Tetracyclines-Resistant bacteria in Shrimps sold in major Supermarkets in Da Nang City, Vietnam

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Abstract
The spread of tetracycline-resistant genes has posed a threat to the therapeutic effectiveness of tetracyclines. Misusing tetracyclines in seafood and shrimp farming has only accelerated this resistance process. Our study aimed to evaluate the status of tetracycline-resistant bacteria in shrimps and to evaluate the probability of spreading these antibiotic-resistant genes. The shrimp purchased from three major supermarkets in Da Nang city were analyzed and the results revealed that the ratio of bacteria resistant to tetracycline and oxytetracycline had a relatively high resistance rate of 4-11%. This study found that all tested supermarkets exhibited resistance with the rate of resistance to oxytetracycline being higher than that of tetracycline.

In situations where bacteria with oxytetracycline-resistant genes and Lactobacillus acidophilus, were co-cultured, the transmission of the resistant genes to Lactobacillus acidophilus occurred. Moreover, the tetracycline resistance of bacteria isolated from shrimp intestines did not decrease even after being cultured in an antibiotic-free medium. The findings of our study strongly indicate the need for immediate action to control the overuse of tetracyclines in shrimp farming and to prevent the further spread of antibiotic-resistant genes.

Keywords: Tetracyclines-resistant bacteria, shrimps, antibiotic-resistant retention, horizontal transfer of antibiotic- resistant gene.

Introduction

Tetracycline is still included in WHO's list of essential medicines for human beings, but only as an eye ointment[15]. It is a broad-spectrum antibiotic that effectively treats many infections such as chlamydia, Lyme disease and anthrax or in cases where penicillin antibiotics do not work and patients are allergic to beta-lactam antibiotics[11]. During the 1970s, owing to its exceptional efficacy, this medication was widely adopted as an over-the-counter drug in Vietnam and various other locations around the world. This abuse has led to widespread resistance to tetracyclines, which has reduced their effectiveness in treating diseases[3]. The advent of a semi-synthetic antibiotic derived from tetracycline has helped to overcome tetracycline resistance and become an essential antibiotic-altering tetracycline in infection treatment. Recent studies have revealed that certain strains of bacteria resistant to tetracycline can develop genes resistant to all these derivations[4]. This resistance has made new antibiotics more challenging and intense.

One of the significant sources of antibiotic-resistant genes is contaminated food containing these bacteria. The shrimp farming and seafood industry are lucrative ventures for many farmers. However, due to climate change, drought and increased salinity, there has been a rise in bacterial infections in seafood livestock. To tackle this challenge, farmers often resort to using tetracycline antibiotics as they are known for their effectiveness, availability and affordability[9]. However, the misuse and improper use of antibiotics may contribute to the emergence of drug-resistant bacteria. This is because constant exposure to antibiotics such as tetracycline or oxytetracyclines at low concentrations led to the development of more resistant genes with more effective resistance mechanisms in bacteria.

Some of these genes are multi-resistant to all antibiotics derived from tetracyclines. The *tet*(X) gene encodes a flavin-dependent monooxygenase that breaks down the antibiotics[10]. This gene is becoming a serious threat to the effectiveness of all tetracyclines against some serious pathogens[7,14]. To combat this emerging threat, continuous monitoring of tetracycline resistance, stricter regulations on the use of tetracyclines (and other antibiotics) in farm animals and the implementation of effective antimicrobial stewardship programs are necessary. Furthermore, it is important to deprioritize the use of tetracyclines for pathogens that may contain the *tet*(X) gene to reduce selective pressure potential.

The aim of this study is to determine the prevalence of tetracycline-resistant aerobic bacteria in shrimps sold at major supermarkets in Da Nang, one of Vietnam's three major cities. Our research also investigated the effectiveness of common food processing methods in destroying these bacteria, as well as their ability to retain antibacterial properties and transfer resistance genes to other species. Our findings provide valuable insights on tetracycline resistance for managers and important safety information for consumers when handling food.

