA			
Tetracyclines					
tetA	tetA-F	GCTGTCGGATCGTTTCGG	55	658	(Chuanchuen et al., 2008b)
	tetA-R	CATTCCGAGCATGAGTGCC			
tetB	tetB-F	CTGTCGCGGCATCGGTCAT	55	615	(Chuanchuen et al., 2008b)
	tetB-R	CAGGTAAAGCGATCCCACC			
tetK	tetKI	TTGAGCTGTCTTGGTTCA	50	352	(Klare et al., 2007)
	tetKII	CAATACCTACGATATCTA			
tetL	tet(L)I	TGGTCCTATCTTCTACTCATTC	53	385	(Werner et al., 2003)
	tet(L)II	TTCCGATTTCGGCAGTAC			
tetM	tet(M)I	GGTGAACATCATAGACACGC	55	401	(Werner et al., 2003)
	tet(M)II	CTTGTTCGAGTTCCAATGC			
tetO	tet(O)I	AGCGTCAAAGGGGAATCACTATCC	55	1723	(Klare et al., 2007)
	tet(O)II	CGGCGGGGTTGGCAAATA			
tetS	tet(S)I	ATCAAGATATTAAGGAC	55	573	(Gevers et al., 2003)
	tet(S)II	TTCTCTATGTGGTAATC			
tetW	TetW-FW	GAGAGCCTGCTATATGCCAGC	52	168	(Aminov et al., 2001)
	TetW-RW	GGGCGTATCCACAATGTTGAC			
tet(C)	tetC-F	CTTGAGAGCCTTCAACCCAG	55	418	(Ng et al., 2001)
	tetC-R	ATGGTCGTCATCTACCTGCC			
tet(D)	tetD-F	AAACCATTACGGCATTCTGC	55	787	(Ng et al., 2001)
	tetD-R	GACCGGATACACCATCCATC			
tet(E)	tetE-F	AAACCACATCCTCCATACGC	55	278	(Ng et al., 2001)
	tetE-R	AAATAGGCCACAACCGTCAG			
tet(G)	tetG-F	GCTCGGTGGTATCTCTGCTC	55	468	(Ng et al., 2001)
	tetG-R	AGCAACAGAATCGGGAACAC			
tet(Q)	tetQ-F	TTATACTTCCTCCGGCATCG	55	904	(Ng et al., 2001)
	tetQ-R	ATCGGTTCGAGAATGTCCAC			

Primers used for detection of AMR genes in this study (Continued)

Gene	Primer	Primer sequences (5' – 3')	Annealing Temp (°C)	Product size (bp)	Reference
tet(X)	tetX-F	CAATAATTGGTGGTGGACCC	55	468	(Ng et al., 2001)
	tetX-R	TTCTTACCTTGGACATCCCG			
tet(30)	tet(30)-F	CCGTCATGCAATTTGTGTTC	60	550	(Call et al., 2003)
	tet(30)-R	TAGAGCACCCAGATCGTTCC			
tet(32)	tet(32)-F	GAACCAGATGCTGCTCTT	57	620	(Melville et al., 2001)
	tet(32)-R	CATAGCCACGCCCACATGAT			
tet(O/W/32/O)	tetWF	GGAGGAAAATACCGACATA	50	729	(Patterson et al., 2007)
	tet32R	CTCTTTCATAGCCACGCC			
Polymyxins					
mcr-1	MCR1-IF	AGTCCGTTTGTTCTTGTGGC	58	320	(Rebelo et al., 2018)
	MCR1-IR	AGATCCTTGGTCTCGGCTTG			
mcr-2	MCR2-IF	CAAGTGTGTTGGTCGCAGTT	58	715	(Rebelo et al., 2018)
	MCR2-IR	TCTAGCCCGACAAGCATACC			
mcr-3	MCR3-IF	AAATAAAAATTGTTCCGCTTATG	58	929	(Rebelo et al., 2018)
	MCR3-IR	AATGGAGATCCCCGTTTTT			
mcr-4	MCR4-IF	TCACTTTCATCACTGCGTTG	58	1116	(Rebelo et al., 2018)
	MCR4-IR	TTGGTCCATGACTACCAATG			
mcr-5	MCR5-IF	ATGCGGTTGTCTGCATTTATC	58	1644	(Rebelo et al., 2018)
	MCR5-IR	TCATTGTGGTTGTCCTTTTCTG			
mcr-6	MCR-6F	GTCCGGTCAATCCCTATCTGT	55	556	(Wang et al., 2018)
	MCR-6R	ATCACGGGATTGACATAGCTAC			
mcr-7	MCR-7F	TGCTCAAGCCCTTCTTTTCGT	55	894	(Wang et al., 2018)
	MCR-7R	TTCATCTGCGCCACCTCGT			
mcr-8	MCR-8F	AACCGCCAGAGCACAGAATT	60	667	(Wang et al., 2018)
	MCR-8R	TTCCCCCAGCGATTCTCCAT			
Vancomycin					
vanA	vanA1	GGGAAAACGACAATTGC	54	732	(Dutka-Malen et al., 1995)
	vanA2	GTACAATGCGGCCGTTA			
vanB	vanB1	ATGGGAAGCCGATAGTC	54	635	(Dutka-Malen et al., 1995)
	vanB2	GATTTCGTTCCTCGACC			
vanC	vanC1	GGTATCAAGGAAACCTC	54	822	(Dutka-Malen et al., 1995)
	vanC2	CTTCCGCCATCATAGCT			

