Assessment of Antimicrobial Activity of Synthetic Peptides on *Escherichia coli* Isolated from Boar Semen

Sophorn Keath

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Animal Science
Prince of Songkla University
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Recently, antimicrobial peptides (AMPs) are known as new potential agent which can inhibit bacteria growth in liquid-preserved boar semen and used to replace the conventional antibiotics. Previous study, our researchers found that nine peptides with derived from the seminal plasma showed the highest antimicrobial activity with *Escherichia coli* ATCC 25922 ranged from 70% to 100% inhibition (not published yet). It is very interesting for further investigation on their activities against *Escherichia coli* isolated from boar semen. The objectives of this study were to i) identify the bacterial contaminants in boar semen ii) discriminate *Escherichia coli* strains and iii) investigate the antimicrobial susceptibility testing and antimicrobial activity of synthetic peptides on *Escherichia coli* isolated from boar semen. The synthetic peptides were prepared by manufacturer as powder and only eight of nine peptides could be synthesized. Eight pooled semen kept in transport media and ten fresh boar semen samples were collected from pig farms located in the central and southern region of Thailand, respectively. The gram staining and biochemical test were conducted to identify the species of both gram-negative and gram-positive bacteria. The remaining unknown species of bacteria were identified by MALDI-TOF MS technique. Antimicrobial susceptibility of *Escherichia coli* isolates was tested by disk-diffusion (penicillin G, ampicillin, gentamicin, amikacin and ceftazidime) and broth microdilution methods (colistin). BOX A1R PCR (primer: 5'-CTACGGCAAGGCGACGCTGACG-3') was used to discriminate *Escherichia coli* strains isolated from boar semen. To determine the minimum inhibitory concentration (MIC), one of isolated *Escherichia* strains, randomly selected, and *Escherichia coli* ATCC 25922 were preliminary investigated with 2 peptides (Sam1 and Sam5) by using a broth microdilution. The MIC value of Sam1 and Sam5 with *Escherichia coli* ATCC 25922 and B05N44 showed higher than 400 µg/ml. So

that, 100 µg/ml of all the peptides were preliminary investigated the antimicrobial activity with 4 strains of *Escherichia coli* selected from each group of clonal relatedness (one replication). The results revealed that Sam1, Sam4, and Sam9 showed higher activity with *Escherichia coli* ATCC 25922 while Sam1, Sam2, and Sam9 showed higher activity with A01N04, A06N16, B05N44, and B07N62. Then, the peptides which showed the highest activity were conducted the time-kill assay by OD measurement and colony count at 0, 4, 8, 16, and 24 h of incubation (triplicate). The results showed that all the samples were contaminated with gram-negative and gram-positive bacteria (n=18, 100%). The dominant species were *Escherichia coli* (100 %, n=18) and *Staphylococcus* spp. (100%, n=18) followed by *Serratia marcescens* (33.33%, n=6), *Klebsiella pneumoniae* (22.22%, n=4), *Enterobacter cloacea, Citrobacter koseri* and *Enterobacter aerogenes* and *Streptococcus* spp. (equally 11.11%, n=2), *Aeromonas hydrophila, Edwardsiella tarda, Providencia stuartii, Providencia rettgeri, Klebseilla oxytoca, Klebseilla aerogenes* and *Pseudomonas aeruginosa* (equally 5.55%, n=1). Moreover, the colony forming unit of gram-positive and gram-negative bacteria isolated from fresh boar semen varied from 4.00×10^2 to 8.50×10^3 and 1.33×10^2 to 4.17×10^3 CFU/ mL, respectively. *Escherichia coli* isolates were resistant to penicillin G (100%), ampicillin (97.96%), gentamicin (12.24%) and colistin (8.16%). All of *Escherichia coli* were sensitive to amikacin (0%) and ceftazidime (0%). According to 75% similarity levels of clonal relatedness, 49 strains of *Escherichia coli* were distinguished into 8 clusters. Sam1, Sam4, and Sam9 showed high antimicrobial activity, in term of inhibition percentage, with *Escherichia coli* ATCC 25922 (99.99%, 98.14%, and 97.57%, respectively) whereas Sam1, Sam2, and Sam9 showed high activity with A01N04 (98.75%, 85%, and 87.5%, respectively), A06N16 (84.29%, 90.86%, and 95.71%, respectively), B05N44 (99.88%, 95.21%, and 98.70%, respectively), and B07N62 (99.79%, 99.97%, and 80%, respectively). In conclusion, Sam1 had the highest antibacterial activity against *Escherichia coli* ATCC 25922 and *Escherichia coli* strains isolated from boar semen.

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Sophorn Keath

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LIST OF ABBREVIATIONS

A. hydrophila	: *Aeromonas hydrophila*
AMK	: amikacin
AMP	: ampicillin
^{o}C	: degree celsius
C. koseri	: *Citrobacter koseri*
CT	: colistin
CTX	: ceftazidime
CFU	: colony forming unit
DMSO	: dimethyl sulfoxide
E. aerogenes	: *Enterobacter aerogenes*
E. cloacea	: *Enterobacter cloacea*
E. coli	: *Escherichia coli*
E. tarda	: *Edwardsiella tarda*
GEN	: gentamicin
K. aerogenes	: *Klebseilla aerogenes*
K. oxytoca	: *Klebseilla oxytoca*
K. pneumoniae	: *Klebsiella pneumoniae*
LBA	: Luria-Bertani agar
MALDI-TOP MS	: matrix-assisted laser desorption ionization time of flight mass spectrometry
MCA	: MacConkey agar
MSA	: Manitol Salt agar
MIC	: minimum inhibitory concentration
OD	: optical density
PEN	: penicillin
P. aeruginosa	: *Pseudomonas aeruginosa*
P. stuartii	: *Providencia stuartii*
P. rettgeri	: *Providencia rettgeri*
S. marcescens	: *Serratia marcescens*

CHAPTER I
INTRODUCTION

1.1 Research background

Artificial insemination (AI) of swine is a great biotechnology application which provide the genetic improvement, increase economic value and prevent disease transmission in swine production (Foote, 2010). Semen preservation therefore plays very important role in longevity and healthy of spermatozoa (Manafi, 2011). However, bacterial contamination is a major problem of using AI which mostly occurred during semen collection and processing. Sources of those microorganism can be classified as being from own body of animal such as preputial ostium, large preputial diverticulum, and long preputial hair and the whole environment including water, air and non-sterilized equipment (Goldberg *et al*., 2013). Both gram-negative and gram-positive bacteria were always found in boar semen which *Escherichia coli* is a dominate species of bacterial contaminants. It caused the negative effects on sperm quality by inducing agglutination and reducing motility and reduced the litter size (Althouse and Lu, 2005; Martín *et al*., 2010).

To solve the problem, antibiotics have being used in semen extenders to control bacterial growth (Sone, 1990, Morrell and Wallgren, 2014,). However, antibiotic usage in the semen extenders can be carrying a risk development of antibiotic resistance and becoming a major concern in artificial insemination. Since, as the report, investigation of bacterial contaminants in extended boar semen and their effects on sperm quality (Althouse *et al*., 2000; Gaczarzewicz *et al*., 2016).

Alternative to conventional antibiotics in semen extenders, recently, antimicrobial peptides (AMPs) is a new potential agent can inhibit bacteria growth in liquid-preserved boar semen (Schulze *et al*., 2014; Speck *et al*., 2014; Puig-Timonet *et al*., 2018; Bussalleu *et al*., 2017). The mechanisms of these AMPs may acts against microorganisms through the cell membrane (Hancock and Rozek, 2002; Zasloff, 2002; Saldit *et al*, 2006). In addition, the results obtained from our

preliminary study (not published yet) showed 9 antimicrobial peptides (named as Sam1, Sam2, Sam3, Sam4, Sam5, Sam6, Sam7, Sam8, and Sam9) collected from boar semen could inhibit *Escherichia coli* ATCC 25922. It is very interesting and worth to do the further study whether these peptides can inhibit the *Escherichia coli* isolated from boar semen or not. The results obtained from this study can benefit the use of these peptides to replace the antibiotics in liquid-preserved boar semen.

1.2 Objectives

1. To isolate and identify the bacterial contaminants in boar semen
2. To discriminate *Escherichia coli* strains isolated from boar semen
3. To investigate the antimicrobial susceptibility testing on isolated *Escherichia coli* from boar semen
4. To investigate the activity of antimicrobial peptides on *Escherichia coli* isolated from boar semen

1.3 Literature review

1.3.1 Bacterial contaminants in boar semen

The original sources of bacterial contaminants in boar semen including own body of animal (fluid, hair, skin, mucus and feces) and environmental effects (water, feed, bedding material, un-sterilized equipment, and housing arrangement system) (Bresciani *et al*., 2014). Both gram-positive and gram-negative bacteria have been reported including *Escherichia coli* , *K. pseudomonas*, *Proteus* spp., *Pseudomonas* spp., *S. marcescens, Streptococcus* spp. as shown in Table 1 (Althouse and Lu, 2005; Bresciani *et al*, 2014; Gaczarzewicz *et al*, 2016). Colony count in fresh semen commonly ranges between 10^4 and 10^6 CFU/ml (Schulze *et al*., 2015). *Escherichia coli* are predominantly major numbers isolated contaminant with the average of bacterial numbers in fresh boar semen ejaculate, approximately $10^3 – 10^5$ CFU/ml (Matin *et al*., 2010; Schulze *et al*., 2012).

1.3.2 *Escherichia coli*

Escherichia coli is a species which was isolated from family Enterobacteriaceae, known as a species which always provides the benefit for digestion food in animal gut but it also causes urinary tract infection (Nataro and Kaper, 1998; Rasheed *et al*., 2014). *Escherichia coli* are normal microbiota in gastrointestinal tract of humans and warm-blood animals, rod shape (Figure 1), oxidase negative, non-spore forming and facultative anaerobic bacteria. Its size is approximately 1.1-1.5 x 2.0-6.0 µm, and movement by peritrichous flagella and fimbriae. Especially, it can grow well at 35 ºC to 37 ºC , pH 5- 9 and can still survive after a short exposure to a pH level as low as 2 (Small *et al*., 1994).

Escherichia coli isolated from contaminated boar semen was found to provoke very strong agglutination of the sperm cells and had a negative effects on litter size. The contaminated semen with > 3.5×10^3 CFU/ ml of *Escherichia coli* was not be recommended to use for artificial insemination (Martín *et al*., 2010). Moreover, *Escherichia coli* -to-sperm ratio of 1:1 has been identified as a threshold level for inducing agglutination and reducing motility of sperm (Diemer *et al*., 1996). *Escherichia coli* adheres to the sperm surface through mannose-binding structures. This receptor-

specific inter-action leads to damage the sperm plasma membrane (Wolff *et al*., 1993; Monga and Roberts, 1994).

1.3.3 Application of antibiotics in boar semen and bacterial resistance

Antibiotics have been used as microorganism growth control in liquid-preserved boar semen. The antibiotic classes mostly present in porcine extenders are aminocyclitols (spectinomycin), aminoglycoside (gentamicin, strepomycin, amikacin and neomycin,), β-lactams (amoxicillin, penicillin and ampicilin), Polypeptides (polymixin B, colistin), macrolides (tylosin), fluoroquinolone (enrofloxacin) and lincosamides (lincomycin) (Althouse and Lu, 2005; Althouse, 2008). The drug-target interactions and associated cell death mechanisms, β-lactams and aminoglycosides, were shown in figure 1. β-lactams inhibit transpeptidation by binding to penicillin-binding proteins (PBPs) on maturing peptidoglycan strands. The decrease in peptidoglycan synthesis and increase in autolysins leads to lysis and cell death. Aminoglycosides bind to the 30S subunit of the ribosome and cause misincorporation of amino acids into elongating peptide (Figure 2). These mistranslated proteins can misfold and incorporation of misfolded membrane proteins into the cell envelope leads to increased drug uptake. This, together with an increase in ribosome binding, has been associated with cell death (Kohanski *et al*., 2010).

Despite the addition of antibiotics to semen extenders, the presence of microorganisms in semen has been reported. Previous research indicated that both gram bacteria include six species of gram negative from Enterobacteriaceae and Pseudomonadaceae family and three species of gram positive from *Staphylococcus* spp. and *Streptococcus* spp. presented in samples of boar semen ranged from 80 to 370×10^6 CFU/ml after preservation at 16 ºC for 5 days. It is shown in Table 2 (Gaczarzewicz *et al*., 2016). In addition, bacterial contaminants was found to be resistant to aminoglycoside gentamicin which was the most common preservative antibiotic used in commercially available porcine semen extenders (Table 3) (Althouse *et al*., 2000).

Table 1 Common bacterial contamination isolated from boar semen

Althouse and Lu. (2005)	Bresciani *et al*. (2014)	Gaczarzewicz *et al*. (2016)
E. coli	*E. coli*	*Pseudomonas* spp.
Pseudomonas spp.	*S. marcescens*	*P. aeruginosa*
Enterococcus spp.	*Staphylococcus* spp.	*P. fluorescens*
A. xylosoxidans	*S. epidermidis*	*Streptococcus* spp.
S. maltophilia	*S. aureus*	*E. coli*
Enterococcus spp.	*Proteus* spp.	*Bacillus* spp.
P. rettgeri	*Streptococcus* spp.	*Staphylococcus* spp.
B. cepacia	*P. aeruginosa*	*Proteus* spp.
E. cloacae		*Enterobacter* spp.
A. lwoffi		
A. xylosoxidans		
S. marcescens		
Corynebacterium spp.		
P. multocida		
P. mirabilis		
Streptococcus suis		

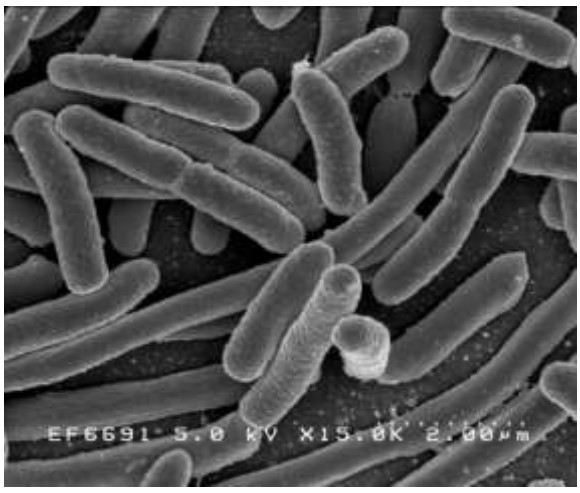

Figure 1 Image of *Escherichia coli* using scanning electron microscope (*Escherichia coli*) NIAID/Rocky Mountain Laboratories

Source: from http://en.wikipedia.org/wiki/Image:*EscherichiaColi*_NIAID.jpg ---- *Escherichia coli*: Scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip. Credit: Rocky Mountain Laboratories, NIAID, NIH Source: [http://www2

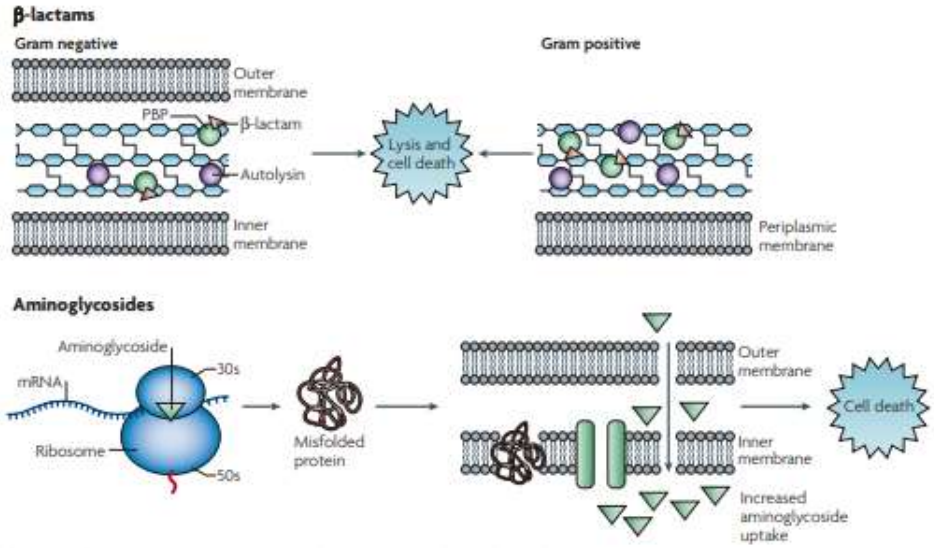

Figure 2 Drug-target interactions and associated cell death mechanisms.
Source: Kohanski *et al*. (2010)

Nevertheless, there were reported on the gentamicin-resistant bacteria isolated from extended semen of 16 of 24 AI boar centers in Germany and Austria (Schulze *et al*., 2015).