Material and Methods

Shrimps were from the three largest supermarkets of Danang city at two distinct time points, marked as C, G and V. Already prepared culture media and chemicals for biochemical characterization were purchased from Himedia, India. Most other chemicals were provided by Xilong Company in China. Tetracycline and oxytetracycline were procured from LGC, England. We are grateful for the tetracycline-sensitive *Lactobacillus acidophilus* strain B1 by the Biotechnology Laboratory at the University of Danang, University of Science and Technology.

Examination of tetracycline resistance of bacteria in shrimp intestines: Shrimps were purchased and stored at -20^oC until used. Shrimps were defrosted at room temperature and wiped clean with cotton pads soaked in ethanol 70% before collecting their intestines in the laminar box. The intestines of 5 shrimps were homogenized with such amount of saline NaCl 0.85% to have 1g of intestine per 1 mL of buffer by pestle. After that, the mixture was centrifuged at 2000 rpm, 5 minutes, 4^oC and then 100 uL of the supernatant was diluted and spread on the Luria–Bertani (LB) agar with antibiotics at 0, 16, 32 ug/mL[12]. The ratio of antibiotic- resistant bacteria was calculated by dividing the number of colonies on LB agar containing 16 ug/mL or 32 ug/mL by the number of colonies on LB agar without antibiotics.

Individual bacteria colonies on the LB agar containing 32 ug/mL were picked and purified by streaking them on an LB agar plate. The isolated bacterial strains were cultured in LB broth medium without antibiotics at 30°C with shaking at 150 rpm in the incubator (Daihan Scientific IS-30, South Korea) to observe the retention of antibiotic-resistant activity. Every 24 hours, 20 uL of broth culture was placed onto an LB agar medium plate containing antibiotics at a concentration of 32 ug/mL. The remaining broth culture was diluted with fresh antibiotic-free LB medium and cultured under the same conditions.

The cross-streak method was to identify the antibiotic-resistant strains that can be co-cultured with the probiotic *L. acidophilus* B1. A 20 uL of the overnight culture of each antibiotic-resistant bacterial strain was streaked parallel onto the LB agar medium. Two streaks of the broth culture of probiotic strain *L. acidophilus* B1 were perpendicular to the streaks of the tetracycline-resistant bacteria above. The bacterial strain is resistant to *L. acidophilus* B1, as shown by the transparent rings around the strain, making the strain unsuitable for further experiments.

Biochemical properties analysis: Gram staining was performed following the standard protocol[5]. Stained microorganisms were then captured under optical microscopy (Olympus CX31, Japan) at 100 X magnification with an oil objective. Indole formation of bacteria by degrading tryptophan presence in the tryptophan peptone broth medium (casein peptone 10.0 g/L, sodium chloride 5.0 g/L, và tryptophan 10.0 g/L) was detected by Kovacs reagent as described[5]. The Lysine decarboxylase test was performed by inoculating cells with the lysine decarboxylase broth medium for 4 days and checking every 24 hours[5].

Data statistics: The results presented are the mean of three replicated independent experiments and the standard deviation. Data is processed and presented using Excel software, Office 360 version.

Results

Examination of tetracycline resistance of bacteria in shrimp intestines purchased from supermarkets in Da Nang city: The digestive system of the shrimps, which houses most of the body's bacteria, was mixed with physiological saline and then spread onto medium agar plates with or without antibiotics. The two antibiotics tested were tetracycline and oxytetracycline at 16 and 32 ug/mL, the minimum inhibitory concentrations for most bacterial species (Figure 1).

Based on the findings, the rate of antibiotic-resistant bacteria varies depending on the time of collecting samples. Despite that, all three supermarkets showed high levels of tetracycline resistance and particularly elevated levels of oxytetracycline resistance in their shrimp samples. Except for supermarket C, the rate of resistance to oxytetracycline was consistently higher than the rate of resistance to tetracycline. For instance, at supermarkets G and V, the rate was up to 9.24% and 11.23% respectively in shrimp samples exposed to an oxytetracycline concentration of 16 ug/mL. Even at a higher antibiotic concentration of 32 ug/mL, the resistance rate remained at 5.94% as observed in supermarket G's samples. The highest rate of tetracycline resistance was 4.52% in the samples from supermarket C. These results align with previous studies showing widespread misuse of oxytetracycline in aquaculture, particularly shrimp farming[8].

