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Detection and Susceptibility of Antibiotic-Resistant *Enterococcus* spp. in Fermented and Pickled Vegetables

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Abstract: *The study was carried out on detection of Enterococcus* spp. *in fermented and pickled vegetable products from markets in Phnom Penh and determination of their antibiotic resistance. Thirty-nine samples were collected from six different sites and classified into six categories of fermented and pickled vegetable products. Samples were subjected to physicochemical and microbiological analysis, followed by isolation and antibiotic susceptibility test conducted by paper disk diffusion method with eight antibiotics such as ampicillin, ciprofloxacin, doxycycline, erythromycin, levofloxacin, tetracycline, tigecycline and vancomycin. The results showed that pH values of fermented vegetable samples ranged from 3.73 to 4.74, followed by total acidity varied from 1.05% to 1.60% and the salt content was in range of 3.31% to 5.41%. Moreover, most of collected samples were contaminated with Enterococcus* spp. *with highest occurrence of 4.22 log CFU/mL in fermented spiderwisp and fermented chilli that originated from orussey market, except three samples were not detected of Enterococcus* spp. *such as fermented mustard greens from orussey market, fermented mustard greens and small fermented cucumber from Aeon supermarket. Among the 144 strains of Enterococcus* spp., *54.17% exhibited their resistance to tigecycline followed by 17.36% resistance to erythromycin, 13.19% resistance to ciprofloxacin, 10.42% resistance to tetracycline, 9.03% resistance to vancomycin, 6.25% resistance to ampicillin, 4.86% resistance to levofloxacin and 3.47% resistance to doxycycline. As conclusion, Enterococcus* spp. *were present and able to survive in fermented and pickled vegetable products. Most of isolated strains of Enterococcus spp. demonstrated their potential resistance to tigecycline.*

Keywords: Antibiotic; Resistance; *Enterococcus* spp.; Fermented vegetable

1. INTRODUCTION

History of humankind through medical point of view as a struggle with infectious of disease. Infectious diseases were the reason of death worldwide at the beginning of twentieth century (Sukmawinata et al., 2018). Antimicrobial resistant bacteria has revealed as a potential issue for human and animal health (Sukmawinata et al., 2018). The resistant mechanism of bacteria includes utilizing an enzymatic target adjustment, changing the bacterial cell divider porousness. Antibiotics resistant bacteria are contaminating in food products and also in environment such as *Enterococcus*, *Pseudomonas aeruginosa*, *Clostridium difficile*, *Klebsiella pneumonia*, *Escherichia coli*, *Acinetobacter baumannii*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae* and *Streptococcus pyogenes* (Imberechts et al., 2013). Enterococci are reported to have innate resistant to antibiotic such as penicillin, monobactam and a little bit amount of aminoglycoside (Nishiyama et al., 2017). Selection of the most appropriate antimicrobial agents to test and report is based on the infectious disease treatment (CLSI, 2017).

Tigecycline is a novel antimicrobial agent which contains broad-spectrum potential of glycylcycline (Pankey, 2005). Tigecycline has influential *in-vitro* activity apposing to most Gram-positive and Gram-negative, aerobic and anaerobic bacteria including *Staphylococcus aureus*, *Enterococcus* spp., *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Neisseria gonorrhoeae*, *Peptostreptococci*, *Clostridium* spp., *Enterobacteriaceae* and *Bacteroides* spp. (Pankey, 2005). *In-vitro* testing has revealed that tigecycline has activity against vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and many species of multidrug-resistant (Pankey, 2005).

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Tigecycline can be utilized as an empiric monotherapy to treat an assortment of genuine bacterial contamination such as complicated appendicitis, infected burns, intra-abdominal abscesses, deep soft tissue infections and infected ulcers (Pankey, 2005). Most of research papers related to enterococci species were reported that the enterococci may cause infections of the urinary tract, bloodstream, endocardium, abdomen, biliary tract, and wound infected (Jett et al., 1994; Tadashi et al., 2013). Relatedly, enterococci, known as a lactic acid bacterium, are able to grow in high salted environment and low pH products, and commonly found in the gastrointestinal tracts of animals, birds, and humans, as well as in soil, water, milk products, and other foods in a high number (Facklam et al., 2002; Hancock and Gilmore, 2006). Moreover, the presence of enterococci in different types of fermented products according to their characteristics that are able to grow in brine solution as mentioned above (Wolfgang et al., 2003). The concerning of enterococci that affect to human beings is related to their enteric habitat, their entering the food chain, their antibiotic resistance and their possible involvement in food-borne disease (Wolfgang et al., 2003). Therefore, investigating the contamination of *Enterococcus* spp. in food products, especially ready-to-eat foods such as fermented foods are crucial for food-borne disease prevention. The objective of this study is to detect *Enterococcus* spp. in fermented vegetable products from markets in Phnom Penh and to determine their antibiotic resistance.