Recently, Keath *et al*. (2019) conducted antibiotic sensitivity with 15 strains of *Escherichia coli* isolated from boar semen and found that most bacteria were resistant to ampicillin (93.33%) and colistin (53.33%). Meanwhile, there were no any strains (0%) resisted to the other antibiotics (Ceftazidime, Cerfotaxime, Imipenem nor Meropenem). Bacteria produce enzyme protein which can develop resistant antibiotics such as β-lactam and aminoglycosides. The major mechanisms of β-lactam and aminoglycosides resistance are the production of β-lactamases and aminoglycoside-modifying enzymes, respectively (Lin *et al*., 2015).

1.3.4 Antimicrobial peptides

Antimicrobial peptides, have a molecular weight under 25-30 kDa with short sequences of 10-50 amino acids, are one of the most widely research for alternative conventional antibiotics from animal production (Sugiarto, and Yu, 2004; Wang and Mishra. 2012). They are produced by various organisms and all classes of fundamental difference existing between prokaryote and eukaryotic cells such as

mammals, arthropod, plants and microorganism. Interestingly, they also have rapid action, broad spectrum of activities against gram-negative and positive bacteria, viruses, fungi, and parasites and major sustain of the innate immune systems for most living organisms (Hancock and Sahl, 2006; Yeung *et al.*, 2011).

The structures and categories of antimicrobial peptides

AMPs are oligopeptides which are available composition of amino acids and number of amino acids (Bahar and Ren, 2013). Antimicrobial peptides can divided into many types depend on different structures, derived sources, and biological activities, based on the structure of amino acids. AMPs are classified into four different structures including alpha-helical peptide, beta-sheet peptide, extended peptide and non-alpha-beta peptide (Figure 3). Alpha-helical peptide is a type of peptide in alpha family mostly consists of Leucine and Lysine (major hydrophobic amino acid and charged amino acid), respectively Magainin 2, LL-37, Bovine lactoferrampin (Wang and Mishra, 2012).

For the beta-sheet peptide, it belongs to beta family which are dominated by Cysteine (polypeptide fold) especially prefer Arginine as the charged amino acid (Bovine Lactoferricin, Protegrin 1, Human Beta-defensin-3). The third family is extended peptides which have a higher presence of Cysteine as hydrophobic component and the same amount of Arginine, Lysine, glycine, proline, tryptophan, arginine (RRWQWR, Tritrpticin, Indocidin). The last family is non-alpha-beta structure (Wang and Mishra, 2012; Sugiarto and Yu, 2004; Hancock *et al.*, 2006). According to Bahar and Ren (2013), different sources of the antimicrobial peptides were also divided into 5 types including mammanlian AMPs (defensin), amphibian AMPs (magainins), insect AMPs (cercropin), plant AMPs (thionin), and microbial AMPs (gramicidin and nisin) (Xiao *et al.*, 2015).

Table 2 Median minimum- maximum (CFU/ml) of total aerobic bacteria (mean ± SEM

	Median Minimum-Maximum (CFU/ml)	Mean ± SEM
Total aerobic bacteria	7.70×10^3 $80\text{-}370.00\times10^6$	$14.52\times10^6 \pm 5.84\times10^6$
Bacillus spp.	90.00×10^3 $10.00\text{-}220.00\times10^6$	$36.07\times10^6 \pm 17.09\times10^6$
Enterobacter spp.	210.00 $20.00\text{-}27.00\times10^3$	$2.80\times10^3 \pm 1.38\times10^3$
E. coli	670.00 $10.00\text{-}10.00\times10^6$	$0.75\times10^6 \pm 0.60\times10^6$
Proteus spp.	260.00 10.00-730.00	313.33 ± 122.03
Pseudomonas aeruginosa	3.60×10^3 $1.00\times10^3\text{-}5.30\times10^3$	$3.37\times10^3 \pm 1.13\times10^3$
Pseudomonas fluorescens	7.10×10^3 $140.00\text{-}2.60\times10^3$	$0.47\times10^6 \pm 0.24\times10^6$
Pseudomonas spp.	700.00 $4.00\text{-}1.00\times10^6$	$4.46\times10^6 \pm 3.25\times10^6$
Staphylococcus spp.	590.00 $14.00\text{-}1.00\times10^6$	$21.59\times10^3 \pm 18.51\times10^3$
Streptococcus spp.	360.00 $10.00\text{-}100.00\times10^3$	$5.91\times10^3 \pm 2.53\times10^3$

Source: Gaczarzewicz *et al*. (2016)

Table 3 Antibiotic resistance with isolated bacteria from 60 samples of boar semen

Antibiotic	*E. coli* (n=20)	*S. marcescens* (n=6)	*Streptococcus* spp. (n=2)	*S. aureus* (n=2)	*S.epidermidis* (n=8)	*P. mirabilis* (n=4)	*Pseudomonas* spp. (n=1)
Amikacin (30 µg)	10 (50%)	4 (66.6%)	0 (0%)	0 (0%)	1 (12.5%)	1 (12.5%)	0 (0%)
Ampicillin (25 µg)	15 (75%)	5 (83.3%)	0 (0%)	1 (50%)	3 (37.5%)	3 (75%)	1 (100%)
Aztreonam (30 µg)	11 (55%)	3 (50%)	0 (0%)	1 (50%)	0 (0%)	1 (12.5%)	1 (100%)
Cefazolin (30 µg)	11 (55%)	5 (83.3%)	0 (0%)	1 (50%)	3 (37.5%)	3 (75%)	1 (100%)
Ceftiofur (30 µg)	4 (20%)	4 (66.6%)	0 (0%)	1 (50%)	1 (12.5%)	0 (0%)	1 (100%)
Cefquinome (30 µg)	7 (35%)	5 (83.3%)	0 (0%)	0 (0%)	3 (37.5%)	3 (75%)	0 (0%)
colistin (10 µg)	19 (95%)	6 (100%)	1 (50%)	2 (100%)	7 (87.5%)	4 (100%)	1 (100%)
Gentamicin (10 µg)	14 (70%)	3 (50%)	1 (50%)	2 (100%)	4 (50%)	2 (50%)	0 (0%)
Oxitetracyclin (30 µg)	17 (85%)	5 (83.3%)	0 (0%)	1 (50%)	3 (37.5%)	4 (100%)	1 (100%)
Penicillin G (10 µg)	17 (85%)	5 (83.3%)	0 (0%)	1 (50%)	3 (37.5%)	4 (100%)	1 (100%)
Streptomycin (10 µg)	17 (85%)	5 (83.3%)	0 (0%)	1 (50%)	3 (37.5%)	3 (75%)	1 (100%)
Tiamulin (30 µg)	20 (100%)	6 (100%)	1 (50%)	2 (100%)	7 (87.5%)	4 (100%)	1 (100%)
Tylosin (30 µg)	20 (100%)	6 (100%)	2 (100%)	2 (100%)	7 (87.5%)	4 (100%)	1 (100%)

Source: Adapted from Bresciani *et al*. (2014)

For biological activities, AMPs are also classified into 4 groups as antiviral peptides (defensins and NP-1), antibacterial peptides (nisin and pyrrhocoricin), antifungal peptides, and antiparasitic peptides (Bahar and Ren, 2013).

Multiple functions of antimicrobial peptides

The original and primary functions of AMPs were proposed to be direct antimicrobial activity against bacteria, fungi, parasites and viruses (Hancock and Rozek, 2002). AMPs are small proteins which have broad-spectrum antimicrobial and immunomodulatory properties (Figure 4) (Frew and Stock, 2011). For instance, microcins are classes of gene-encode low molecular-mass, contributing to microbial competitions within the intestinal microorganisms (Duquesne *et al*., 2007).

1.3.5 Mechanism of antimicrobial peptides

Antibacterial activities

The action of AMPs with target cells is based on cell surface and composition of amino acids (Guilhelmelli *et al*., 2013). According to Xiao *et al.* (2015) also demonstrated that there are two types of AMPs' mechanism including membrane-active and active intracellular. To kill bacteria, AMPs works through several mechanisms such as the modification of membrane permeability, depolarization of membrane ion gradients and the degradation of nucleic acids (Duquesne *et al*., 2007). For cationic AMPs act only by disrupting the integrity of the bacteria membrane as revealed through one of four proposed models including barrel-stave, aggregate, carpet and toroidal pore (Powers and Hancock. 2003; Hallock *et al*., 2003). Antimicrobial peptides result in deportation of metal, destroying the outer membrane, and facilitating the additional molecule from the exterior.

Moreover, peptides also get successful in accessing to the periplasmic space and integrating into cyto-plasmic membrane of bacteria (Zasloff, 2002). According to Friedrich *et al*. (2000), AMPs have been proposed and widely believed that the peptides disrupt the cytoplasmic membrane that lead to the dissolution of the proton motive force and leakage of essential molecules, resulting in cell death.

Antifungal activities

In addition, antimicrobial peptide can exert strong antifungal activity and could be a potential in addressing fungal infections. Their mechanism involved in fungal cell lysis and interference with cell wall synthesis. For instance, Cathelicidin peptides (synthetic) include SMAP-29, BMAP-27, BMAP-28, Protegrin-1 and indolicidin, rapidly damaged *Candida albicans* and *Cryptococcus neoformans*.

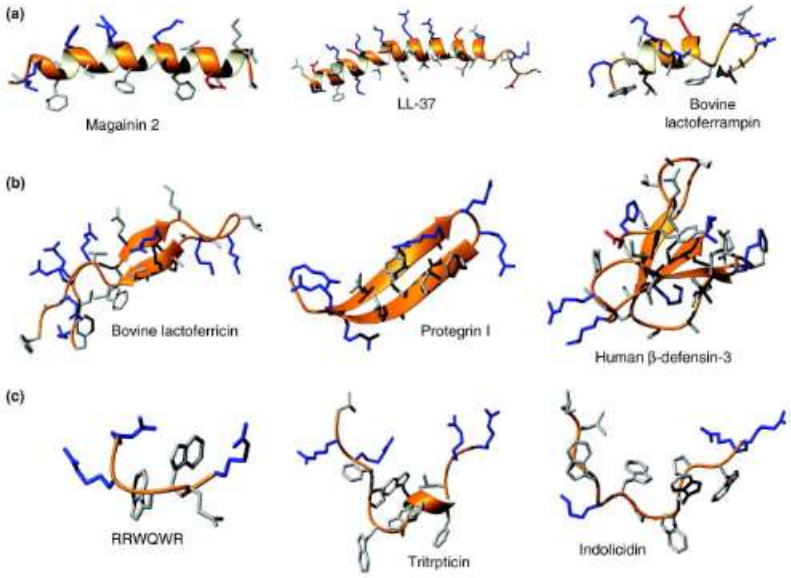

Figure 3 Structure of the peptides

Source: Nguyen *et al*. (2011)

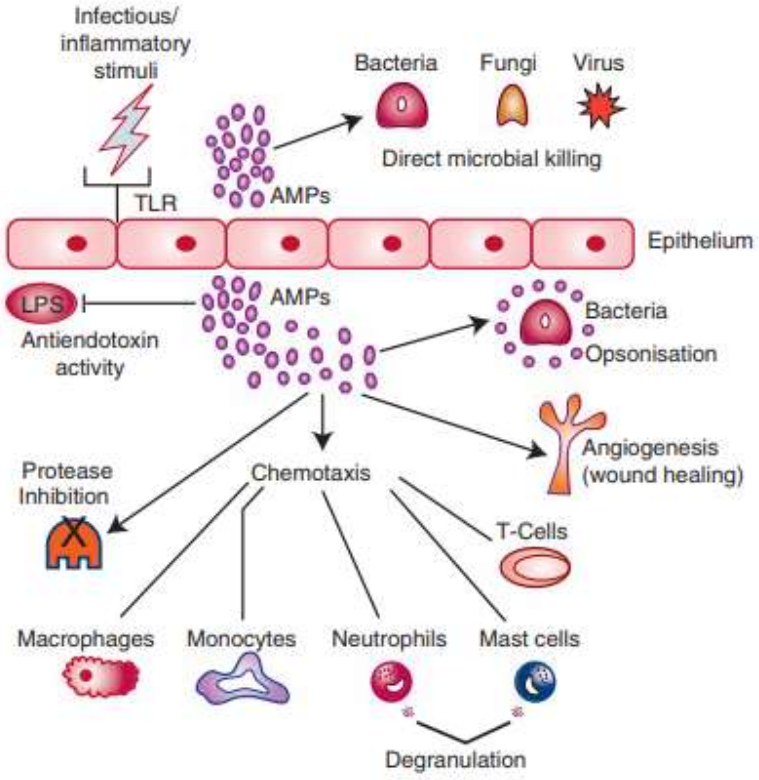

Figure 4 Multiple function of peptides

Source: Frew and Stock. (2011)

1.3.6 The application of AMPs

Application on bacterial contamination in boar semen

The previous research therefore indicated the effects of two antimicrobial peptides include synthetic cyclic hexapeptide (c-WWW 2 µM, c-WFW, 4 µM) and one synthetic helical magainin II amide derivative (MK5E) by preservation boar semen at 16 ºC for 4 days (Table 4). As a result, cyclic hexapeptide 2 µM (c-WWW) was able to achieve normal fertility rate after artificial insemination (Speck et al., 2014). In addition, the study of Puig-Timonet *et al*. (2018) indicated that 3 µM defensins-1 and -2 had more effective for 10 days boar semen preservation at 17 °C.

Applications on multiple drug resistant bacteria

According to Costa *et al*. (2018) indicated that synergic effect of pelgipeptins could inhibit drug resistant *K. pneumoniae*, *S. aureus* ATCC 14458,

Escherichia coli ATCC 11229, *K. pneumoniae* ATCC 13883, and two multi-drug resistant strains (MDR) including *K. pneumoniae* LACEN 3259271 and *Escherichia coli* LACEN 3789319 (all strains were isolated from patients' blood sample from Brasilia and Brazil). As a result, synergic effects of Pelgipeptin A-D were sensitive with bacteria but no synergic effect on the MDR strains between penicillin G and Pelgipeptin (Table 5).

Application of antimicrobial peptide as growth and health promoters

Xiao *et al*. (2015) reported that types of AMPs indicated positive effect on performance, nutrient digestibility, intestinal morphology, fecal microflora in swine production, were mentioned as A3, P5, Colicin E1, Cecropin AD, and CipB-lactoferricin-lactoferrampin (Table 6). Similarly, previous research about dietary supplementation of synthetic peptides reported that pig diets supplemented with AMP-A3 and P5 increased in the evident tract digestibility (Yoon *et al*., 2013).