The following study aimed to determine the efficacy of various cooking methods in eliminating total aerobic and antibiotic-resistant bacteria in shrimp samples. Our test involved two types of shrimp - large shrimps (symbol B, weighing 40-50 shrimps/kg) and small shrimps (symbol S, weighing 70-80 shrimps/kg) from supermarkets in Da Nang City, Vietnam. We examined the bacteria count in the intestines of shrimps after steaming (symbol S), stir-frying (symbol F) and grilling (symbol G) compared with uncooked ones (Figure 2).

Steaming was the most effective method of sterilizing shrimps, with over 90% of bacteria destroyed. Grilling and stir-frying, on the other hand, only eliminated 61.00 – 71.00 % respectively. When dealing with bacteria resistant to tetracycline, grilling and stir-frying worked well on small shrimps. Meanwhile, the bactericidal effect of stir-frying and grilling on large shrimps is poor, only eliminating 34.32% and 24.81% respectively (Figure 2). This effect is because steam is a faster and more efficient heat transfer than oil (used in frying) or air (used in grilling). Therefore, it is recommended to stir-fry or grill large shrimp for a long time, rather than just cooking them until they turn evenly pink, to increase the disinfectant effect.

Oxytetracycline-resistant bacteria were relatively eliminated by food processing methods with bactericidal efficiency greater than 90%, except for the stir-frying large shrimps where the efficiency drops to 75.41 %. Our results provide valuable insights into the best cooking methods to kill bacteria in shrimp samples.

The retention of antibiotic-resistant property in bacteria: To comply with regulations on antibiotic residues in shrimp, farmers typically cease using illegal antibiotics for a certain period before selling. However, the absence of antibiotic residue does not necessarily mean a decline in the risk of antibiotic-resistant bacteria in shrimp. In the study, bacterial strains resistant to tetracycline and oxytetracycline (6 strains) isolated from shrimp intestines were then cultured in a nutrient-rich medium without antibiotics for a period with a fresh medium replaced daily. The broth culture of the bacteria was carefully placed onto agar medium, both with and without antibiotics and allowed to incubate for 24 hours. The cell density observed after 24 hours of incubation in the incubator on two medium agar plates with and without antibiotics shows the retention or loss of antibiotic resistance activity after living in a medium without antibiotics.

The study found that the strains of bacteria resistant to tetracycline did not change after being cultured for three continuous days in an environment free of antibiotics (Figure 3). However, all the strains (6 in total) of bacteria resistant to oxytetracycline showed a decline in antibiotic resistance when comparing the number of colonies grown on media supplemented with oxytetracycline at a concentration of 32 ug/mL to those grown on un-supplemented media. The results suggested that tetracycline resistance was more durable than oxytetracycline resistance, even though the rate of oxytetracycline resistance was higher. It is worth considering that temporarily discontinuing antibiotics could lead to a decline in resistance toward certain antibiotics while maintaining resistance to others. Further, large-scale studies are needed to evaluate the reduction level of antibiotic-resistance genes in microorganisms and to provide better recommendations on antibiotics used in livestock production.