2. METHODOLOGY

2.1 Sample collection

Samples were collected form six different markets located in Phnom Penh city and kept in ice box during transportation to laboratory at the Institute of Technology of Cambodia. The collected samples were immediately subjected to microbiological and physicochemical analyses. Thirty-nine samples that took from six different sites such as Central market (CTM), Orussey market (ORS), Kilo No.4 market (K4M), Aeon supermarket (AEON), Institute of Technology of Cambodia (ITC) and Royal Phnom Penh University (RUPP). The collected samples were classified into six categories by the types of samples such as first group of sample refer to small fermented cucumber (SFC) contained 8 samples, the second group refer to fermented mustard greens (FGM) that had 11 samples, third group contained 7 fermented cucumber (FCC) samples, followed by the fourth group of 5 fermented Spiderwisp (FS), fifth group of 3 samples of fermented chilli (FC), and the last group of 5 samples of cucumber pickle (CCP). The different number of collected samples were based on the seller in each site. The samples were subjected to analysis in duplicate with physicochemical parameters and testing of cell density of *Enterococcus* spp.

2.2 Physicochemical properties analysis

2.2.1. Measurement of pH

Samples were mixed and filtered with filter paper to separate between solid phase ansd liquid phase, the filtered sample were subjected to pH measurement using a portable pH meter (MA-260) calibrated with certified buffer solutions that bracket the range of the sample pH values. Buffers ,commonly used as standards for testing the pH of fermented and acidified vegetable products, are pH of 4.00 and 7.00.

2.2.2. Determination of acidity

The filtrated samples were titrated with a standard solution of diluted alkali such as NaOH. The indicator phenolphthalein was used in colorless or slightly colored samples to indicate the titration end-point at pH 8.3. Sample volume and alkali concentration were established based on the expected acid concentration of the samples. Five milliliters of sample were diluted with 45 mL of distilled water. Five milliliters of samples were titrated with standardized 0.1 N NaOH until a light pink color of the phenolpthlalein indicator was observed and maintained for 30 seconds. Blank sample was conducted with the same procedure, whereas the titrated volume of blank was used to minus by the volume of sample titration (AOAC, 1995).

2.2.3. Determination of salt content

The determination of salt content was determined using Volhard method. The presence of silver nitrate ($AgNO_3$) in brine solution was formed in to silver chloride (AgCl) within the presence of concentrated nitric acid 65% as a catalyst at boiling temperature. The remained Ag^+ would be formed in to Siliver Thiocynate (AgSCN) after titrated with potassium thiocynate (KSCN). The indicator $FeNH_4(SO_4)_2.12H_2O$ was used to indicate the equilibrium point when the solution changed to dark orange. One milliliters of sample added with 5ml of $AgNO_3$ and 15ml of HNO_3 before burning, followed by addition of distilled water to reach to 100ml, solution was kept cool at room temperature. Sample solution was added by 2.5 mL of ($FeNH_4(SO_4)_2.12H_2O$) and titrated with KSCN until it reached to dark orange. Blank was conducted as same as the sample whereas the titrated volume of blank was used to minuses the titrate volume of sample (AOAC, 1995).

2.3 Isolation of Enterococcus spp.

A volume of 100 µL of each sample was spread on the m-*Enterococcus* agar (Difco, USA) and incubated at 37°C for 24 h. Two colonies were selected randomly based on morphology from colony-grown agar plate and individually transferred into an eppendorf containing 1 mL of Enterococcusel broth (Difco, USA) and incubated at 37°C for 24 h. Broth in tube was observed in dark color when there was a presence of *Enterococcus* spp. The analysis has been conducted in duplicate.