In addition, the bacteriocin has a broad inhibitory spectrum that can inhibit members of gram-positive bacteria. Furthermore, the synthesized bactericidal peptides were produced by lactic acid bacteria (LAB) in general such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Sang and Blecha, 2008; Hammami *et al*., 2010; Lagha *et al*., 2017). For instance, garvicin KS, a new bacteriocin produced by *Lactococcus garvieae* which was combined with polymyxin B can against *Acinetobacter* spp. and *Escherichia coli* (Chi and Holo, 2018). According to Laukova *et al*. (2000). Bacteriocin could decrease the numbers of bacteria in waste water and manure, including CBE 24 could be used to manage animal excrement and waste water. Antimicrobial peptide Lactoferrin also reduced the total viable counts of *Escherichia coli and Salmonella*, and increased the *Lactobacillus* and *Bifidobacterium* (Wang *et al*., 2016).

Pharmaceutical applications

Beside of animal production, cationic antimicrobial peptides including gramicidin S has been used in the clinic especially as topical over-the-counter medicines (Hancock and Sahl, 2006). Based on a review by Zhang and falla, 2006, and

updated by Hancock *et al*., (2006) revealed that several peptides were improved through pharmaceutical applications in private company such as hLF-1-11 (Lactoferrin) which were used for Allogeneic bone marrow stem, HB-50 (Cecropin) uses for anti-infective, and HB-107 (19-amino-acid fragment of Cecropin B) uses for wound healing. There are 65 separate peptide products on the market. The distribution of the peptide sizes, in terms of number of amino acids, are presented in the Figure 5. For future developments, the size of peptide will be bridge up to 50 amino acids using a various technologies including mimicking elements of secondary structures in proteins (Vlieghe *et al*., 2010).

1.3.7 Disadvantages of antimicrobial peptides

Though, the antimicrobial peptides have potential for broad-spectrum activity, rapid bactericidal activity and low propensity for resistance development in the clinical application. On the contrary, they have some disadvantages including high cost, limited stability (composed of L-amino acids), and unknown toxicology and pharmacokinetics (Marr *et al*., 2006). For instance, Saar *et al*. (2005) investigated membrane toxicity of five peptides with well-documented cell-penetrating properties including pAntp(43–58), pTAT(48–60), pVEC(615–632), model amphipathic peptide (MAP), and transportan 10, on two human cancer cell lines, K562 (erythroleukemia) and MDA-MB-231 (breast cancer), as well as on immortalized aortic endothelial cells. The results showed that the higher membrane toxicity of MAP and transportan 10 compared with pAntp(43–58), pVEC(615–632), and pTAT(48–60).

Table 4 Antimicrobial peptide used to control bacterial contamination

Bacteria	MIC (µM) determined for			MIC (µg/mL) determined for gentamicin when combined with	
	c-WFW	c-WWW	MK5E	c-WWW (2 µM)	MK5E (1 µM)
E. coli ATCC 25922	6.3-12.5	50	25-50	0.6–0.7	0.6–0.8
E. coli DH5α	6.3	12.5-25	25-50	0.3–0.5	0.2–0.5
E. coli (hemolytic)	6.3-12.5	50	25-50	0.8–0.9	0.9
E. cloacae	25	25	25	0.2–0.4	0.3–0.4
K. pneumoniae	12.5-25	25-50	50	0.4–0.7	0.5–0.6
P. myxofaciens	>100	>100	>100	0.7–0.9	0.7–0.9
P. vulgaris	>100	>100	>100	0.6–0.8	0.5–0.8
B. subtilis DSM 347	6.3	6.3	6.3-12.5	0.05–0.1	0.1
S. aureus ATCC 29213	25	50	>100	0.6–0.7	0.5–0.6

Source: Speck *et al*. (2014)

Table 5 Minimum inhibitory concentration of Pelgipeptin A-D (µg/ml)

Bacteria	PelgipeptinA	PelgipeptinB	PelgipeptinC	PelgipeptinD
E. coli ATCC 11229	8	32	32	8
E. coli LACEN 3789319	16	16	8	8
S. aureus ATCC 14458	128	16	32	16
K. pneumoniae ATCC 13883	8	64	64	8
K. pneumoniae LACEN 3259271	8	32	16	16

Source: Costa *et al*. (2018)

Figure 5 Size distributions of peptide drugs on the market in 2010

Source: Vlieghe *et al*. (2010)

Table 6 Summary of studies on the applications of AMPs in swine nutrition

Antimicrobial	Application effects	References
Antimicrobial peptides-A3, 60 or 90 mg/kg	Effects on performance, total tract apparent digestibility of nutrients, intestinal morphology and intestinal and fecal microflora	Yoon *et al.* (2012) Cited by Xiao *et al*. (2015)
Antimicrobial peptide-P5 (AMP-P5); 40 or 60 mg/kg	Improves the performance and apparent total tract digestibility of nutrients and reduces coliforms	Yoon *et al.* (2012) Cited by Xiao *et al*. (2015)
Synthetic antimicrobial peptide-A3 or P5 (AMP-A3 and P5); 60 mg AMP-A3 or 60 mg AMP-P5/kg	Improves the performance, nutrient digestibility, intestinal morphology and to reduces pathogenic bacteria	Yoon *et al.* (2014**)** Cited by Xiao *et al*. (2015)
Antimicrobial peptide colicin E1; 11 or 16.5 mg/kg	Improves the performance, reduces incidence of post weaning diarrhea	Cutler *et al*. (2007) Cited by Xiao *et al*. (2015)
Antimicrobial peptide cecropin AD; 400 mg/kg	Enhances pig performance through increasing immune status and nitrogen and energy retention as well as reducing intestinal pathogens	Wu *et al*. (2012) Cited by Xiao *et al*. (2015)

Antimicrobial	Application effects	References
cipB-lactoferricin-lactoferrampin (cipB-LFC-LFA); 100 mg/kg	Improves performance through an antibacterial effect, the regulation of immune function, improvement of the absorption of Fe and a reduction in the incidence of diarrhea	Tang *et al.* (2009) Cited by Xiao *et al*. (2015)
Recombinant Lactoferrampin-Lactoferricin; 100 mg/kg	Improves performance and affects serum parameters	Tang *et al.* (2012) Cited by Xiao *et al*. (2015)
Composite antimicrobial peptides (CAP, consist mainly of antibacterial lactoferrin peptides, along with plant defensins and active yeast); 400 mg/kg	Improves feed efficiency, immune function, and antioxidation capacity and alleviates organ damage	Xiao *et al*. (2013) Cited by Xiao *et al*. (2015)
A mixture of lactoferrin, cecropin, defensin, and plectasin	Improves performance, reduces the incidence of diarrhea, and increases the survival rate of weaned pigs	Xiong *et al*. (2014) Cited by Xiao *et al*. (2015)

Source: Adapted from Xiao *et al*. (2015)

CHAPTER II
MATERIALS AND METHODS

As mention above, the results obtained from previous studies showed 9 antimicrobial peptides (Sam1, Sam2, Sam3, Sam4, Sam5, Sam6, Sam7, Sam8 and Sam9) can inhibit *Escherichia coli* ATCC 25922. For this study, we continued to assess the antimicrobial activity of these 9 peptides on *Escherichia coli* isolated from boar semen.

In this study, isolation and identification of bacterial contaminants were conducted. The antimicrobial susceptibility test of *Escherichia coli* isolated from boar semen was then performed. The distribution of clonal relatedness of *Escherichia coli* isolated from boar semen was analyzed. Especially, this study assessed the antimicrobial activity of peptides on one strain from each clonal relatedness group of *Escherichia coli*. The investigation was performed as the following flowchart in figure 6.

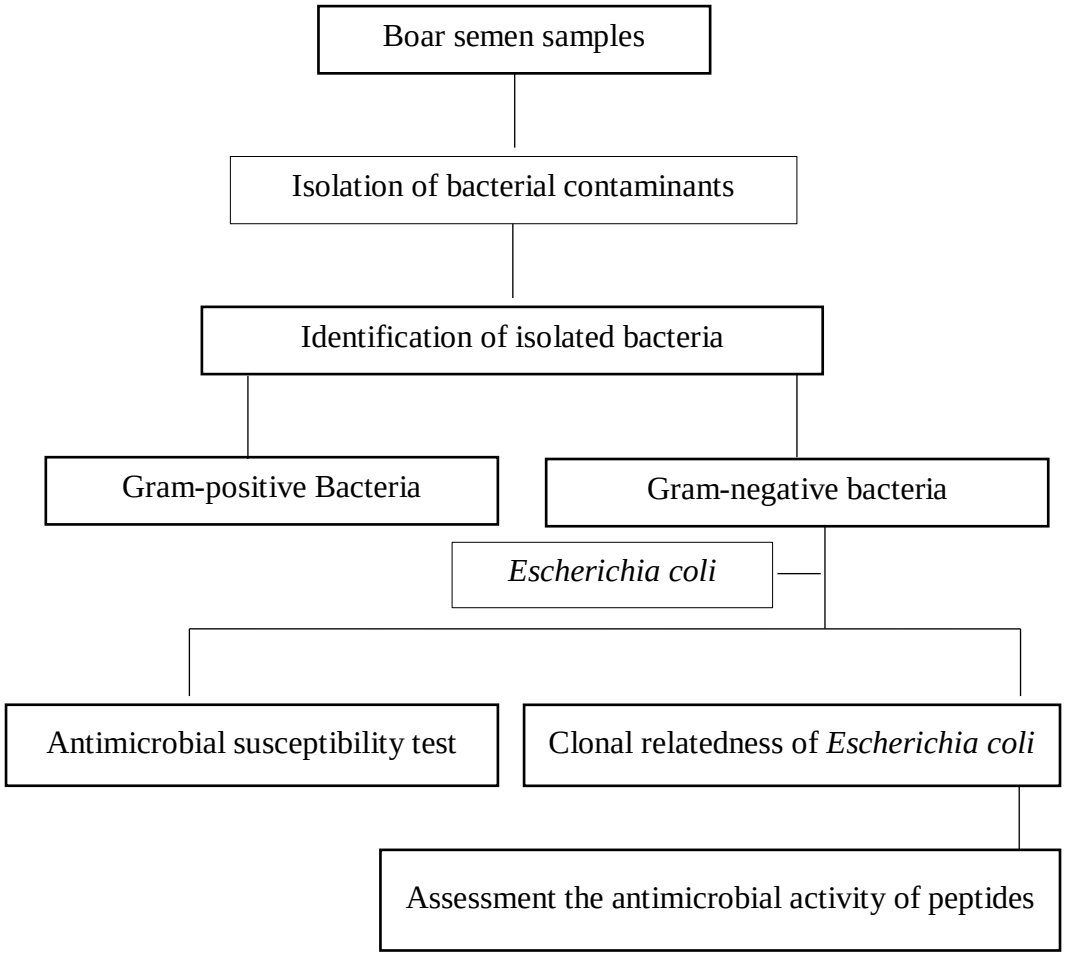

Figure 6 Flowchart of experiment procedure

2.1 Preparation of antimicrobial peptides

The sequence of 9 peptides was sent to the manufacturer (GenScript, Leiden, Netherlands) to synthesize. The 8 peptides could be synthesized by the manufacturer while another peptide could not be synthesized. Thus, the 8 peptides were synthesized as powder and were used in the study. The concentration of 200 µg/ ml of each peptide was prepared by dissolving with 100% Dimethyl Sulfoxide (DMSO) and stored at -20 °C.

2.2 Isolation and identification of bacterial contaminants in boar semen

2.2.1 Semen samples

Semen samples were provided by 9 different private pig farms. Ten fresh semen samples (from 10 boars) were collected from a farm located in the southern Thailand and were transported on ice. The other 8 pooled semen samples were collected from the 8 farms located in the central region of Thailand and were kept in transported media before transportation. The gloved hand technique was used for semen collection and filter to remove the bulbo-urethral gland gel secretion. After collection, the samples were sent to laboratory for isolation of bacterial contamination.

2.2.2 Isolation of the bacterial contaminants

The bacterial contaminants were isolated from all semen samples using direct method by plating on the different three agar media including Mac Conkey Agar (MCA), Manitol Salt Agar (MSA), Luria-Bertani Agar (LBA) (Martin *et al*., 2010 and Kateete *et al*., 2010). For colony count, bacteria were plated on each agar media by preparing 5-fold serial dilutions. After incubation at 37 ºC for 24 h and 48 h, the colony was counted and calculated as colony forming units per milliliter (CFU/ ml).

2.2.3 Identification of isolated bacteria

Isolated bacteria were identified using standard microbiological procedures. The gram staining and catalase test was used for identification of gram-positive bacteria, likewise, biochemical test and MALDI-TOF were used for gram-negative bacterial identification. In brief:

2.2.4 Catalase test

Small amount of isolated bacteria, colony of bacteria growing on the MSA media, was mixed to a drop of 3% of hydrogen peroxide on the clean slide. After 3-5 second, the result was obtained by observing the bubble (positive) and no bubble (Negative) comparing to positive control *Staphylococcus* spp. and negative control *Streptococcus* spp. (M'leod and Gordon, 1923; Reiner, 2010).

2.2.6 Biochemical test

Gram-negative bacteria identification, single colony of bacteria growing on the MCA media was selected for purification using TSA and test for indole, Methyl Red, Voges-Proskauer, citrate utilization, hydrogen sulfide, urea hydrolysis, lysine decarboxylase, motility, gas from glucose, lactose fermentation, sucrose fermentation, and metallic sheen by following the standard biochemical test (Kovacs, 1956; Lowrance *et al*., 1969; Sutter and Carter, 1972; Miller and Wright, 1982; Leclercq *et al*., 2001). In addition, Enterobacteriaceae was analyzed on website Identification of Enterobacteriaceae members according to P.N.SridharRao (http://www.microrao.com/entero_ident.htm?fbclid=IwAR0IU_z9Q3lLwVQVU1F_wdNf0rjBHnPfwGmMrgafGqHrCyUvg6LwAzyJ9xg).

Gram staining

The bacterial isolates were stained with crystal violet on the grass slide for 1 min and washed with running water for 2 sec. After washing, one drop of gram's iodine was added on the bacterial staining, left for 1 min, and then washed with running water for 2 sec. To remove the color, acetone-alcohol was dropped on the slide until decolorizing. Finally, the slide was stained with safranin for 1 min and then washed with running water. The gram-positive bacteria were observed under microscope at 1000x magnification comparing to the standard bacteria of gram staining (Hucker and Conn, 1923).

Indole and hydrogen sulfide test

The formulation of Sim medium is designed to allow the detection of sulfide production, indole formation and motility. The colony of the bacteria was stabbed in the middle of the Sim media in the tube then inoculated for overnight. After incubation, hydrogen sulfide production is detected when ferrous sulfide, a black precipitate, is produced as a result of ferrous ammonium sulfate reacting with H_2S gas. Meanwhile, KOVAC's reagent was added 3-5 drops to test for indole. A positive indole test was determined by formation of red color on the top of the media within 2 s of adding reagent (Hemraj *et al*., 2013).

Methyl Red and Voges-Proskauer test (MR-VP)

This test is used to determine which fermentation pathway is used to utilize glucose. Four milliliter of the MR/VP broth with bacterial colony was cultured and 5-6 drops of a-naphthol and potassium hydroxide (KOH) were added after overnight incubation at 37 ^{0}C. The Voges-Proskauer test detects the presence of acetoin. Thus, 40% potassium hydroxide and 6% naphthol were used as a reagent for Voges-Proskauer test and Methyl Red test, respectively. The positive results were shown brownish-red to pink. In contrast, if the culture was turned to brownish-green to yellow, it was negative for acetoin.