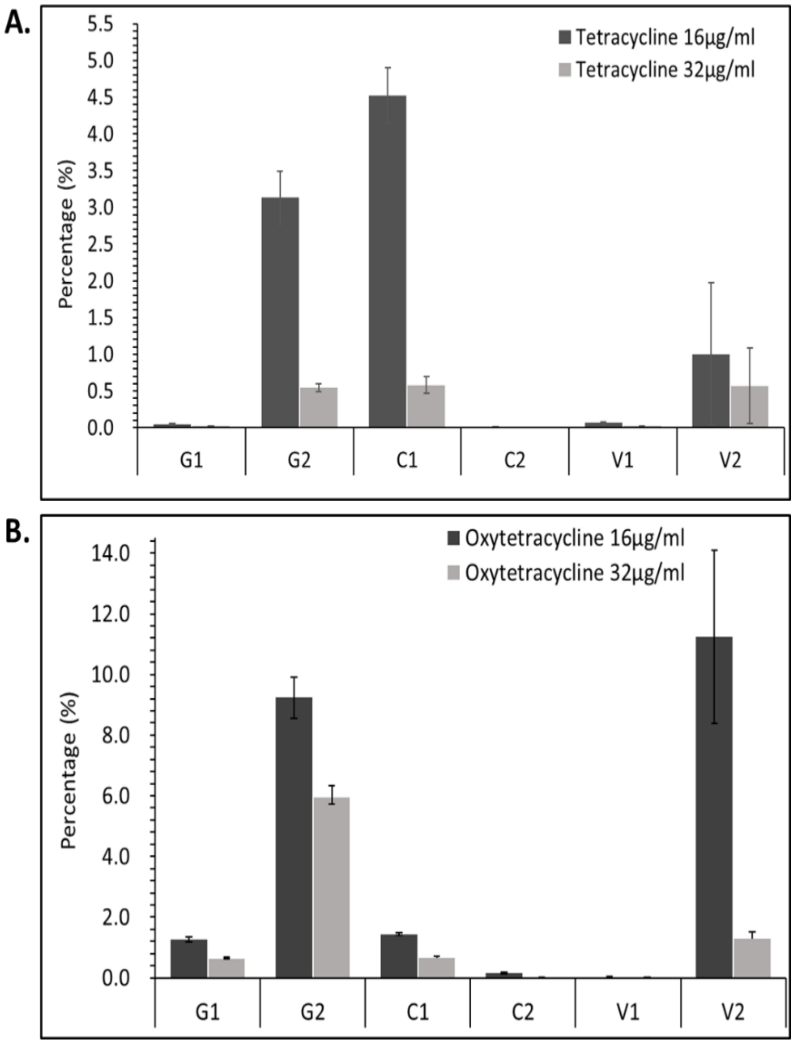

Figure 1: The proportion of antibiotic-resistant bacteria against total aerobic bacteria were determined in 1g of digestive system from shrimps purchased at three supermarkets at two different time points. Figure A displays the prevalence of tetracycline resistance and B for oxytetracycline resistance. The mean and standard deviation of 2 boxes of shrimps, randomly selected at the same time and supermarket, are shown.