APPENDIX D

MIC (μg/ml) distribution of all isolates including *Bacillus* (n=54), *Lactobacillus* (n=4) and *Enterococcus* (n=4)

Product	Isolate	Species	MIC (μg/ml													
			AMP	STR	KAN	GEN	CHL	TET	ERY	TRI	SUL	CIP	VAN	CLI	MER	RIF
P1	B1.1	*B. subtilis*	0.0625	1	1	0.125	8	8	0.25	0.125	1	0.015625	0.25	0.5	0.0625	0.125
	B1.3	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	1	0.015625	0.25	0.5	0.0625	0.125
	B1.5	*B. sphaericus*	0.125	1	1	0.125	16	0.125	>128	0.125	1	0.015625	0.25	8	0.0625	0.125
P2	B2.1	Other *Bacillus* spp.	4	1	1	0.125	4	0.25	0.5	0.5	16	0.0625	4	0.5	0.0625	0.125
	B2.2	*B. subtilis*	0.0625	1	1	0.125	4	4	0.25	0.125	0.5	0.015625	0.25	1	0.0625	0.125
	B2.3	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	0.125	0.25	0.125	4	0.015625	0.25	0.25	0.0625	0.125
P3	B3.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	0.125	0.5	0.125	4	0.015625	0.25	0.25	0.0625	0.125
	B3.3	*B. subtilis*	0.0625	1	1	0.125	4	4	0.25	0.125	0.5	0.015625	0.25	1	0.0625	0.125
P4	B4.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	1	0.015625	0.25	0.5	0.0625	0.125
P5	B5.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	4	0.015625	0.25	0.5	0.0625	0.125
P6	B6.1	*B. licheniformis*	0.125	1	1	0.125	16	0.125	>128	0.125	0.5	0.03125	0.25	64	0.0625	0.125
	E.6.1	*E. faecium*	1	1	128	4	2	0.125	1	0.125	2048	0.25	0.5	2	4	0.125
P7	B7.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.25	4	0.015625	0.25	0.25	0.0625	0.125
	L7.1	*L. casei*-group	8	16	64	2	8	1	0.125	32	128	2	>256	0.03125	4	8
	L7.2	*L. plantarum*	8	32	64	4	8	16	0.125	>512	128	16	>256	0.25	0.25	0.5
	L7.4	*L. rhamnosus*	0.5	32	64	8	4	1	0.125	16	128	1	>256	0.03125	2	0.5

Grey-shaded boxes show MICs of antibiotics of bacteria that were considered as resistance. AMP, ampicillin; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin; MER, meropenem; RIF, rifampicin.

MIC (µg/ml) distribution of all isolates including *Bacillus* (n=54), *Lactobacillus* (n=4) and *Enterococcus* (n=4)