Citrate utilization test

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen. The colony of bacteria was cultured on the agar and incubated at 37 ^{0}C for overnight. The positive result was indicated by turning from green to blue color of the media.

2.2.7 MALDI-TOF MS

For MALDI-TOF MS analysis, samples were prepared using a single colony of fresh sample, cultured with Tryptic Soybean Agar for overnight. Classification was followed the formic acid extraction procedure and measured by using Microflex III instrument the automatic acquirement bases on the linear positive mode ranging from 2–20 kDa (Bruker Daltonik, Bremen, Germany). Then colonies were analyzed by MALDI-TOF MS Run Identifier: 190703-2120. The MALDI-TOF identifies bacteria species by comparing their mass spectral protein pattern to the reference expression patterns in a database (Blondiaux *et al*., 2010).

In addition, the closeness of the match to the reference is reflected in score values $P \geq 2.0$ can be considered as a possible identification. For instance, 2.00-3.00 is high confidence identification, 1.70-1.99 is low confidence identification, and 0.00-1.69 is no organism identification possible (Alvarez-Buylla *et al*., 2012).

2.2.8 Stock of bacteria isolated from boar semen

To make a bacterial stock, the purification colony was grown in 500 µl of Tryptic Soybean Broth (TSB) and incubated by shaking incubator at 150 rpm and 37ºC for overnight. Then the bacterial suspension was re-suspended in 40% glycerol solution, stored at -80 ºC for further antibiotic susceptibility test and antimicrobial activity of synthetic peptides.

2.3 Antimicrobial susceptibility testing of *Escherichia coli* isolated from boar semen

2.3.1 Bacterial suspension

The colony of each *Escherichia coli* was streaked on the Tryptic Soybean Agar (TSA) and incubated at 37ºC for overnight. One single colony was picked from TSA media and added into 4 ml of Mueller–Hinton Broth (MHB), then incubated at 37ºC for 3 h. The bacteria suspension was prepared to get the final concentration at 1 x 10^8 CFU/ml with 0.85% normal saline measuring by 0.5 McFarland turbidity standard.

The samples of *Escherichia coli* ATCC 25922 and *Escherichia coli* isolated from boar semen were tested with ampicillin (AMP), ceftazidime (CTX), penicillin G (PEN), gentamicin (GEN) and amikacin (AMK) by performing the disk-diffusion method whereas polymycin E (colistin) was conducted by broth microdilution according to CLSI recommendation.

2.3.2 Disk-diffusion method

The antibiotics were selected from family of β–lactams (10 µg ampicillin and 10 µg penicillin G), aminoglycosides (10 µg gentamicin and 30 µg amikacin) and carbapenems (30 µg ceftazidime). The bacterial suspension was spread on the MHA by sterile cotton swap and then antibiotics disk were plated on the media and incubated at 37 °C. Finally, the result was read after 16 or 18 h of incubation and measured the inhibition zone diameters by Vernier Caliper based on Criteria of CLSI (CLSI, 2016).

2.3.3 Minimum Inhibitory Concentration

The disk diffusion method for polymyxins (colistin and polymycin B) are inadequate for use as the only screening test for susceptibility. It should be confirmed with MIC measurement by E-test or broth dilution method (Tan and Ng, 2006). So the recent study of antibiotic resistant colistin sulfate was designed by broth microdilution method and result obtained from minimum inhibitory concentration of colistin sulfate was compared to the MIC quality control (QC) which was given by CLSI guideline.

A total of isolated *Escherichia coli* were investigated on colistin resistance by using a broth microdilution. Colistin was diluted with DMSO to be 128 µg/ml and 100 µl of two fold serial dilutions was prepared into 96 wells plates. Bacterial suspension was prepared to be 10^6 CFU/ml. An equal volume of bacterial inoculum was mixed with colistin into 96 wells plates. Two control groups were included (1) positive control (bacteria + water) and (2) negative control (MHB + water). The 96 wells plate was incubated at 37 °C for 18-24 hours. The breakpoint was determined as the lowest concentration of the antibiotic can inhibit the visible growth of the inoculum (CLSI, 2016).

2.4 Clonal relatedness of *Escherichia coli* isolated from boar semen

2.4.1 Bacterial suspension

The samples of *Escherichia coli* ATCC 25922 and isolated *Escherichia coli* were collected from the stock. The colony of each bacteria were streaked on the Tryptic Soybean Agar (TSA) and incubated at 37 ºC for overnight. One single colony was picked from TSA media and added into 4 ml of Mueller–Hinton Broth (MHB), then incubated at 37ºC for 3 hours.

2.4.2 DNA Extraction

A single colony was cultured in 1 ml of Tryptic soy broth (TSB) and inoculated in incubator shaker at 37 ^{0}C, 150 rpm for overnight. One ml of bacterial culture was centrifuged at 10000 rpm for 2 min and then supernatant was discarded.

Genomic DNA of each *Escherichia coli* strain was extracted using DNA extraction kit following the manufacturers' instructions (PrestoTM Mini gDNA Bacteria Kit).

Briefly, the pellet of bacteria was added with 180 µl GT buffer and 20 µl of proteinase K. The tube was heat at 60 ^{0}C for 10 mn by invert the tube every 3 min. After that, 200 µl of GB buffer was added into the tube (by invert the tube every 10 sec) and then heated again for 10 min (invert the tube in each 3 min). For DNA binding, 200 µl of absolute ethanol was adjusted in the tube (mixed) and placed to GD column in a 2 ml collection tube then centrifuged at 12000 rpm for 2 min. The supernatant was discarded and moved GD column to the new collection tube. For washing step, 400 µl of W1 was added, centrifuged at 12000 rpm for 30 min. Supernatant therefore was discarded and 600 µl of wash buffer was added into GD column, centrifuged at 12000 rpm for 30 min. The supernatant was discarded and centrifuged again at 12000 rpm for 3 min to dry the GD column. GD column was moved to 1.5 ml tube. Finally, 30 µl of deionized water was added and centrifuged at 12000 rpm for 30 sec to get genomic DNA (PrestoTM Mini gDNA Bacteria Kit).

2.4.3 Box A1R PCR

Box A1R PCR was performed in a 25-µl reaction mixture consisting of 0.2 µM of BOX A1R primer (CTACGGCAAGGCGACGCTGACG), 5× My Taq reaction buffer (comprises 5 mM dNTPs, 15 mM of MgCl2), 0.25 units of GoTaq DNA polymerase, and 50 ng of DNA template. Thermal cycler condition was performed with an initial denaturation step (95 ºC for 3 mn) following by 35 cycles of denaturation at 94 ºC for 3 sec, annealing at 50 ºC for 1 mn, and extension at 65 ºC for 8 mn (Versalovic *et al*., 1994).

2.4.4 Gel electrophoresis

Thirteen microliters of the amplification product were mixed with 1 µl of loading dye and loaded into the wells. Three microliters of 1 kb of DNA ladder I was loaded into the first well as a standard comparison. Meanwhile, electrophoresis was run at 50 V in 1× TAE buffer for 1.5 h. Dendrogram (Box A1R genomic profiles) of *Escherichia coli* was constructed using unweighted pair-group method of arithmetic average (UPGMA), Bio-numeric program version 7.3.

2.5 Assessment of the antimicrobial activity of synthetic peptides on *Escherichia coli* isolated from boar semen

2.5.1 Determination of Minimum Inhibitory Concentration

One of isolated *Escherichia coli* trains, randomly selected, and *Escherichia coli* ATCC 25922 were preliminary investigated the minimum inhibitory concentration (MIC) with 2 peptides by using a broth microdilution. 100 µl of two fold dilution series (400 µg/ml) of antimicrobial peptide was prepared into 96 wells plates. An equal volume of bacterial inoculum was mixed with peptide dilution up to the final concentration of 5 x 10^5 CFU/ml. Two control groups were included (1) positive control (bacteria + DMSO) and (2) negative control (MHB + DMSO). The 96 wells plates was incubated at 37 °C for 18-24 hours. DMSO at a final concentration of 2.93 % used as control group. The breakpoint was determined as the lowest concentration of the antibiotic can inhibit the visible growth of the inoculum (CLSI, 2016).

2.5.2 Time-kill assay

The results obtained from MIC determination showed that 400 µg/ml of peptides could not kill the *Escherichia coli* ATCC 25922 and *Escherichia coli* strain isolated from boar semen. However, 100 µg/ml could inhibit bacterial growth after 24 h of OD measurement. Thus, 100 µg/ml of 8 peptides was tested the antimicrobial activity with 4 isolates of *Escherichia coli* by OD measurement at 0 h, 4 h, 8 h, 16 h, and 24 h after incubation (one replicate). Then the peptides which had the highest activity were selected to conduct time-kill assay by OD measurement and colony count (triplicate).

The bacterial suspension was adjusted to 1 ×10^8 CFU/ ml with Normal Saline Sterile (NSS) by using McFarland turbidity standard. To get concentration of bacteria 1×10^6 CFU/ ml, the diluted bacteria was diluted with MHB.

In this study, 200 µg/ ml of each peptide was prepared from stock solution. After that 100 µl of them was pipetted into 96 well-plates with triplication of each. Then, 100 µl of *Escherichia coli* ATCC 25922 and each *Escherichia coli* strain suspension was pipetted into the 3 wells of each peptide. Final concentration

of each peptide was 100 µg/ ml. The antibiotic Amikacin and MHB were used to be the positive and negative control, respectively. After 0, 4, 8, 16 and 24 h of incubation at 37 ^{0}C, OD_{600} measurement was conducted using Microplate reader. Bacteria colony was plated after measuring the OD_{600}. Ten micro-litter of the samples was diluted 10- folds serially diluted in NSS (90 µl) before plating on the cultural media (TSA) and incubated at 37 ºC for overnight.

The log of colony forming unit was plotted against time using Microsoft Excel 2013. The percentage of growth inhibition was calculated as following equation:

% Growth inhibition = 100 × (mean C- mean T)/ mean C

Where C is mean of colony count in negative control within time measurement and T is mean of colony count in treatments within time measurement.

The bacterial growth inhibition was accepted as ≥ 50% of inhibition.

CHAPTER III
RESULTS

3.1 Occurrence of bacteria isolated from boar semen

All samples (10 fresh and 8 pooled semen samples) were found to be contaminated with both gram-negative and gram-positive bacteria. *Escherichia coli* and *Staphylococcus* spp. were the most frequently species which isolated from boar semen. The other species were *S. marcescens* (n = 6, 33.33%), *K. pneumoniae* (n = 4, 22.22%), *E. cloacea* (n = 2, 11.11%), *C. koseri* (n = 2, 11.11%), *E. aerogenes* (n = 2, 11.11%), *Streptococcus* spp. (n = 2, 11.11%), *A. hydrophila* (n = 1, 5.55%), *P. stuartii* (n = 1, 5.55%), *P. rettgeri* (n = 1, 5.55%), *K. oxytoca* (n = 1, 5.55%), *K. aerogenes* (n = 1, 5.55%), *P. aeruginosa* (n=1, 5.55%), and *E. tarda* (n = 1, 5.55%). The result of bacterial identification isolated from boar semen is shown in Table 7. The amount of gram-positive and gram-negative bacteria isolated from fresh varied from 4.00×10^2 to 8.50×10^3 CFU/ ml respectively. The raw data of colony count of bacterial contamination isolated from boar semen is shown in table 8.

Identification of gram negative bacterial revealed 49, 24, 8, 6, 2, 2, 1, 1, 1, 1, 1 and 1 strain(s) of *Escherichia coli* , *S. marcescens, C. koseri, K. pneumoniae, E. cloacea, E. aerogenes, K. aerogene, A. hydrophila, P. stuartii, P rettgeri, E. tarda, K. oxytoca* and *P. aeruginosa* were identified, respectively. For gram-positive bacterial identification, 90 and 5 strains were *Staphylococcus* spp. and *Streptococcus* spp., respectively.

Table 7 Occurrence of bacterial identification isolated from 18 boar semen samples (10 fresh and 8 pooled semen samples)

Organism	Prevalence (N = 18)	
	Gram-negative bacteria	Gram-positive bacteria
E. coli	18 (100%)	
Staphylococcus spp.		18 (100%)
S. marcescens	6 (33.33%)	
K. pneumoniae	4 (22.22%)	
C. koseri	2 (11.11%)	
E. cloacea	2 (11.11%)	
E. aerogenes	2 (11.11%)	
K. aerogenes	2 (11.11%)	
Streptococcus spp.		2 (11.11%)
A. hydrophila	1 (5.55%)	
E. tarda	1 (5.55%)	
K. oxytoca	1 (5.55%)	
P. aeruginosa	1 (5.55%)	
P. stuartii	1 (5.55%)	
P. rettgeri	1 (5.55%)	

Table 8 Colony count of bacterial contamination isolated from fresh boar semen samples

Sample	Gram negative bacteria		Gram positive bacteria	
	Total bacteria/ sample (CFU/ ml)	Example of bacterial contamination	Total bacteria/ sample (CFU/ ml)	Example of bacterial contamination
1	1.67×10^3	*E. coli* *K. pneumoniae*	3.83×10^3	*Staphylococcus* spp.
2	1.67×10^2	*E. coli* *K. pneumoniae*	4.17×10^3	*Staphylococcus* spp.
3	1.67×10^2	*E. coli* *A. hydrophila*	4.00×10^2	*Staphylococcus* spp.
4	1.33×10^2	*E. coli* *E. cloacae*	4.67×10^3	*Staphylococcus* spp.
5	2.33×10^2	*E. coli*	6.00×10^2	*Staphylococcus* spp.
6	1.00×10^3	*E. coli*	4.50×10^3	*Staphylococcus* spp.
7	1.00×10^3	*E. coli* *P. rettgeri*	8.50×10^3	*Staphylococcus* spp.
8	1.17×10^3	*E. coli*	1.00×10^3	*Staphylococcus* spp.
9	3.83×10^3	*E. coli* *E. tarda*	5.67×10^3	*Streptococcus* spp. *Staphylococcus* spp.
10	4.17×10^3	*E. coli* *P. stuartii*	3.67×10^3	*Staphylococcus* spp.

3.2 Antibiotic susceptibility test of *Escherichia coli* isolated from boar semen

The susceptibility to antimicrobial agents was determined for all of *Escherichia coli* strains isolated from boar semen (n = 49). The result revealed that *Escherichia coli* were resistant to penicillin G (100%), ampicillin (97.96%) and gentamicin (12.24%) with the exception of amikacin and ceftazidime. Antibiotic susceptibility test of *Escherichia coli* isolated from boar semen is displayed in table 9.

Of 49 isolates, 3 (A05N13, B04N33 and B04N34) were resistant to colistin with MIC 4 µg/ml and 1 isolate (A04N10) was with MIC 16 µg/ml, respectively.