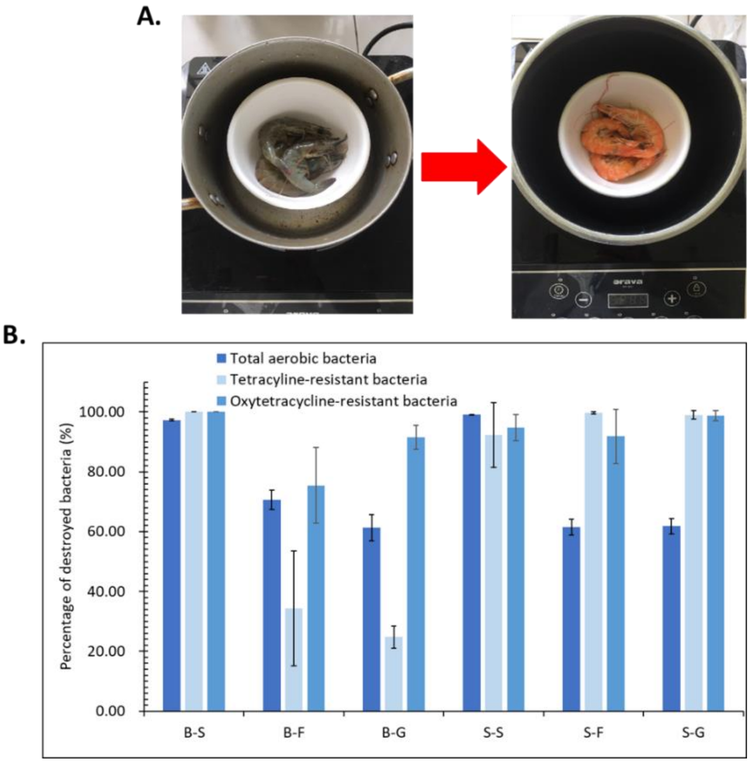

Figure 2: The effectiveness of different cooking methods in killing total aerobic bacteria, tetracycline- and oxytetracycline- resistant bacteria in shrimp of varying sizes. Shrimps cooked until a pink color appeared (Figure A) were used for evaluation. The symbols in each group represent the size of the shrimp - the method of cooking used (Figure B). Specifically, large shrimps (symbol B, weighing 40-50 shrimps/kg) and small shrimps (symbol S, weighing 70-80 shrimps/kg) were cooked by steaming (symbol S), stirring frying (symbol F) and grilling (symbol G). The means and standard deviations are shown.

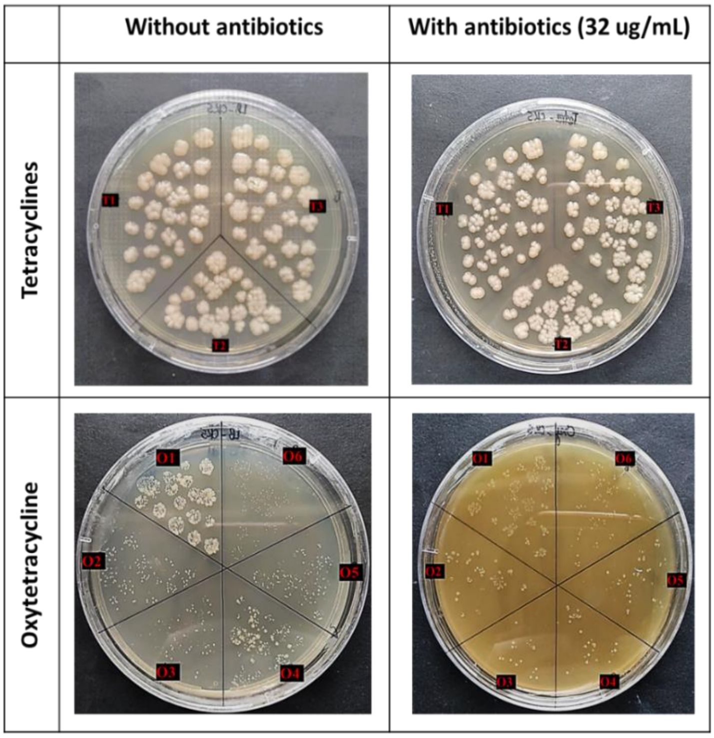

Figure 3: The maintenance of tetracyclines resistance of isolated bacteria in shrimp intestines. Out of the strains tested, 3 were found to be resistant to tetracycline and 6 were resistant to oxytetracycline when exposed to a high concentration of 32 ug/mL. To test for resistance, 20 uL of the overnight culture in LB broth medium of single strains were dropped onto a media plate containing antibiotics (right column) and no-antibiotics (left column) after 3 days of continuous culture (with medium renewal).