2.4 Antibiotic susceptibility test by paper disk diffusion method

Antibiotic susceptibility testing was conducted by paper disk diffusion method using eight antibiotics such as ampicillin (AMP) 10 µg/disk, ciprofloxacin (CIP) 5 µg/disk, doxycycline (DOX) 30 µg/disk, erythromycin (EM) 15 µg/disk, levofloxacin (LVX) 10 µg/disk, tetracycline (TC) 10 µg/disk, tigecyline (TIG) 15 µg/disk, vancomycin (VCM) 30 µg/disk followed by Clinical Laboratory Standard Institute guidelines (CLSI, 2017). Accordingly, 144 isolated strains of *Enterococcus* spp. were individually streaked on Todd Hewitt Broth agar (Difco, USA) and incubated at 37°C for 24 h. White small colonies were appeared on the agar and colonies were homogenized in sterilized saline to give an equivalent turbidity of 0.5 based on McFarland Standard (equivalent to a growth of 10^8 CFU/mL). Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, sterilized cotton was dipped in to the inoculum suspension to swab on the Mueller-Hinton agar (MHA) (Himedia, India). The antibiotic-containing paper disk was placed onto the seeded MHA agar. Each disk was distributed evenly no closer than 24 mm from center to center; the plate was inverted and incubated at 37°C for 24 h. The diameter of inhibitory zone was measured using digital calliper Top Craft (Globaltronics GmbH & Co. KG, Hamburg, Germany) and compared to the CLSI standard. The result was interpreted to the percentage of Resistance (R), Intermediate (I) and Susceptibility (S) of total strain in each category of samples within different 8 antibiotics.

3. RESULTS AND DISCUSSION

3.1 Physicochemical properties

A total samples of 39 fermented and pickled vegetables, classified into six groups such as small fermented cucumber (SFC), fermented mustard greens (FGM), fermented cucumber (FCC), fermented spiderwisp (FS), fermented chilli (FC), and cucumber pickle (CCP), were analyzed for their physicochemical properties. As results, the pH of collected fermented vegetables were ranged from 3.73 to 4.74 (Table 1-6).

Table 1. Physicochemical contents in small fermented cucumber

Sample ID	pH	**Acidity(% V/V)**	**Salt (%V/V)**
CTM-SFC001	4.46 ± 0.01	1.55 ± 0.15	4.36 ± 0.03
CTM-SFC002	4.02 ± 0.01	1.60 ± 0.01	4.36 ± 0.03
ORS-SFC-002	4.11 ± 0.01	1.45 ± 0.05	4.01 ± 0.03
ORS-SFC-003	4.46 ± 0.01	1.65 ± 0.05	3.89 ± 0.03
ORS-SFC-004	4.02± 0.01	1.50 ± 0.01	3.89 ± 0.03
ORS-SFC-005	4.44 ± 0.01	1.60 ± 0.01	4.86 ± 0.06
K4M-SFC-002	3.76 ± 0.02	1.70 ± 0.01	4.71 ± 0.03
AEON-SFC-001	4.06 ± 0.01	1.25 ± 0.05	4.77 ± 0.03

In fact, pH in fermented vegetable or pickle was reported to be in range of 3.2-4.2 (Pérez, 2013), while another study was reported that pH of fermented product should range from 3.47-4.77 (Susilowati et al., 2018). In terms of acidity, it is found that the acidity level were in range of 1.05 to 1.70%. According to previous study of Pérez (2013), the concentration of acidity of fermented vegetable should be present in a range of 0.8-2.5%. In addition to pH and acidity level, the concentrations of salt contained in fermented vegetables and pickles were also determined in the current study and found in range of 3.31%-5.41%. This finding was in accordance to the concentration of salt in fermented vegetables it was reported in a range of 2.5-7.5% (Susilowati et al., 2018), while another study has been reported the concentration of salt should be range from 2% to 10% (Pérez, 2013). In fermentation process, the lactic acid bacteria need to adjust to the environmental conditions including vary in temperature, pH and salinity, depending on the specific application (Manas et al., 2014). Lactic acid has produced during fermentation when acetic acid was generally added in form of vinegar to acidify in the pickle products (Pérez, 2013). Acidity had been reported plays as a number of protons equivalence of organic acid anion presented in the sample (Bibiana et al., 1980). Determination of acidity was routinely measured to estimate the amount of free acid in fermenting and fermented or acidified products (Pérez et al., 2013). pH of brine in fermented vegetable could be decreased by purging with carbon dioxide (CO_2), when the lower initial pH was able to release the excess CO_2 formed during the process of fermentation by selected the growth of lactic acid bacteria, then inhibiting the growth of the acid sensitive enterobacteria (Pérez et al., 2013). The concentration of salt has played as an important role to supporting homo-fermentative lactic acid bacteria but inactivate the growth of hetero-fermentative lactic acid

bacteria which produced a bit of carbon dioxide that necessary for flushing out entrapped air from raw material shreds (Ratan et al., 2016). In this study the determination of physicochemical properties were conducted to prove that the condition of fermented vegetable are allowed colonies of enterococci to growth. when the enterococci were reported to be involved in fermentation of meat products, dairy products and even vegetables (Hanchi et al., 2018)