Table 9 Antimicrobial Susceptibility test with isolated *Escherichia coli* from boar semen

Antibiotics	Disc Content	Antimicrobial Susceptibility Testing (n = 49)		
		Susceptible	Intermediate	Resistant
Ampicillin	10 µg	1 (2.04%)	0 (0%)	48 (97.96%)
Ceftazidime	30 µg	49 (100%)	0 (0.00%)	0 (0.00%)
Penicillin G	10 µg	0 (0.00%)	0 (0%)	49 (100%)
Gentamicin	10 µg	37 (75.51%)	6 (12.24%)	6 (12.24%)
Amikacin	30 µg	49 (100%)	0 (0.00%)	0 (0.00%)

3.3 Clonal relatedness of *Escherichia coli* isolated from boar semen

All 49 strains of *Escherichia coli* were performed by BOX A1R to differentiate the DNA fingerprinting. The results showed that the 49 strains were very diverse and clonally related. According 75% similarity levels of clonal relatedness, 49 strains of *Escherichia coli* were distinguished into VIII clusters. At the cluster I, including B06N51, B06N52, B06N53, and B06N54 showed 100% similarity DNA profile which isolated from the same sample at the same time while B02N12 and B04N31 were showed 100% similarity DNA profile which were isolated from different samples at the same time. Interestingly, A06N14 and A07N19 at cluster III and A01N04 and A01N29 at cluster V were determined 100% similarity DNA profile in the different samples. In group IV, 100% similarity cluster of *Escherichia coli* strains were isolated from the same samples. The highest diverse group was found in group V. It was determined as the largest population comparing to others which obtained 21 isolates of *Escherichia coli*. After resulting to clonal relatedness, 100% similarly of DNA profile for the same samples and at the same time was cut off from the cluster analysis (13 strains). Thus, 36 strains were reanalyzed. The cluster analysis exhibited the identical DNA fingerprint among strains, which were isolated from different samples and different times were found in cluster I (B02N12 and B04N24), cluster III (A06N14 and A07N19) and cluster V (A01N04 and A10N29). These results showed that clonal relatedness of *Escherichia coli* strains isolated from the same sample at the same time were closer than *Escherichia coli* isolated from the different sample at the different time which dispersed in semen condition.

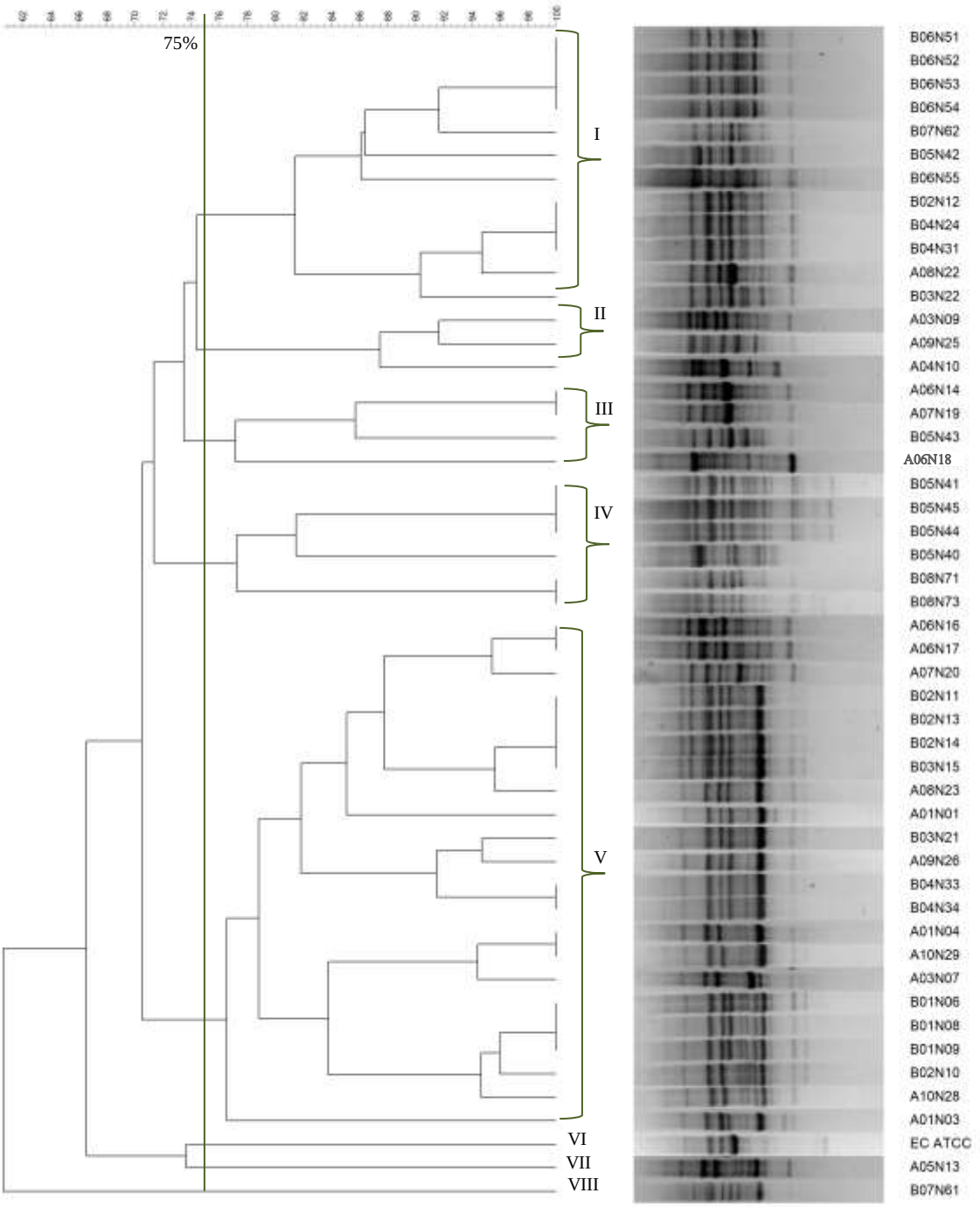

Figure 7 BOX-PCR-based dendrogram of 49 strains of *Escherichia coli* isolated from boar semen samples collected on 20th November 2018 and 4th February 2019, fresh semen and pooled semen, respectively. *Escherichia coli* surrogates are classified into VIII clusters, based on 75% similarity (analyzed by Bio-numeric software version 7.3).

3.4 Assessment of the antimicrobial activity of synthetic peptides on *Escherichia coli*

Minimum inhibitory concentration (MIC) of peptides

One of isolated *Escherichia coli* (B05N44) was randomly selected to investigate minimum inhibitory concentration with 2 randomly selected peptides (Sam1 and Sam5). The *Escherichia coli* ATCC 25922 was tested at the same time as a control group. The highest final concentration 400 µg/ml of Sam1 and Sam5 were determined MIC by using a broth microdilution. MIC of Sam1 and Sam5 for both strains were higher than 400 µg/ml (MIC > 400 µg/ml).

However, 100 µg/ml of these peptides therefore could reduce the value of optical density, based on OD_{600} measurement at 24h incubation. So 100 µg/ml of the 8 peptides were further investigated on their activity against time inhibition at 0h, 4h, 8h, 16h and 24h. The result showed Sam1, Sam4 and Sam9 were higher activity inhibit with *Escherichia coli* ATCC 25922 while Sam1, Sam2 and Sam5 were reported as the higher activity to inhibit *Escherichia coli* strains including A01N04, A06N16, B05N44 and B07N62 at 0, 4, 8, 16 and 24 h of incubation.

Time-kill assay

According to above results, 100 µg/ml of 3 peptides, Sam1, Sam4 and Sam9 were further tested with referent *Escherichia coli* ATCC 25922 (triplicate). It reported that Sam1 showed high inhibitory activity against *Escherichia coli* ATCC 25922. A percentage of bacterial growth inhibition of Sam1 was 99.99%, 99.99%, 99.99%, 99.99% at 4, 8, 16 and 24 h, respectively. Meanwhile, Sam4 was 87%, 89.59%, 96.63%, 98.14% and Sam9 was 70%, 71.43%, 91.81%, 97.57% at 4, 8, 16 and 24 h, respectively. Sam1, Sam4 and Sam9 could inhibit referent *Escherichia coli* ATCC 25922 were defined as ≥ 50% of inhibition. The growth curves of Sam1, Sam4 and Sam9 is shown in Figure 8.

Sam1, Sam2 and Sam5 were further tested with 4 representative of *Escherichia coli* strains selected from clonal relatedness of *Escherichia coli*. The results revealed that Sam1, Sam2, and Sam9 showed high activity with A01N04 (98.75%, 85%, and 87.5%, respectively), A06N16 (84.29%, 90.86%, and 95.71%, respectively),

B05N44 (99.88%, 95.21%, and 98.70%, respectively), and B07N62 (99.79%, 99.97%, and 80%, respectively). In addition, Sam5 showed high activity with A06N16 (96%, 91.11%, 95.2% and 95.71% at 4, 8, 16, and 24 h, respectively) compared to Sam1 and Sam2. The results of Sam1, Sam2 and Sam5 with the 4 strains reduced colony forming unit per milliliter of the *Escherichia coli* compared to negative control group at 0 h, 4 h, 8 h, 16 h and 24 h of incubation. Sam1, Sam2 and Sam5 could inhibit 4 isolates of *Escherichia coli* strains which were defined as ≥ 50% of inhibition. The growth curves of Sam1, Sam2 and Sam5 are shown in Figure 9, 10 and 11, respectively.

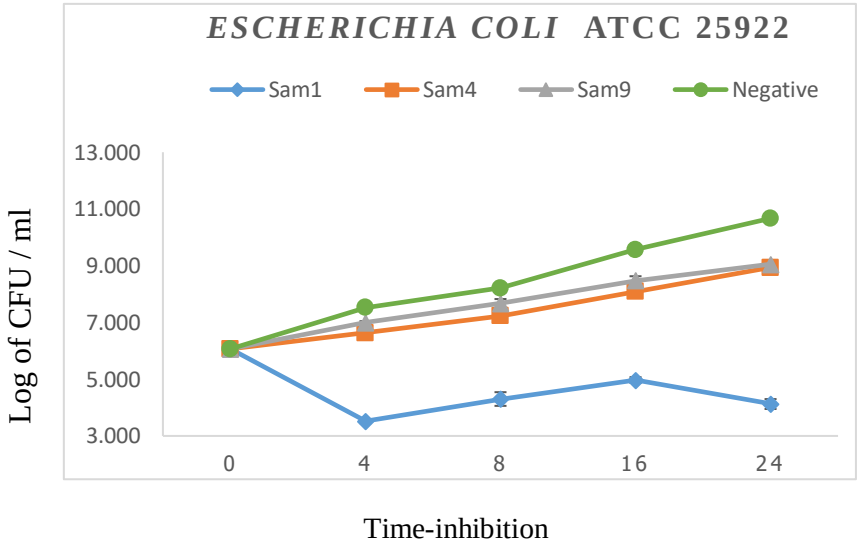

Figure 8 Growth curves of Sam1, Sam4, Sam9, and their combination against *Escherichia coli* ATCC 25922 monitoring using plate count. The colonies were counted at 0, 4, 8, 16 and 24 h of incubation at 37 ^{0}C after grown in MHB media and calculated to log of CFU/ ml.

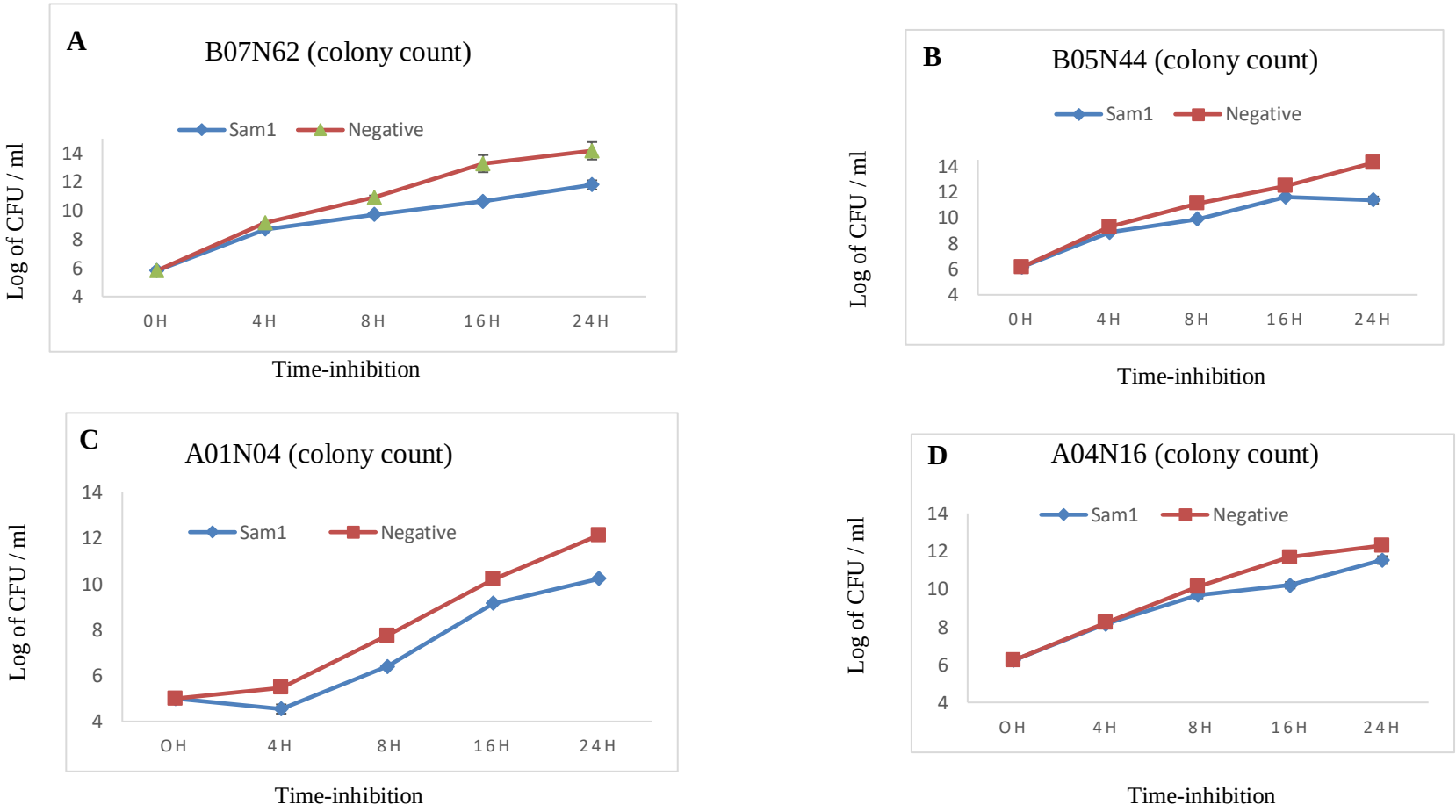

Figure 9 Growth curves of Sam1, Log CFU/ml, A (B07N62), B (B05N44), C (A01N04) and D (A04N16)