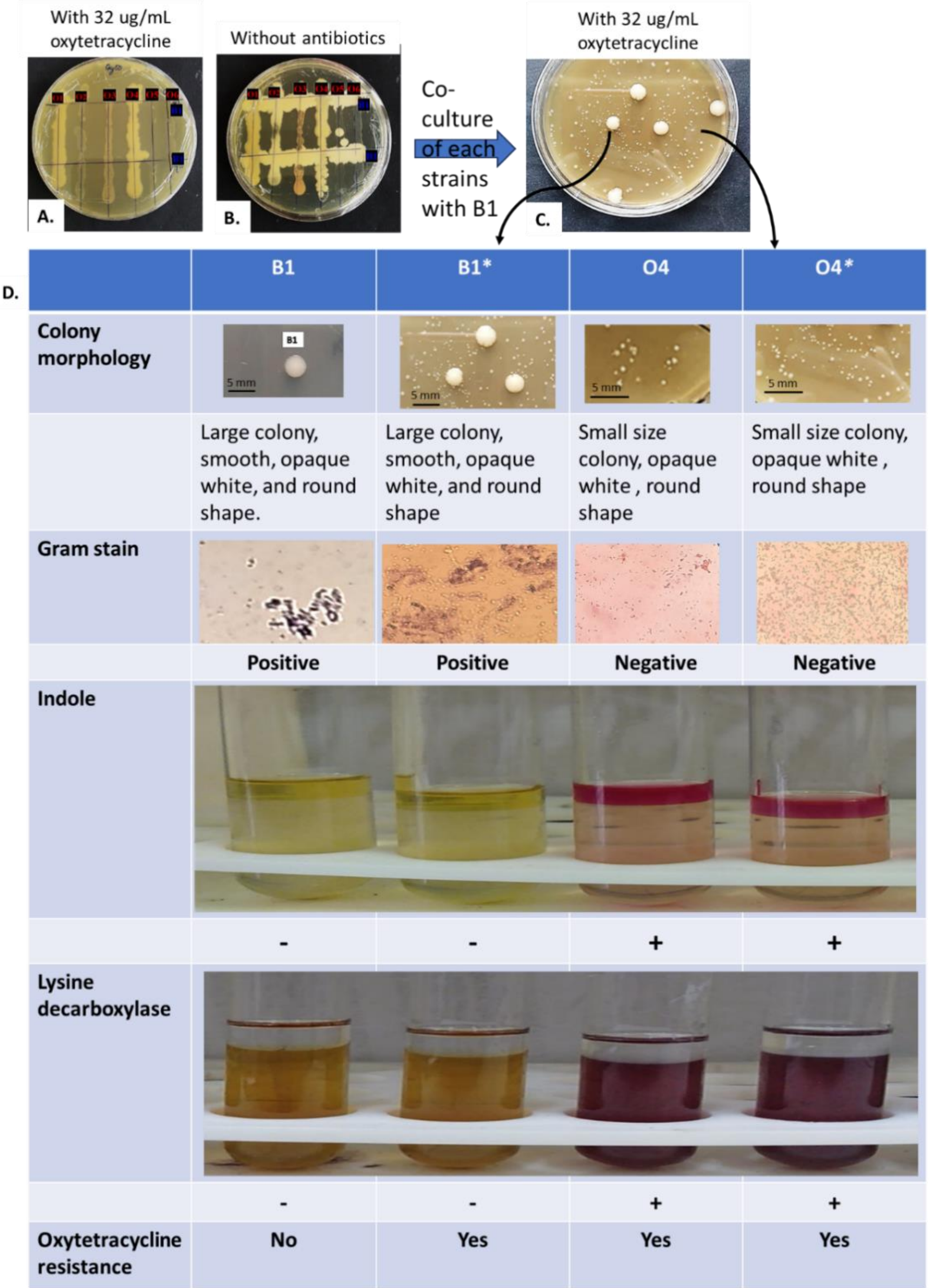

Figure 4: Characteristics of *L. acidophilus* strain B1 and oxytetracycline-resistant bacterial strain O4 before and after co-culturing. Cross-streak culture of 6 oxytetracycline-resistant bacteria with *L. acidophilus* strain B1 on the medium with (A) 32 ug/mL oxytetracycline and (B) without antibiotics. The appearance of two different colonies on the medium agar with 32 ug/mL oxytetracycline after 5 days co-culturing of (C) B1 and O4 strains in free-antibiotic broth medium. (D) showed the biochemical characteristics of tested strains. B1 and O4 symbols for original bacterial strains. B1* and O4* denote bacteria from co-culture broth spreading on an agar medium supplemented with 32 ug/mL oxytetracycline.