Table 2. Physicochemical property contents in Fermented Green Mustard

Sample ID	pH	**Acidity(% V/V)**	**Salt (%V/V)**
CTM-FGM-001	4.44 ± 0.01	1.70 ± 0.01	4.01 ± 0.03
CTM-FGM-004	4.09 ± 0.01	1.35 ± 0.15	3.31 ± 0.03
ORS-FGM-001	3.73 ± 0.01	1.40 ± 0.10	4.36 ± 0.03
ORS-FGM-002	4.27 ± 0.01	1.50 ± 0.20	4.36 ± 0.03
ORS-FGM-003	4.36 ± 0.00	1.55 ± 0.05	4.07 ± 0.03
K4M-FGM-001	3.93 ± 0.01	1.35 ± 0.05	4.48 ± 0.03
K4M-FGM,C-001	4.07 ± 0.03	1.60 ± 0.01	4.77 ± 0.03
K4M-FGM,S-003	4.05 ± 0.01	1.45 ± 0.05	5.27 ± 0.01
K4M-FGM,B-003	4.59 ± 0.01	1.35 ± 0.05	4.62 ± 0.06
AEON-FGM,S-001	3.98 ± 0.01	1.30 ± 0.01	3.63 ± 0.06

Table 3. Physicochemical property contents in Fermented Cucumber

Sample ID	pH	**Acidity(% V/V)**	**Salt (%V/V)**
CTM-FCC-001	4.36 ± 0.01	1.50 ± 0.20	4.48 ± 0.03
CTM-FCC-003	4.36 ± 0.01	1.55 ± 0.05	4.50 ± 0.06
ORS-FCC-002	4.29 ± 0.01	1.35 ± 0.05	4.48 ± 0.03
ORS-FCC-005	3.82 ± 0.01	1.60 ± 0.01	4.71 ± 0.03
K4M-FCC-003	4.69 ± 0.01	1.45 ± 0.05	5.41 ± 0.03
K4M-FCC-003	4.39 ± 0.01	1.35 ± 0.05	4.04 ± 0.01
AEON-FCC-001	4.03 ± 0.01	1.30 ± 0.01	4.07 ± 0.03

Table 4. Physicochemical property contents in Fermented Cleome gynandra

Sample ID	pH	**Acidity(% V/V)**	**Salt (%V/V)**
ORS-FCG-002	4.36 ± 0.01	1.70 ± 0.01	3.33 ± 0.06
ORS-FCG-003	4.65 ± 0.00	1.35 ± 0.15	3.45 ± 0.06
ORS-FCG-004	4.36 ± 0.00	1.40 ± 0.10	4.33 ± 0.01
ORS-FCG-005	4.28 ± 0.01	1.50 ± 0.20	4.01 ± 0.03
K4M-FCG-001	4.13 ± 0.00	1.55 ± 0.05	4.30 ± 0.09

Table 5. Physicochemical property contents in Fermented Chilli

Sample ID	pH	**Acidity(% V/V)**	**Salt (%V/V)**
ORS-FC-002	4.49 ± 0.01	1.35 ± 0.05	3.31 ± 0.03
ORS-FC-003	4.48 ± 0.02	1.60 ± 0.01	3.92 ± 0.06
ORS-FC-004	4.52 ± 0.00	1.45 ± 0.05	4.83 ± 0.03

Table 6. Physicochemical property contents in Cucumber Pickle

Sample ID	pH	**Acidity(% V/V)**	**Salt (%V/V)**
ITC-CCP-001	4.29 ± 0.00	1.70 ± 0.01	4.07 ± 0.03
ITC-CCP-002	4.74 ± 0.01	1.35 ± 0.15	4.01 ± 0.03
RUPP-CCP-001	4.36 ± 0.01	1.05 ± 0.05	4.04 ± 0.06
RUPP-CCP-002	4.50 ± 0.01	1.50 ± 0.20	4.07 ± 0.03
AEON-CCP-001	4.24 ± 0.03	1.55 ± 0.05	3.98 ± 0.01