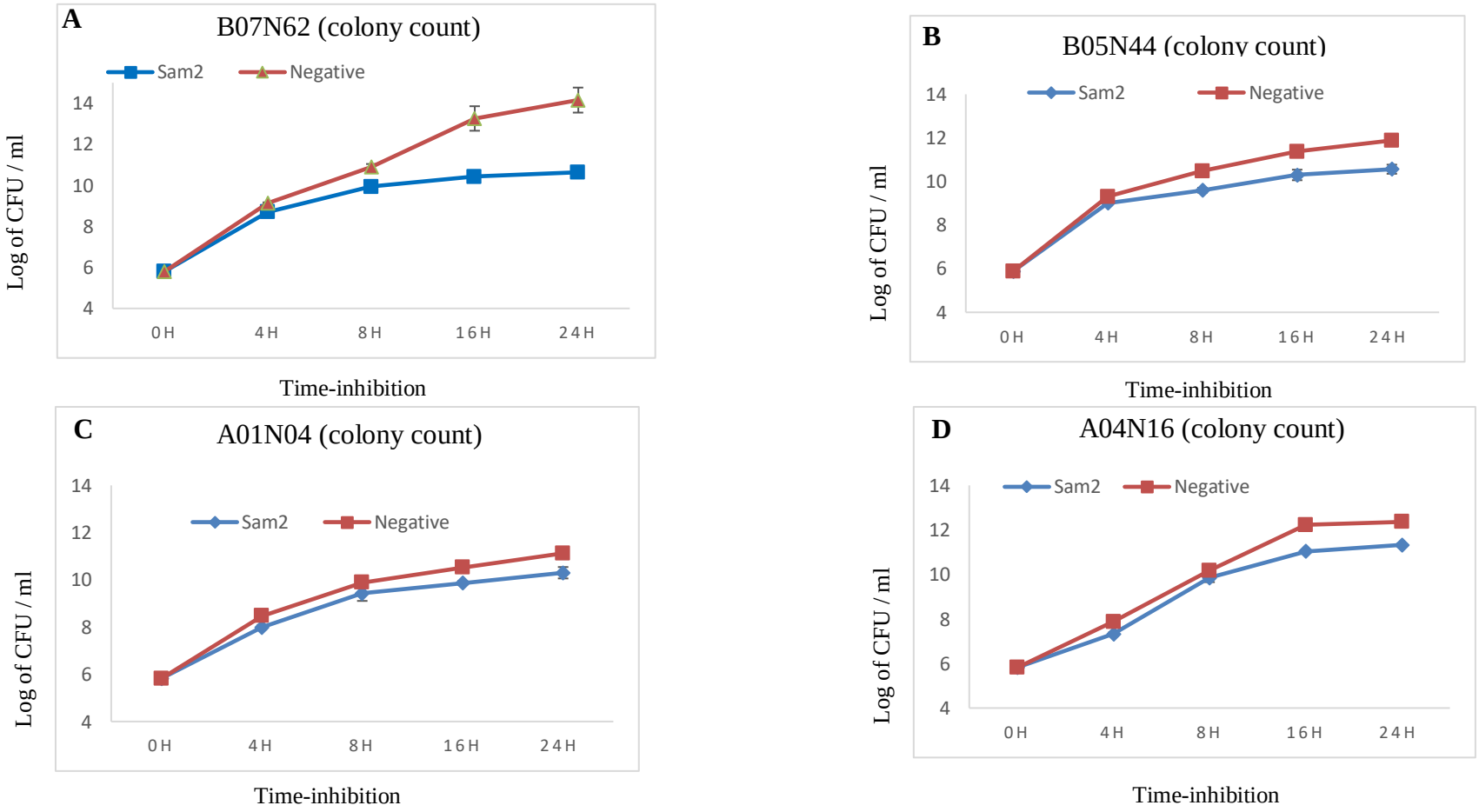

Figure 10 Growth curves of Sam2 against A (B07N62), B (B05N44), C (A01N04) and D (A04N16), Log CFU/ml

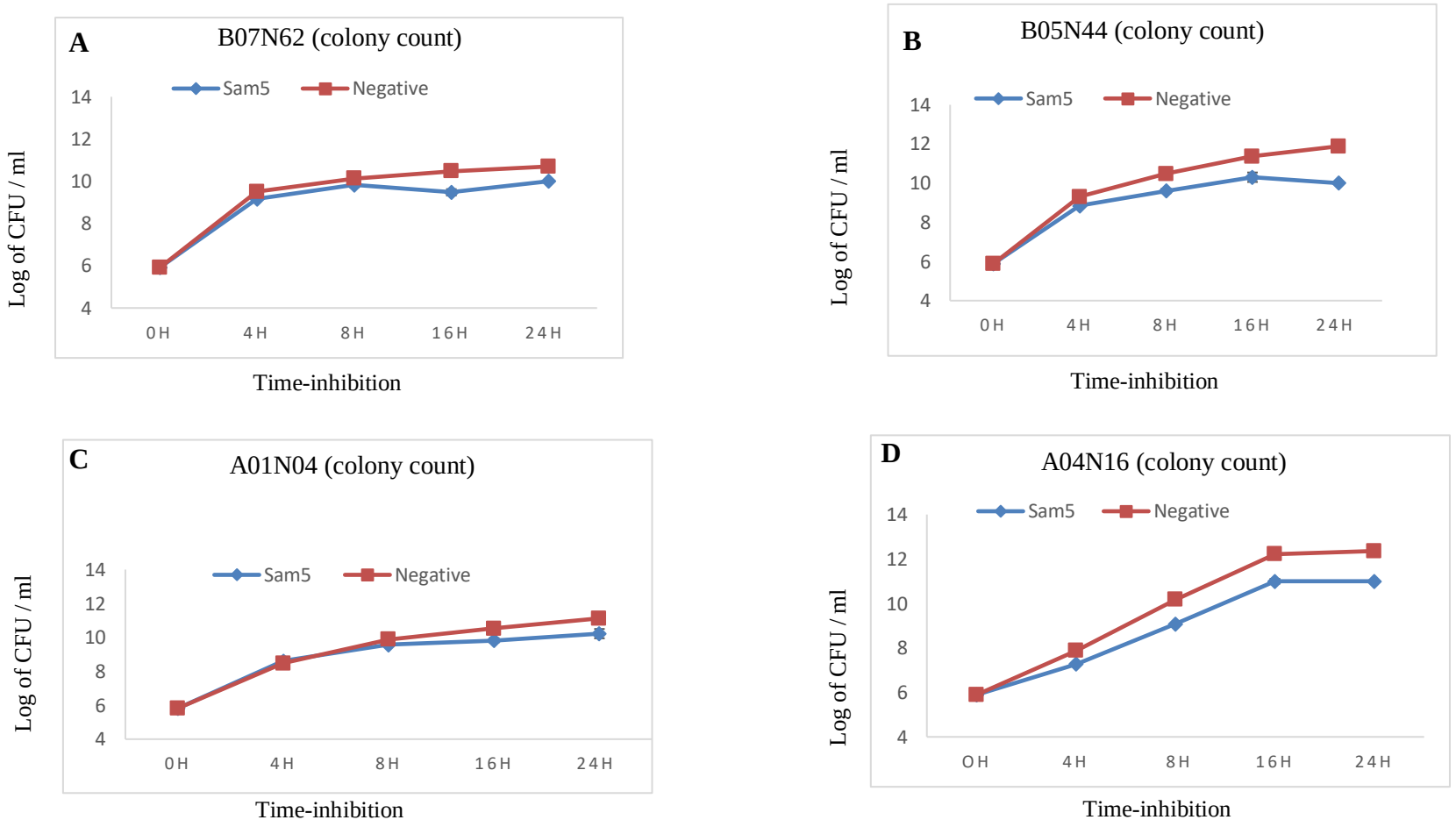

Figure 11 Growth curves of Sam5 against A (B07N62), B (B05N44), C (A01N04) and D (A04N16), Log CFU/ml

CHAPTER IV
DISCUSSION

The most frequently isolates of gram-negative and gram-positive bacteria in boar semen were *Escherichia coli* and *Staphylococcus* spp., respectively. This study is consistent with the discovery of previous researches (Arredondo et al, 2001; Martín *et al*, 2010; Bussaleu *et al*, 2013, Bresciani et al, 2014; Gaczarzewicz et al., 2016). One study reported that at least 75% of the samples were contaminated with one type of bacterium. Therefore, *Escherichia coli* was the most common species which was known as bacterial contamination in boar semen (Martín *et al*., 2010). However, Althouse and Lu (2005) found that *Enterococcus* spp. was the most prevalent bacteria isolated from boar semen. Furthermore, sex species of gram-negative were classified from family Enterobacteriaceae and Pseudomonadaceae while 3 species were identified from gram-positive bacteria including *Staphylococcus* spp. and *Streptococcus* spp. (Gaczarzewicz *et al*., 2016). In a recent study, 13 species of gram-negative bacteria were identified and the most species were from family Enterobacteriaceae and one species was from family Pseudomonadaceae. *Staphylococcus* spp. and *Streptococcus* spp. were also identified as gram-positive bacteria. Similarly, 7 species were found (*E. coli* , *S. marcescens*, *S. epidermidis*, *Streptococcus* spp., *Proteus* spp., *S. aureus*, *Pseudomonas spp*.) while other studies have counted additional 18 species in North America, Brazil, Cuba, Korea, and Thailand (Althouse *et al*., 2000; Maroto Martín *et al*., 2010; Suwimonteeraburt *et al*., 2011; Bussalleu *et al*., 2013).

For colony count of gram-positive and gram-negative bacteria isolated from fresh boar semen varied from 4.00×10^2 to 8.50×10^3 and 1.33×10^2 to 4.17×10^3 CFU/ mL, respectively. It was nevertheless consistent with the previous report which showed that a total aerobic bacteria count varied from 10^3 to 10^5 CFU/mL (Schulze *et al.*, 2015) and the higher level was up to 10^9 bacteria/mL (Althouse *et al*., 2000; Baracaldo and Ward, 2008).

Considering both the gram-negative and gram-positive bacteria of bacterial contaminants in boar semen, it had deleterious effect on liter size

(Gaczarzewicz *et al*., 2016), a decrease in sperm motility, viability (Althouse, 2008; Bussalleu *et al*., 2011) and damage on the membrane integrity of spermatozoa (Sepúlveda *et al*., 2013). Especially, Althouse *et al*. (2008) and Althouse and Lu (2005) reported that *Escherichia coli* strains demonstrated the adherence to spermatozoa via mannose-binding structures, a decreased percentage progressive motility and vitality of sperm and deleterious to the sperm plasma membrane. *Escherichia coli* isolated from boar semen provoke very strong agglutination of isolated sperm cells. The concentration of *Escherichia coli* had a positive correlation with the sperm agglutination and a negative correlation with litter size (Martín *et al*., 2010). Similarly, Bussalleu *et al*. (2011) reported that adverse effects on boar sperm quality were observed from 10^3 CFU/ mL *Escherichia coli* in the experiment performed at 37°C.

Moreover, *Escherichia coli* -to-sperm ratio of 1:1 has been identified as a threshold level for inducing agglutination and reduced motility of sperm (Diemer *et al*., 1996). In addition, *Escherichia coli* was therefore determined to adhere the sperm surface through mannose-binding structures of human spermatozoa. This receptor-specific inter-action led to damage the sperm plasma membrane (Wolff *et al*., 1993; Monga and Roberts, 1994). In addition, in the study of bacterial contamination in bovine semen and ram semen, *Escherichia coli* showed to cause the negative effect on the motility and viability of spermatozoa (Corona and Cherchi, 2009; Yániz *et al*, 2010). Furthermore, Sepulveda *et al*. (2013) therefore indicated that the presence of *P. aeruginosa* could spread infectious diseases and negative impact on sows, reducing the longevity and fertilizing ability of boar sperm. In our study, a species of *P. aeruginosa* was identified which was isolated from pooled semen. It might be effective on boar semen quality as *Escherichia coli* as mentioned above.

Semen extenders are important for maintaining fertilizing and the different types of semen extenders are commercially available for boar, bull, equine, and humans. To control bacterial growth, antibiotics are also used in the extenders. However, antibiotics pose a threat of antibiotic-resistant bacterial strains in artificial insemination centers (AI) and assisted reproductive technology (ART) laboratories

(Vickram *et al*., 2017). Ampicillin and penicillin resistance were commonly discovered in the previous study of *Escherichia coli* isolated from boar semen. In our study, we determined that ampicillin and penicillin were harmful antibiotics which could not be used in boar semen preservation.

Drug resistant *Escherichia coli*, isolated from boar semen, was a common discovery. The high prevalence of resistance might be the cause of using antibiotic for improving growth rate, efficiency of feed utilization, improving reproductive performance, and treatments in commercial pig farms (Cromwell, 2002). Penicillin and ampicillin are known as old drugs which have not been for available use for clinical treatment in humans and animals. Since the1980s, penicillin–streptomycin combinations of antibiotics had to be abandoned because of bacterial resistance problems (Sone *et al*., 1982). The previous study also reported that penicillin use in the extender might be effective on sperm viability during semen preservation (Fang 2017). It was similar to our study that the high prevalence of penicillin and ampicillin resistance were determined. The most common preservative antibiotic used in commercially available boar semen extenders was aminoglycoside gentamicin (Althouse *et al*., 2000). A recent study found that 6 strains of *Escherichia coli* were resistant to gentamicin while 4 strains of *Escherichia coli* were resistant to colistin. Therefore, two strains of *Escherichia coli* were isolated from the same sample and 2 other strains were isolated from different sample. Colistin has been known as the last option for treatment of carbapenem-resistant bacterial infection in humans. In animal farm, colistin has been used for over 50 years for treatment of digestive disorders and as growth promoter (feed additive). Recently, the high prevalence of colistin resistance increased in veterinary medicine and livestock and it became a major concern in many countries. In China, colistin has been banned for use in animal feeds since Nov 1, 2016 because of the high frequency of colistin resistance to *Escherichia coli* isolated from food animals (Huang *et al*., 2017). It might be caused of widely use colistin in veterinary medicine for many years. However, our result showed that all *Escherichia coli* isolates were still sensitive to ceftazidime and amikacin. Thus, the use of aminoglycoside amikacin and ceftazidime (3^{rd} generation) might not be widely use in veterinary medicine and livestock. Increases in rates of resistance to different antimicrobials have been reported in many

studies. To decrease the development of resistant bacteria, a reduction in antibiotic use globally may lead to a reduction of drug-resistant bacteria. The results obtained from this study were in agreement with the reports of other authors (Althouse *et al*., 2000 and Bresciani *et al*., 2014). Parallel with the study of Bresciani *et al*. (2014), ampicillin 75%, colistin 95%, penicillin G 85%, and gentamicin 70% were resistant to *Escherichia coli* isolated from boar semen while the other study in North America farm examined that *Escherichia coli* was resistant 100% of gentamicin, ampicillin, and polymicin B (Althouse *et al*. 2000).

Some of the bacterial species have special function to produce enzyme protein (extended spectrum beta – lactamase) which can develop antibiotic resistance such as colistin, carbapenem, and other third generation cephalosporins (Cheng *et al*., 2014; Bitrus, Chuanchuen and Luangtongkum, 2018). These resistances in recent study might be associated with bacteriolysis based on their antibiotic action and the release of soluble spermatotoxic factors of bacterial species (Okazaki *et al*., 2010). There are many forms that bacteria can acquire antibiotic resistance which are bacteria with intrinsic, adaptive and associated resistance.

DNA fingerprint generated by PCR targeting repetitive sequence regions has given a reliable for individual bacteria strains (Versalovic *et al*., 1994). In this study, the Box A1R was selected to differentiate all *Escherichia coli* strains isolated from boar semen. Especially, one representative of *Escherichia coli* in the larger clusters was selected for further study. Both of Pulsed-field Gel Electrophoresis (PFGE) and BOX-PCR were popular for DNA fingerprinting technique which can distinguish closely-related strains of *Escherichia coli*. Moreover, the protocol to generate the DNA fingerprint using BOX-PCR proved to have good discrimination power, and proved to be cheap, easy to conduct, and time-saver compared to PFGE (Versalovic *et al*., 1994; Cesaris *et al*., 2007). The diversity and the similarity indexes of *Escherichia coli* have indicated a similarity between population structures from different sources, according to Carlos *et al*. (2010). *Escherichia coli* strains isolated from boar semen in our study were very diverse. Moreover, the largest group in our study was found in cluster V. It might be because these *Escherichia coli* strains of the cluster had similar DNA pattern and

characteristic. When average similarity is used, an isolate is classified within the source group which shares the highest average similarity. Hence, the use of maximum similarity can be advantageous (Hassan *et al*., 2005).