Antibiotic-resistant genes being transmitted to other bacterial species: There is a risk of antibiotic-resistant genes transmitted to other species. This transmission can happen due to the possibility of gene transfer. In this experiment, a mixture of oxytetracycline-sensitive *Lactobacillus acidophilus* strain B1 and purified oxytetracycline-resistant bacteria was co-cultured in a culture broth without antibiotics. After culturing for 24 hours, the mixture was diluted in a fresh medium and spread on an agar medium supplemented with antibiotics. After three days in culture, two different colonies in the co-culture of oxytetracycline-resistant strain O4 and *L. acidophilus* B1 occurred on the medium agar containing 32 ug/mL oxytetracycline (Figure 4). These two colonies were isolated and tested to compare their biochemical properties with the original strains.

The results showed that the colonies were identical to the original strains in colony morphology, gram staining and other biochemical characteristics. That means *L. acidophilus* B1 acquired resistant activity to oxytetracycline after co-culturing with oxytetracycline-resistant bacteria strain O4. This result suggests that antibiotic-resistant genes can transmit between different bacterial species. This awareness needs to be concerned to develop effective strategies for controlling and preventing the spread of antibiotic-resistant genes.

Discussion

Tetracycline antibiotics were discontinued for nearly two decades due to the limits on treatment effectiveness when resistance spread. They were only returned when more effective semi-synthetic antibiotics with more advantages than natural tetracyclines were developed[11]. However, the risk of increasing antibiotic resistance is alarming. Since all tetracycline antibiotics have the same origin, bacteria can develop resistance mechanisms for all of them. The emerging *tet*(X) gene codes for enzymes that decompose all antibiotics of the tetracycline group[10]. Among the six ESKAPE pathogens, which are notorious for causing hospital-acquired infections across the globe, *tet*(X) gene has been discovered in four of them - namely *Enterococcus faecium*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*[1,6]. Overuse of tetracyclines in animal husbandry and treatment can promote the rapid spread of these resistance genes, which poses a risk of reducing the therapeutic effectiveness of all antibiotics in this family again.

Despite policies prohibiting the use of antibiotics in shrimp farming, our study shows that bacteria resistant to tetracyclines still exist at high rates. The rate of bacteria resistant to tetracycline and oxytetracycline in shrimp intestines purchased from supermarkets was up to 4.4% and 11.5% respectively (Figure 1). These results support previous surveys on the illegal use of antibiotics in shrimp farming and aquaculture, where oxytetracycline is the most abused antibiotic[8]. Our research has uncovered that oxytetracycline resistance genes spread and transmit to other bacterial species (*L. acidophilus*) more advantageous than tetracycline resistance genes, as illustrated in figure 4. This could be a key factor contributing to the spread of oxytetracycline resistance genes in shrimp ponds and the environment.

It is worth noting that *L. acidophilus* is a probiotic strain commonly found in human bodies to offer health benefits to humans[2]. The ability of this probiotic strain to acquire antibiotic-resistance genes from antibiotic-resistant bacterial strains that enter the human body, can result in the spread, retention and circulation of the gene between strains including those that cause human diseases. As a result, this reduces the effectiveness of antibiotics in killing those pathogens. It is reassuring that steaming, a widely used shrimp cooking method, can effectively kill most tetracyclines-resistant bacteria (Figure 2B). However, this method does not eliminate the risk of antibiotic resistance genes spreading due to antibiotic overuse. Antibiotic resistance could have a direct impact on the health of farmers who have little protective equipment and lack awareness of personal hygiene when caring for the pond. Additionally, there is a risk of indirect transmission when shrimp pond water is discharged into the environment without proper treatment[13].

Conclusion

According to our research, the number of bacteria resistant to tetracyclines in shrimp purchased from supermarkets in Danang, Vietnam, is still high. Compared to other cooking methods, steaming shrimp is effective against total aerobic and antibiotic-resistant bacteria. These bacteria can transfer antibiotic-resistance genes to other bacteria residing in human bodies. Furthermore, tetracycline resistance is persistent in the absence of antibiotics for some time. Consequently, discontinuing antibiotic use before sale does not necessarily translate to a decline in the antibacterial resistance activity of these bacterial strains.

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