3.2 Cell density of Enterococcus spp.

Thirty-nine samples were conducted for microbiological analysis to enumerate colonies of *Enterococcus spp.* in local fermented vegetables collected from six markets in Phnom Penh, Cambodia. The results of microbiological analysis were shown in Figures 1-6 and expressed as log10 of colonies forming unit (CFU) per mL. Among the collected samples of different categories of fermented vegetables, 36 were found contaminated by *Enterococcus* spp. with concentration ranged from 1 to 4.22 log10 CFU/mL with highest occurrence in samples of ORS-FS-004 and ORS-FC-004. Three samples including AEON-SFC-002, ORS-FGM-001 and AEON-FGM-001 were not detected with the enterococci. The presence of these *Enterococcus* spp. in the fermented food products could be related to the lack of good hygienic practice of local producers and handlers because the gram-positive enterococci are well known as one of the fecal contamination indicators (Giraffa, 2001; Hanchi et al., 2018). However, there were still no clear information with regards to the contamination source of such bacteria in the fermented vegetables whether it came from an endogenous contamination of plant itself or environmental contamination including soil and water (Giraffa, 2001; Hanchi et al., 2018). Being part of lactic acid bacterial group, the enterococci were reported to be involved in fermentation of meat

products, dairy products and even vegetables. Some enterococcal strains associated to plants were identified as *E. faecium*, *E. mundtii*, *E. casselifavus*, *E. faecalis* and *E. sulfurous* (Hanchi et al., 2018). It could be the fact that the enterococci were detected and able to survive in our local fermented vegetables with low pH ranged from 4.36 to 4.52 (Hanchi et al., 2018)and high salt content ranged from 4.33 to 4.83% (Hanchi et al., 2018). These bacteria were also reported for their ability to resist in the food with low to high range of pH and with high salt concentration up to 6.5% (Wolfgang et al., 2003; Byappanahalli et al., 2012; Hanchi et al., 2018). Even though, these bacteria may contribute in some way in fermentation process of certain fermented products, the Genus *Enterococcus* was not allowed to be used due to its prevalence of virulence factors and antibiotic resistance genes as well as the potential ability to cause disease in human (Hanchi et al., 2018).

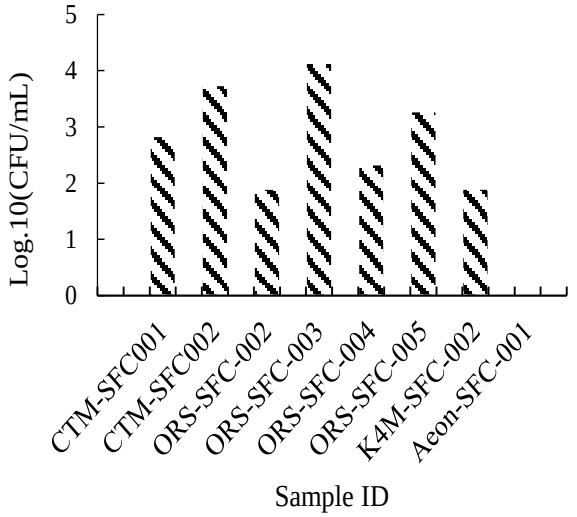

Fig.1. Cell density of *Enterococcuss* spp. in small fermented cucumber. SFC: Fermented Cucumber; FGM: Fermented Mustard Greens; FCC: Fermented Cucumber; FS: Fermented Spider wisp, FC: Fermented Chilli, CCP: Cucumber Pickle. Central Market (CTM), ORS: Orussey Market, Kilo No.4 Market (K4M), AEON Mall 2 (Aeon), Canteen in Institute of Technology of Cambodia (ITC), Canteen in Royal University of Phnom Penh (RUPP)