Recently, antimicrobial peptides (AMPs) become a major interest as an alternative conventional antibiotics. AMPs are produced by various organisms and all classes of fundamental difference existing between prokaryote and eukaryotic cells. Interestingly, they have rapid action, broad spectrum of activities against gram-negative and gram-positive bacteria, viruses, fungi, parasites and the innate immune systems for most living organisms (Hancock and Sahl, 2006; Yeung *et al*., 2011). Their applications are available for pharmaceutical applications and growth and health promoters of animals (Hancock and Sahl, 2006; Vlieghe *et al*., 2010; Xiao *et al*., 2015)**.** To remove the antibiotics in semen extenders, antimicrobial peptides (AMPs) are a new potential agent that can inhibit bacteria growth in liquid-preserved boar semen (Schulze *et al*., 2014; Speck *et al*., 2014; Bussalleu *et al*., 2017; Timonet *et al*., 2018). Especially, antimicrobial peptides derived from seminal plasma of human, bovine, and mud crab samples have been reported in previous studies (Sceit *et al*., 1988; Jayasankar and Subramoniamm, 1999; Edström *et al*., 2008).

Based on one study in 2017, it was reported that boar seminal plasma contained at least one of 9 peptides including Sam1, Sam2, Sam3, Sam4, Sam5, Sam6, Sam7, Sam8, and Sam9. This study revealed that 100 µg/ml of 9 antimicrobial peptides derived from boar seminal plasma could inhibit *Escherichia coli* ATCC 25922, ranging from 70-100%, while there have not been any reported about boar semen peptides yet (not published yet). This low molecular weight fraction had antibacterial activity and might be target of bacterial membrane which were similar to the study of Hancock and Rozek, (2002), Zasloff, (2002), and Saldit (2006). Demonstrating the mechanisms of some AMPs which may acts against microorganisms through the cell membrane.

In a recent study, four representative *Escherichia coli* strains were selected to test with peptides. The results showed that the Sam1, Sam2, Sam4, Sam5, and Sam9 synthesis from boar semen peptides are not bactericidal. They could only inhibit *Escherichia coli* during time incubation. This finding is the first report and

the study found that all of the peptides have different targets of bacterial inhibition. Furthermore, Sam1 was reported as the highest activity against *Escherichia coli* ATCC 25922 and *Escherichia coli* strains which showed high prevalence resistance to common antibiotic penicillin G and ampicillin. Parallel with Junkes *et al*. (2011), the study found that Cyclic R-, W-rich peptides were against gram-negative bacteria including LPS mutant *Escherichia coli*. It can be implied that peptide Sam1 has the same mechanism as penicillin, ampicillin, and gentamicin to destroy *Escherichia coli* ATCC 25922 and isolated *Escherichia coli*. With supporting evidence, Zurfluh *et al*. (2013) reported that one of the most relevant resistance mechanisms in Enterobacteriaceae is the production of enzymes that leads to modern expanded-spectrum cephalosporin and carbapenem resistance. Synthetic magainin derivative and cyclic hexapeptide were investigated for semen preservation. As a result, the combination of antibiotics and cyclic hexapeptides was reported as a new candidate for future development of antimicrobial agent for boar semen preservation (Schulze *et al*., 2014; Speck *et al*., 2014). Similarly, three different AMPs, PMAP-36, PMAP-37, PR-39 and both porcine beta defensing-1 (PBD1) and -2 (PBD2) were evaluated about their effects on sperm quality and bacterial growth in the study of Bussalleu *et al*. (2017) and Puig-Timonet *et al*. (2018). PR-39 (10 µM) and PMAP-37 (3 µM) and both PBD1 and PBD2 had an inhibitory effect on bacterial growth but PMAP-36 could not reduce bacterial growth. In a study of Sancho *et al*. (2017), protegrin-1 (PG1) could control the bacteria load in all the assessed concentrations. However, the antibiotic use is more effective. Further investigations should deal with synergistic effect of different peptides or combination with conventional antibiotics.

One ability of peptides are to work through several mechanisms such as the modification of membrane permeability, depolarization of membrane ion, gradients, and the degradation of nucleic acids of bacterial membrane (Duquesne *et al*., 2007). Gram-negative bacteria have outer membrane made by lipopolysaccharide (LPS) including magnesium, calcium, and ions which bridge phosphosugars. Antimicrobial peptides result in deportation of metal, destroying the outer membrane, and facilitating the additional molecule from the exterior (Zasloff, 2002).

CHAPTER V
CONCLUSIONS

` It can be concluded from this study that all the samples were contaminated with gram-negative and gram-positive bacteria which were known as *Escherichia coli* and *Staphylococcus* spp., respectively. Moreover, penicillin G and ampicillin showed higher prevalent resistance to *Escherichia coli*. Especially, all of *Escherichia coli* strains isolated from boar semen were closely related genetic pattern. Sam1 showed the highest antibacterial activity against *Escherichia coli* ATCC 25922 and *Escherichia coli* strains isolated from boar semen.

SUGGESTIONS

This study recommended to combine Sam1 with antibiotics to find the synergic effect. For further investigation of antibacterial activity of synthesis peptide, Sam1 should be tested with all bacterial species isolated from boar semen. It should be investigated in boar semen preservation to evaluate semen quality. Due to the restriction of antibiotic use, Sam1 is suggested to be an alternative methods.

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APPENDIX

APPENDIX A. Guideline for using BioNumeric software Version 7.3

Creating a fingerprint type experiment, according to Applied Maths. (2011)

1. Install BioNumeric software
2. Create new database:

2.1. Press key database name (specify the name of fingerprint type)

2.2. Press create a new database or open exiting database

2.3. In the main window, click on in the toolbar of experiment type panel and select **Fingerprint type** from the list (See figure 12)

3. Press > **OK** then enter a name, for example PFGE-XbaI and press > Next
4. In the next window, make sure that **Two-dimensional TIFF files** and **8 bit (256 gray values)** are selected and press **> Next**
5. In the next dialog box, select **Yes** for fingerprint with inverted densitometric values
6. Press **> Next** to process
7. In the final step, leave **No** selected for applying the background subtraction
8. Press **> Finish** to complete the creation of the new fingerprint type, figure 12 (A-E)

Import a fingerprint gel file

1. To add a new fingerprint file to database, select **File** > **Import** and select
2. Press > **Import** to call the select **Fingerprint file** dialog box
3. Select the **file ec-XbaI-001**. Tiff in BioNumerics Tutorial data

The dialog also asks if you want to edit the image by opening the image editor. Uncheck the Open in image editor option if you are sure the file is an uncompressed gray scale

TIFF image. For the conversion to an uncompressed gray scale **TIFF file** make sure the option is checked.

4. Since the example file is uncompressed gray scale **TIFF file**, uncheck the option and press > **OK**.

Processing a fingerprint gel file

The fingerprint processing window opens. In this window is going to process **TIFF file** in 4 important steps:

- Strip (defining lane)
- Curves (extracting densitometric curves)
- Normalization
- Band (defining bands and quantification)

Step 1: Strip

The first step in processing a gel is to crop the image to remove empty space, and to define the lanes. Delineate the area of gel lanes by click dragging the nodes of rectangle to adjust it. Exclude the wells from the rectangle (Figure 13)

Next, we will edit the tone curve in improve the band visibility by select **Edit** > **Edit tone curve** and press **Linear**, the visible gray scale interval showed (figure 14)

Step 2: Define curves

The lanes have been defined, the software can generate densitometric curves describing the optical density across the spline along each lane.

Background scale: Estimation of disk size for background subtraction

- Close the spectral analysis window
- Open the fingerprint processing setting dialog box again
- Check apply least square filtering and specify a least square filtering cut off as indicated by the Wiener cut-off scale in the spectral analysis window (use the percentage value). Least square filtering removes very small peaks from the curves
- Check **Apply** in the background subtraction panel and specify a background subtraction disk size as indicated by the background scale in the spectral analysis window (use the percentage value). Background subtraction remove large background trend from the curves (figure 14).

Step 3: Normalize the gel

- Press the normalized review
- To assign a reference lane select **References** > **Use as reference lane**
- Choose the most suitable standard lane for creating the reference system
- Right-click on the top of the band and add external reference position
- Enter 582.6 and press **Ok**

- Repeat the process for each band in the lane
- Then select **Normalization** > **Auto assign**
- Make sure Using bands is selected and press **OK**
- If the band assignment is incorrect, select the band and press the **Del-key**
- Press next to proceed the last step. Alternatively press and band tab (figure 15)

Step 4: Define bands

- Usually, assigning bands in the sample lane is done with software automatic band search, followed by manual corrections.
- Press > **Search all lanes** > to execute the band search with these setting
- After satisfying with the band assignment, press save the file **Linking fingerprint data to entry** (figure 16)

Linking fingerprint data to entries

- In the **Fingerprint file panel**, double click on file name to open **Fingerprint window**.
- Select lane and **Database** > **Link lane.**
- Enter EC001 and press **OK,** create the new entry.
- We can let the program create new entries and link the gel lane automatically by selecting **Database** > **Add all lanes to database.**
- After linking, we close the fingerprint window and open the gel strip for one of the entries in the database by clicking on a color dot in the experiment presence panel.

The fingerprint still misses a standard pattern, so we will link a standard pattern following:

- Close the Fingerprint type window.
- In the fingerprint file panel, double click on ec-XbaI-001 to open fingerprint window.
- In fingerprint window, add lane **Marker** to the database.
- In the dialog box, enter **REF** and press **OK**

- Select **Yes** to create the new entry in the database then close the fingerprint window

Fingerprint type experiment settings

- Open the fingerprint experiment type **Box A1R** by double click on the experiment type in the **Experiment type panel.**
- Press Next to standard in the setting panel and drag it over to **REF** database entry.
- In the **Fingerprint type window**, select settings > **Edit referent system** or double click on **R01.**
- Copy molecule weight by selecting **Metrics** > Copy markers from referent system**.**
- Designate a metric unit with **Metrics** > **Assign units,** enter **kb** and press **OK** then lose the window (figure 17)**.**
- Open the gel strip for one of entries in the database by clicking on the color dot in the Experiment presence panel
- Increase or decrease the size of the card

Importing and processing a second gel file

For the second gel, we can use the same fingerprint type, reference system and conversion settings used for the first gel. We can skip the assignment of the reference position. Finally, it can show the comparison window with group defined (figure 18).

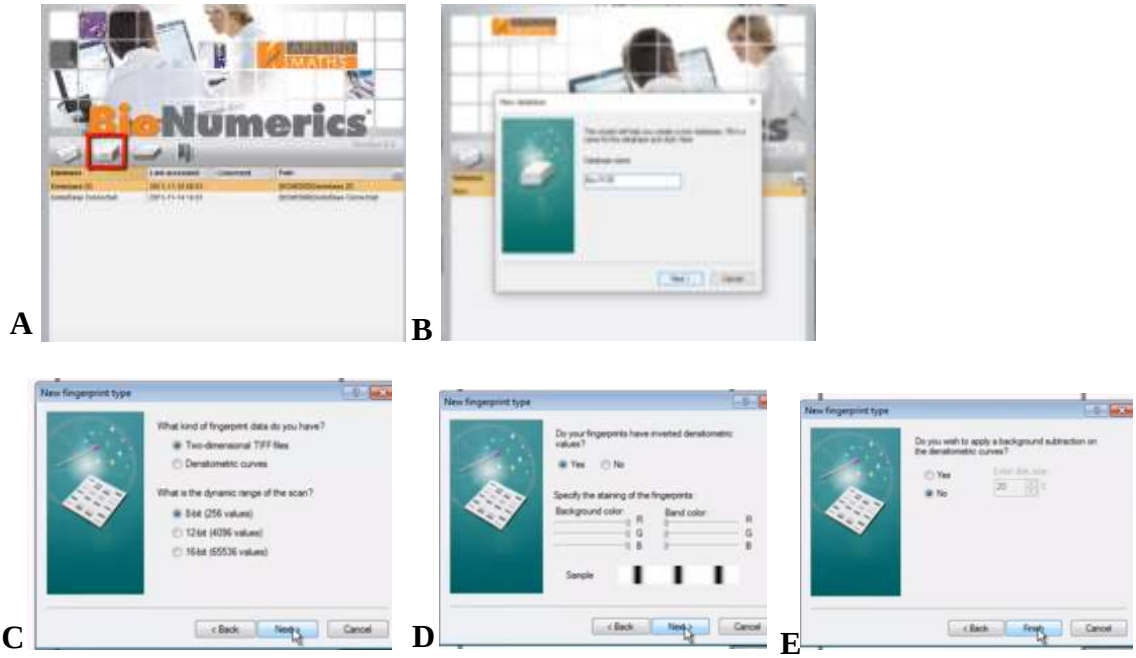

Figure 12 Create new data base and name of data base

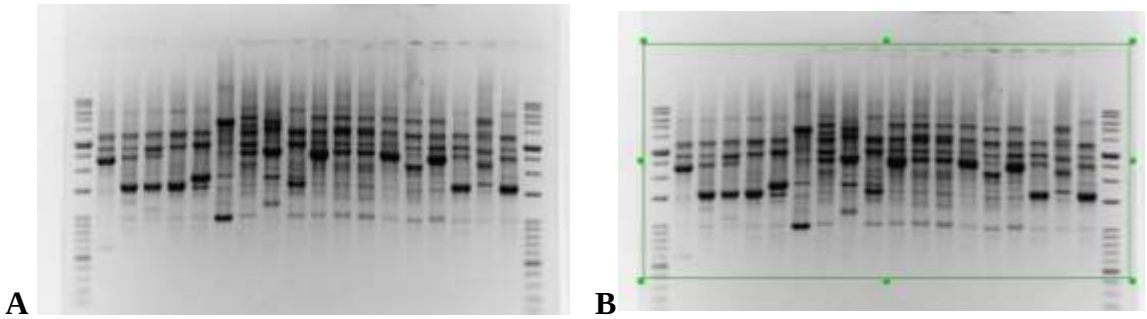

Figure 13 Import a fingerprint gel file

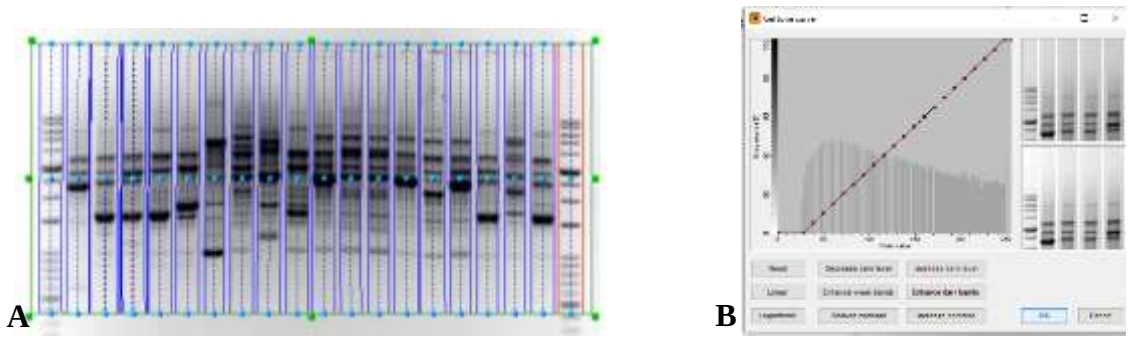

Figure 14 (A) Adjusting the thickness and (B) adjust positions and gel tone curve