Product	Isolate	Species	MIC (µg/ml													
			AMP	STR	KAN	GEN	CHL	TET	ERY	TRI	SUL	CIP	VAN	CLI	MER	RIF
P8	E8.1	*E. faecium*	1	1	128	4	2	0.125	1	0.125	2048	0.25	0.5	2	4	0.125
P9	B9.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	0.125	0.25	0.25	4	0.015625	0.25	0.25	0.0625	0.125
P10	B10.1	Members of *B. subtilis* cluster	0.0625	2	1	0.125	4	4	0.25	1	4	0.015625	0.25	0.125	0.0625	0.125
P11	B11.1	*B. licheniformis*	0.125	1	1	0.125	16	2	>128	0.125	0.5	0.03125	0.25	16	0.0625	0.125
P12	B12.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	4	0.25	0.5	4	0.03125	0.25	0.25	0.0625	0.125
	B12.2	*B. sphaericus*	2	4	1	0.125	4	0.25	0.25	256	2048	0.0625	0.5	1	0.0625	0.125
P13	B13.1	*B. licheniformis*	0.125	1	1	0.125	16	4	>128	0.125	1	0.03125	0.25	32	0.0625	0.125
	B13.3	*B. subtilis*	0.0625	1	1	0.125	4	0.125	0.25	0.125	1	0.015625	0.25	0.5	0.0625	0.125
P14	E14.1	*E. faecium*	1	1	128	4	2	0.125	1	0.125	2048	0.25	0.5	2	4	0.125
P18	B18.1	*B. licheniformis*	0.25	1	1	0.125	16	0.125	0.25	0.125	0.5	0.03125	0.25	32	0.0625	0.125
P19	B19.1	Other *Bacillus* spp.	0.0625	1	1	0.125	4	4	0.25	0.5	0.5	0.03125	0.25	0.125	0.0625	0.125
P20	B20.1	*B. subtilis*	0.0625	1	1	0.125	4	0.125	0.25	0.125	0.5	0.015625	0.25	0.125	0.0625	0.125
P21	B21.1	*B. subtilis*	0.0625	1	1	0.125	4	0.125	0.25	0.125	0.5	0.015625	0.25	0.125	0.0625	0.125
P22	B22.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	2	0.25	0.125	1	0.03125	0.25	0.25	0.0625	0.125
P23	B23.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	2	0.25	0.125	1	0.0625	0.25	0.25	0.0625	0.125
P24	B24.1	Other *Bacillus* spp.	0.0625	2	1	0.125	64	64	1	>256	2048	0.0625	1	0.25	0.0625	0.125

Grey-shaded boxes show MICs of antibiotics of bacteria that were considered as resistance. AMP, ampicillin; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin; MER, meropenem; RIF, rifampicin.

MIC (µg/ml) distribution of all isolates including *Bacillus* (n=54), *Lactobacillus* (n=4) and *Enterococcus* (n=4)

Product	Isolate	Species	MIC (µg/ml													
			AMP	STR	KAN	GEN	CHL	TET	ERY	TRI	SUL	CIP	VAN	CLI	MER	RIF
P25	B25.1	Other *Bacillus* spp.	0.0625	2	1	0.25	64	64	1	>256	2048	0.0625	1	0.25	0.0625	0.125
P26	B26.1	*B. licheniformis*	0.125	1	1	0.25	16	2	>128	0.125	0.5	0.015625	0.25	16	0.0625	0.125
P27	B27.1	*B. subtilis*	0.0625	1	1	0.125	4	4	0.25	0.125	0.5	0.03125	0.25	0.5	0.0625	0.125
	B27.2	Members of *B. subtilis* cluster	0.0625	1	1	0.125	2	0.125	0.25	0.125	1	0.015625	1	0.25	0.0625	0.125
P28	B28.1	Other *Bacillus* spp.	0.0625	1	1	0.125	4	4	0.25	0.25	4	0.015625	0.25	0.125	0.0625	0.125
	B28.5	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.25	4	0.03125	0.25	0.125	0.0625	0.125
P29	B29.1	Other *Bacillus* spp.	0.0625	1	1	0.125	4	4	0.25	0.25	4	0.015625	0.25	0.125	0.0625	0.125
P30	B30.2	*B. subtilis*	0.0625	1	1	0.125	4	0.125	0.25	0.125	0.5	0.015625	0.25	0.25	0.0625	0.125
	B30.4	Other *Bacillus* spp.	0.0625	1	1	0.125	2	0.125	0.25	0.125	1	0.015625	0.25	0.25	0.0625	0.125
	B30.5	Members of *B. subtilis* cluster	0.0625	1	1	0.125	0.5	0.125	0.25	0.125	4	0.015625	0.25	0.5	0.0625	0.125
P31	B31.1	Other *Bacillus* spp.	0.0625	1	1	0.125	4	8	0.25	0.125	4	0.015625	0.25	0.5	0.0625	0.125
	B31.4	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	1	0.015625	0.25	0.5	0.0625	0.125
P32	B32.1	Other *Bacillus* spp.	0.0625	1	1	0.125	2	8	0.25	0.125	0.5	0.015625	0.25	0.25	0.0625	0.125
	B32.4	Members of *B. subtilis* cluster	0.0625	1	1	0.125	2	8	0.25	0.125	2	0.015625	0.25	0.25	0.0625	0.125
P33	B33.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	0.125	0.25	0.125	0.5	0.015625	0.25	2	0.0625	0.125
	B33.3	Other *Bacillus* spp.	0.0625	1	1	0.125	2	0.125	0.25	256	2048	0.03125	0.5	0.5	0.0625	0.125
	B33.4	*B. sphaericus*	0.0625	1	1	0.125	4	0.125	0.25	256	2048	0.03125	0.5	0.5	0.0625	0.125
P34	B34.1	*B. sphaericus*	0.0625	1	1	0.25	8	0.125	0.25	128	2048	0.03125	0.5	0.5	0.0625	0.125

Grey-shaded boxes show MICs of antibiotics of bacteria that were considered as resistance. AMP, ampicillin; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin; MER, meropenem; RIF, rifampicin.