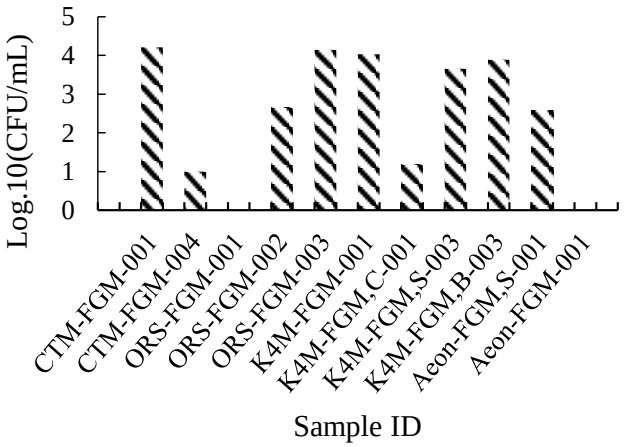

Fig.2. Cell density of *Enterococcuss* spp. in fermented mustsard greens

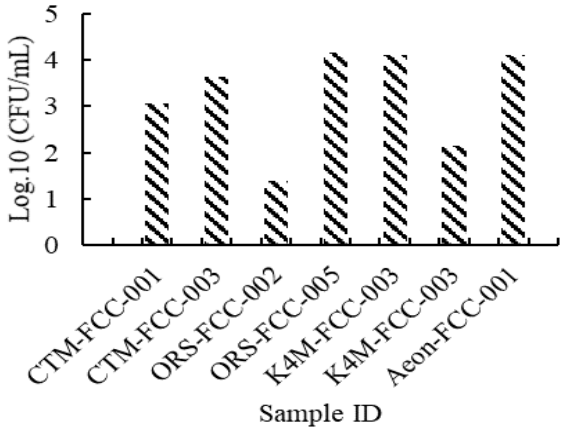

Fig.3. Cell density of *Enterococcuss* spp. in fermented cucumber

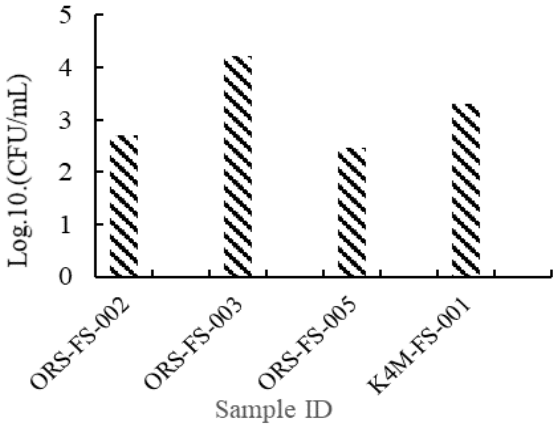

Fig.4. Cell density of *Enterococcuss* spp. in fermented spider wisp

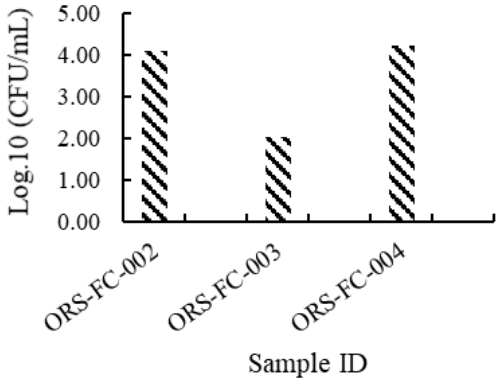

Fig.5. Cell density of *Enterococcuss* spp. in fermented chilli

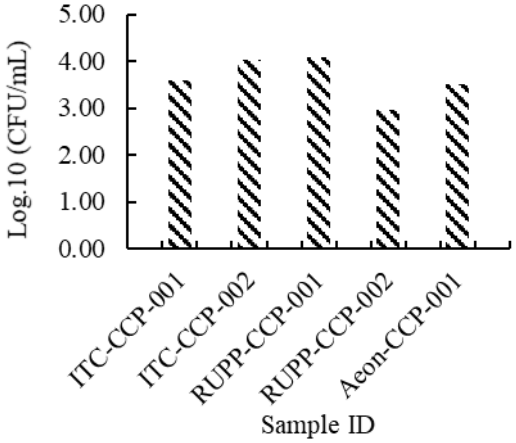

Fig.6. Cell density of *Enterococcuss* spp. in cucumber pickle

3.3 Antimicrobial susceptibility test

Isolation of enterococcal strains were conducted in the current study. A total number of 144 strains of *Enterococcus* spp. isolated from the six selected groups of fermented and pickled vegetables as shown in Figure 7. Strains of *Enterococcus* were mostly collected from fermented mustard greens (36 strains), followed by small fermented cucumber (28 strains), fermented cucumber (28 strains), fermented cleome gynandra (20 strains), cucumber pickle (20 strains) and fermented chilli (12 strains). All the isolated strains of *Enterococcus* spp. were tested for their susceptibility to eight antibiotics such as ampicillin (AMP), ciprofloxacin (CIP), doxycycline (DOX), erythromycin (EM), levofloxacin (LVX), tetracycline (TC), tigecyline (TIG) and vancomycin (VCM). Antibiotics susceptibility results were interpreted as resistance (R), intermediate (I), Susceptible (S), while the percentage of resistance was calculated by resistant strains compared with the total strains of samples. Distribution of susceptibility test of *Enterococcus* spp. against eight types of antibiotics was shown in Figure 7**.** Most of isolated stains *Enterococcus* spp. showed their resistance to tigecycline (54.17%), followed by erythromycin (17.36%), ciprofloxacin (13.19%), tetracycline (10.42%), vancomycin (9.03%), ampicillin (6.25%), levofloxacin (4.86%) and doxycycline (3.47%). According to the previous study, *Enterococcus spp.