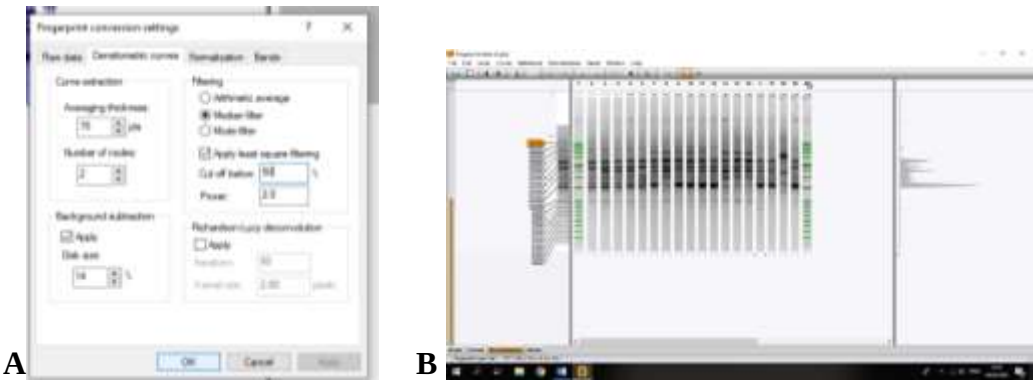

Figure 15 (A) Splines and median filtering and (B) normalize the gel

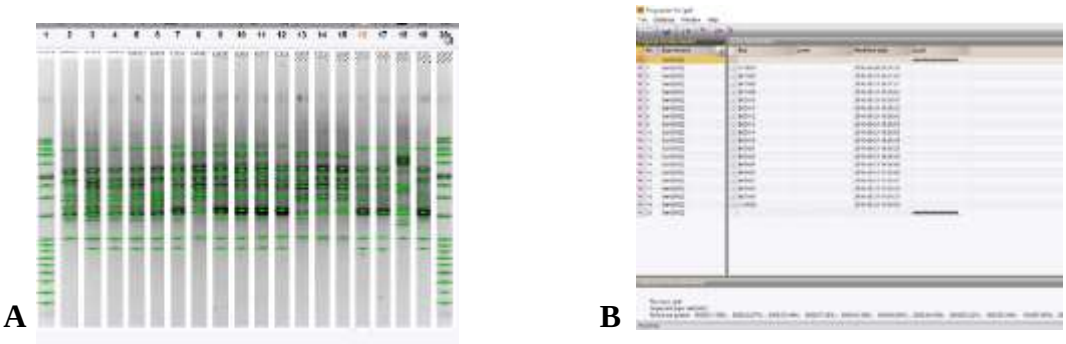

Figure 16 (A) Reference position assigned and (B) fingerprint file with linked lanes

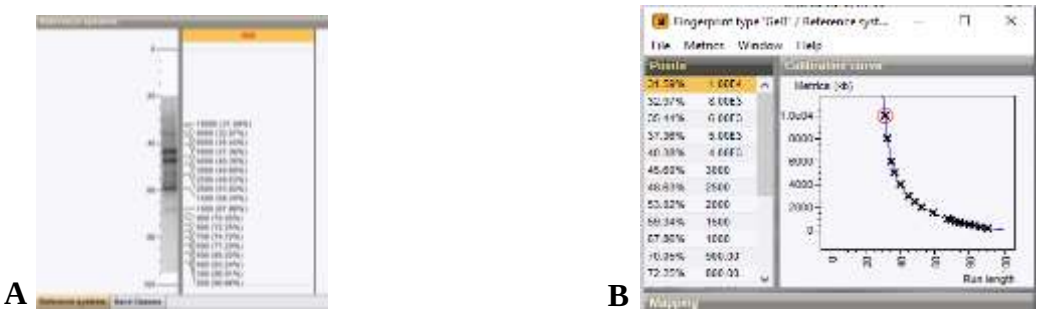

Figure 17 (A) The fingerprint type window and (B) calibration curve calculated

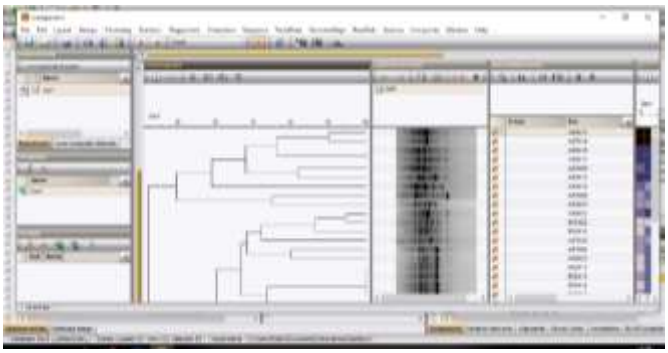

Figure 18 The comparison window

APPENDIX B: Identification of 104 strains bacteria by Biochemical test

Table 10 Results of biochemical test for gram-negative bacterial identification, fresh semen

No	Indole	MR	VP	Citrate	H_2S	Urea	LDC	Motility	Glucose, Gas	Lactose	Sucrose	EMB	Bacteria
A01N01	+	+	–	–	–	–	+	–	+G	+	–	+	*E. coli* 86.52%
A01N02	–	+	–	+	–	+K	+	–	+G	+	+G	–	*K. pneumoniae* 95.4%
A01N03	+	+	–	–	–	–	+	+	+G	+	+	+	*E. coli* 99.99%
A02N04	+	+	–	–	+	–	+	+	+G	+	+G	+	*E. coli* 99.99%
A02N05	+	+	–	–	–	–	+	–	+G	–	–	–	*E. coli* (inactive) 89.87%
A02N06	–	–	+	+	–	+K	+	–	+G	+	+G	–	*K. pneumoniae* 99.11%
A03N07	+	+	–	–	+	–	+	–	+G	+	+	+	*E. coli* 97.32%
A03N08	+	+	–	–	–	+	+	+	+G	–	+G	+	*A. hydrophila*
A03N09	+	+	–	–	–	–	+	+	+G	+	–	–	*E. coli* 99.96%
A04N10	+	+	–	–	–	–	–	+	+	+	+	+	*E. coli*
A04N11	+	+	–	–	–	–	–	–	+G	–	–	–	*E. cloacae*
A04N12	+	+	–	–	+	–	+	–	+G	–	+G	–	*E. coli* (inactive)
A05N13	+	+	–	–	–	–	+	+	+G	+	+G	+	*E. coli* 99.99%
A05N14	+	+	–	–	+	–	+	–	+G	+	–	+	*E. coli* 86.44%
A05N15	+	+	–	–	–	–	–	+	+	+	–	–	*E. coli* (inactive)
A06N16	+	+	-	-	-	-	+	+	+G	+	-	+	*E. coli* 99.96%
A06N17	+	+	-	-	-	-	+	-	+G	+	-	+	*E. coli* 86.52%
A06N18	+	+	-	-	-	-	+	-	+G	-	-	+	*E. coli* 89.87%
A07N19	+	+	-	-	-	-	+	-	+G	+	-	+	*E. coli* 86.52%
A07N20	+	+	-	-	-	-	+	-	+G	+	-	+	*E. coli* 86.52%
A07N21	-	+	-	+	-	+	-	+	+	-	-	-	*P. rettgeri*
A08N22	+	+	-	-	-	-	+	-	+	+	-	+	*E. coli* 86.52%
A08N23	+	+	-	-	-	-	+	-	+	+	-	+	*E. coli* 86.52%
A08N24	+	+	-	-	-	-	+	-	+G	-	+	-	*E. coli (inactive)*
A09N25	-	+	-	-	+	-	+	-	+G	+	-	+	*E. coli*
A09N26	+	+	-	-	-	-	+	+	+G	+	+G	-	*E. coli* 99.99%
A09N27	+	+	-	-	+	-	+	+	+G	-	-	-	*E. tarda* 99.99%
A10N28	+	+	-	-	-	-	+	-	+	+	-	+	*E. coli* 86.52%
A10N29	+	+	-	-	-	-	+	-	+	+	-	+	*E. coli* 86.52%
A10N30	+	+	-	+	-	-	-	+	+	-	+	+	*P. stuartii* 87.98%

Table 11 Results of biochemical test for gram-negative bacterial identification, pooled semen

No	Indole	MR	VP	Citrate	H_2S	Urea	LDC	Motility	Glucose, Gas	Lactose	Sucrose	EMB	Bacteria
B01N01	–	+	–	+	–	+	+	–	+G	–	+G	–	*K. pneumoniae*
B01N02	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B01N03	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B01N04	–	+	–	+	–	+	+	–	+G	–	+G	–	*K. pneumoniae*
B01N05	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B01N06	+	+	–	–	+	–	+	–	+G	+	+	+	*E. coli* 99.99%
B01N07	+	–	+	–	–	–	+	–	+G	+	+	+	*K. oxytoca* 100%
B01N08	+	+	–	–	+	–	+	–	+G	+	+	+	*E. coli* 99.99%
B01N09	+	+	–	–	+	–	+	–	+G	+	+	+	*E. coli* 99.99%
B01N10	+	+	–	–	+	–	+	–	+G	+	+G	+	*E. coli* 99.99%
B02N11	+	+	–	–	–	–	+	+	+G	–	+	+	*E. coli* 99.56%
B02N12	+	+	–	–	+	–	+	+	+G	–	+	+	*E. coli* 99.99%
B02N13	+	+	–	–	–	–	+	+	+G	–	+	+	*E. coli* 99.56%
B02N14	+	+	–	–	–	–	+	+	+G	–	+	+	*E. coli* 99.56%
B02N15	+	+	–	+	–	–	+	+	+G	–	+	+	*E. coli* 89.74%
B03N16	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B03N17	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B03N18	–	+	–	+	–	–	+	+	+G	–	+G	–	*K. aerogenes*
B03N19	–	+	–	+	–	–	–	+	+G	–	+G	–	*K. aerogenes*
B03N20	–	–	+	+	–	–	–	+	–	–	–	–	*S. marcescens* 99.56%
B03N21	+	+	–	–	–	–	–	+	+	+	+	+	*E. coli* 93.99%
B03N22	+	+	–	–	–	–	–	+	+	+	+	+	*E. coli* 93.99%
B03N23	+	–	+	–	–	–	–	+	+	+	+	+	*S. marcescens* 100%
B03N24	+	+	–	–	–	–	+	+	+G	+	+G	+	*E. coli* 97.32%
B03N25	–	+	–	+	–	–	+	+	+G	+	+G	+	*E. aerogenes* 88.1%
B03N26	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B04N27	–	+	–	+	–	–	–	+	+	–	+	–	*E. cloacae*
B04N28	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B04N29	–	–	+	+	–	–	–	+	–	–	–	–	*S. marcescens* 99.56%
B04N30	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%

Table 12 Results of biochemical test for gram-negative bacterial identification, pooled semen

No	Indole	MR	VP	Citrate	H_2S	Urea	LDC	Motility	Glucose, Gas	Lactose	Sucrose	EMB	Bacteria
B04N31	+	+	–	–	–	–	+	+	+G	+	+	+	*E. coli* 97.32%
B04N32	–	+	–	+	–	+	+	–	+G	+	+G	+	*K. pneumoniae* 95.4%
B04N33	+	+	–	–	–	–	+	+	+G	+	+	+	*E. coli* 97.32%
B04N34	+	+	–	–	–	–	+	+	+G	+	+	+	*E. coli* 97.32%
B04N35	–	+	–	+	–	+	+	–	+G	+	+G	–	*K. pneumoniae* 95.4%
B05N36	–	–	+	+	–	–	+	–	–	–	–	–	*S. marcescens* 99.7%
B05N37	–	–	+	+	–	–	+	–	–	–	–	–	*S. marcescens* 99.7%
B05N38	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 99.7%
B05N39	–	–	+	+	–	–	–	+	–	–	–	–	*S. marcescens* 99.56%
B05N40	+	+	–	–	–	–	+	+	+G	–	+	–	*E. coli* 99.56%
B05N41	+	+	–	–	+	–	+	+	+G	+	+	+	*E. coli* 97.48%
B05N42	+	+	–	–	–	–	+	–	+G	+	+	+	*E. coli* 97.09%
B05N43	+	+	–	–	+	–	+	+	+G	+	+	+	*E. coli* 99.99%
B05N44	+	+	–	–	+	–	+	+	+	+	+	+	*E. coli* 99.99%
B05N45	+	+	–	–	+	–	+	+	+	+	–	+	*E. coli* 99.84%
B06N46	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B06N47	–	–	+	+	–	+	–	–	–	–	–	–	*P. aeruginosa*
B06N48	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B06N49	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B06N50	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B06N51	+	+	–	–	–	–	+	–	+G	+	+G	+	*E. coli* 97.09%
B06N52	+	+	–	–	–	–	+	–	+G	+	+G	+	*E. coli* 97.09%
B06N53	+	+	–	–	–	–	+	–	+	+	+	+	*E. coli* 97.09%
B06N54	+	+	–	–	–	–	+	–	+	+	+	+	*E. coli* 97.09%
B06N55	+	+	–	–	–	–	+	–	+	+	+	+	*E. coli* 97.09%
B07N56	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B07N57	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B07N58	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%
B07N59	–	+	–	+	–	–	–	+	–	–	–	–	*E. aerogenes* 88.1%
B07N60	–	–	+	+	–	–	–	–	–	–	–	–	*S. marcescens* 98.67%

Table 13 Results of biochemical test for gram-negative bacterial identification, pooled semen

No	Indole	MR	VP	Citrate	H_2S	Urea	LDC	Motility	Glucose, Gas	Lactose	Sucrose	EMB	Bacteria
B07N61	+	+	–	–	–	–	+	–	+G	+	+	+	*E. coli* 99.99%
B07N62	+	+	–	+	+	–	+	+	+	+	–	+	*E. coli*
B07N63	–	+	–	–	–	–	+	–	–	+	–	–	*E. coli* (inactive)
B07N64	–	–	+	+	–	–	+	–	–	–	–	–	*S. marcescens* 99.7%
B07N65	+	+	–	+	–	–	–	+	+G	–	–	–	*C. koseri*
B08N66	+	+	–	+	–	–	–	+	+G	–	+	–	*C. koseri*
B08N67	+	+	–	+	–	–	–	+	+G	–	+	–	*Citrobacter koseri*
B08N68	+	+	–	+	–	–	–	+	+G	–	+	–	*Citrobacter koseri*
B08N69	+	+	–	+	–	–	+	+	+G	–	+	–	*Citrobacter koseri*
B08N70	+	+	–	+	–	–	+	+	+G	–	+	–	*Citrobacter koseri*
B08N71	+	+	–	–	+	–	–	–	+G	+	+	+	*Escherichia coli*
B08N72	+	+	–	+	+	–	–	+	+G	+	+	–	*Citrobacter koseri*
B08N73	+	+	–	–	+	–	–	+	+G	+	+	+	*Escherichia coli*
B08N74	+	+	–	+	–	–	–	+	+G	+	+	–	*Citrobacter koseri*

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List of Publication and Proceeding

Keath. S, S. Chumtong, R. Pomwised, N. Am-in, S. Roytrakul and T. Thepparat. 2019. Bacterial Contamination and Antibiotic Resistance of *Escherichia coli* Isolated from Boar Semen. KHON KAEN AGR. J. 47 SUPPL. 2:105-11

Keath. S, S. Chumtong, R. Pomwised, N. Am-in, S. Roytrakul and T. Thepparat. 2020. Identification of Bacteria and Antimicrobial Susceptibility of *Escherichia coli* Isolated from Boar Semen. KHON KAEN AGR. J. (submitted)