MIC values (µg/ml) of all isolates including *Bacillus* (n=54), *Lactobacillus* (n=4) and *Enterococcus* (n=4)

Product	Isolate	Species	MIC (µg/ml)													
			AMP	STR	KAN	GEN	CHL	TET	ERY	TRI	SUL	CIP	VAN	CLI	MER	RIF
P35	B35.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	2	8	0.25	0.125	4	0.015625	0.25	0.5	0.0625	0.125
P36	B36.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	2	8	0.25	0.125	2	0.015625	0.25	0.25	0.0625	0.125
P37	B37.1	*B. licheniformis*	0.125	1	1	0.125	16	8	>128	0.125	2	0.015625	0.25	16	0.0625	0.125
	B37.3	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	4	0.03125	0.25	0.5	0.0625	0.125
P38	B38.1	*B. licheniformis*	0.125	1	1	0.25	16	0.125	0.25	0.125	4	0.03125	0.25	32	0.0625	0.125
P39	B39.1	*B. licheniformis*	0.125	1	1	0.25	16	0.125	0.25	0.125	0.5	0.015625	0.25	32	0.0625	0.125
P40	B40.1	*B. licheniformis*	0.125	1	1	0.25	32	0.125	0.25	0.125	0.5	0.015625	0.25	32	0.0625	0.125
P41	L41.1	*L. delbruekii*	2	128	64	2	4	256	0.125	256	64	32	>256	0.03125	1	0.125
P43	B43.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	2	4	0.25	0.125	2	0.03125	0.25	0.25	0.0625	0.125
	E43.1	*E. faecium*	1	1	128	4	2	0.125	1	0.125	2048	0.25	0.5	0.5	8	0.125
P44	B44.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	2	0.015625	0.25	0.25	0.0625	0.125
	L44.1	Other *Lactobacillus* spp.	1	32	32	4	4	32	0.125	>512	128	64	>256	0.03125	0.25	0.5
P45	B45.1	Members of *B. subtilis* cluster	0.0625	1	1	0.125	4	8	0.25	0.125	4	0.015625	0.25	0.25	0.0625	0.125
	B45.1	Other *Lactobacillus* spp.	4	64	128	2	4	32	0.125	64	128	64	>256	0.03125	0.5	0.5

Grey-shaded boxes show MICs of antibiotics of bacteria that were considered as resistance. AMP, ampicillin; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; TRI, trimethoprim; SUL, sulfamethoxazole; CIP, ciprofloxacin; VAN, vancomycin; CLI, clindamycin; MER, meropenem; RIF, rifampicin

OUTPUTS

The results from this study were presented as oral presentation at the 3rd International Symposium on Alternatives to Antibiotics (ATA, 2019) from 16th December to 19th December, 2019. The abstract for our research was published in the proceeding of ATA conference.

> M.H. Tran and R. Chuanchuen, 2019. Microbiological quality and possible role as a source of antimicrobial resistance genes of commercial probiotic products for livestock and aquatic animals.

The results were also presented as poster presentation at the 19th Chulalongkorn University Veterinary Conference (CUVC, 2020) from 22nd April – 24th April. The abstract for our research was published in the proceeding of Thai Journal of Veterinary Medicine

> Hoang My Tran and Rungtip Chuanchuen, 2020. Screening of antimicrobial resistance genes in commercial probiotic products for food animal production in Thailand. Thai J Vet. Suppl (5): 367-368.

VITA

NAME	Hoang My Tran
DATE OF BIRTH	25 October 1993
PLACE OF BIRTH	Tra Vinh, Vietnam
INSTITUTIONS ATTENDED	Faculty of Veterinary Science, Chulalongkorn University, Thailand Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Vietnam
HOME ADDRESS	Room C1, 2th Floor, Baan Somboon, 70/24, soi 7, Phetchaburi Road, Thung Phaya Thai, Ratchathewi, Bangkok, Thailand