* which isolated from Swedish retailed chicken were resistant to one or more different antibiotics such as tetracycline, erythromycin and vancomycin (Giraffa, 2002). Furthermore, *Enterococcus* that isolated from another food product such as dairy product, aquatic and raw meat were reported to be resistance to erythromycin within 46% to 55% of isolated strains (Malik and Gupta, 2007). Another reference was proved that enterococci were able to resist to ciprofloxacin, ampicillin and tetracycline by 12%, 8% and 16% respectively (Sukmawinata et al., 2017). Enterococci that were isolated from raw fish and seafood in Swish land were reported that none resistance to vancomycin (Boss et al., 2016). Resistance to tigecycline was remarkable, while it has been reported as a broad spectrum of antimicrobial and able to inhibit the bacteria growth by inactivate protein synthesis in organism cell (Zanzel et al., 2004). The resistance of tigecycline may cause by the bacteria which were able to produce a ribosome to protect their protein during synthesis (Hiroshi and Ruoichi, 2006).

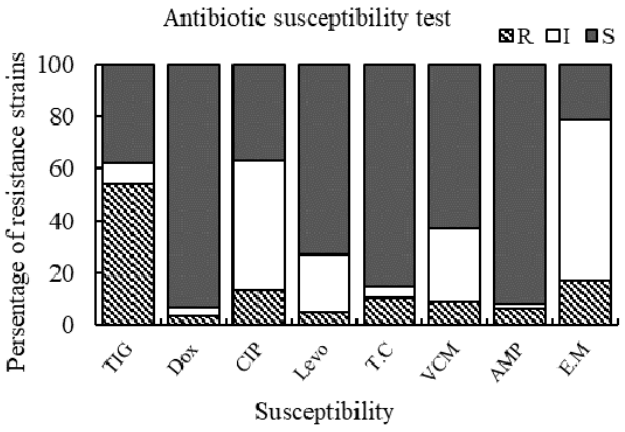

Fig.7. Antibiotic susceptibility test of *Enterococcus* spp. of 144 isolated strains with eight antibiotics R: resistance; I: intermediate; S: susceptible

4. CONCLUSIONS

A total number of thirty-nine fermented vegetables products were collected from three local markets, one supermarket and two school canteens in Phnom Penh. The physicochemical properties of vegetable products were determined including pH with a range of 3.73-4.74, followed by acidity ranged from 1.05-1.60%, and salt content of 3.31%-5.41%. The opportunistic *Enterococcus* spp. were

detected in 92.3% of collected vegetables, reflecting the high contamination of such fecal indicator with highest concentration of 4.22 log10 CFU/mL. The current study also revealed that all isolated *Enterococcus* spp. from fermented vegetables products were resistant at least to one of the eight antibiotics with 54.17% to Tigecyline and less than 20% to other seven antibiotics. Overall, the finding of this study revealed high contamination of enterococci in fermented vegetables products and different magnitude of resistance to the tested antibiotics. It could be a warning sign to producers to reinforce their hygiene practice to reduce contamination level and for safer consumption. In addition, an identification of *Enterococcus faecalis* needs to be carried out in the next phase of study to confirm whether antibiotic resistant strains are *Enterococcus faecalis* or other *Enterococcus* species.

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