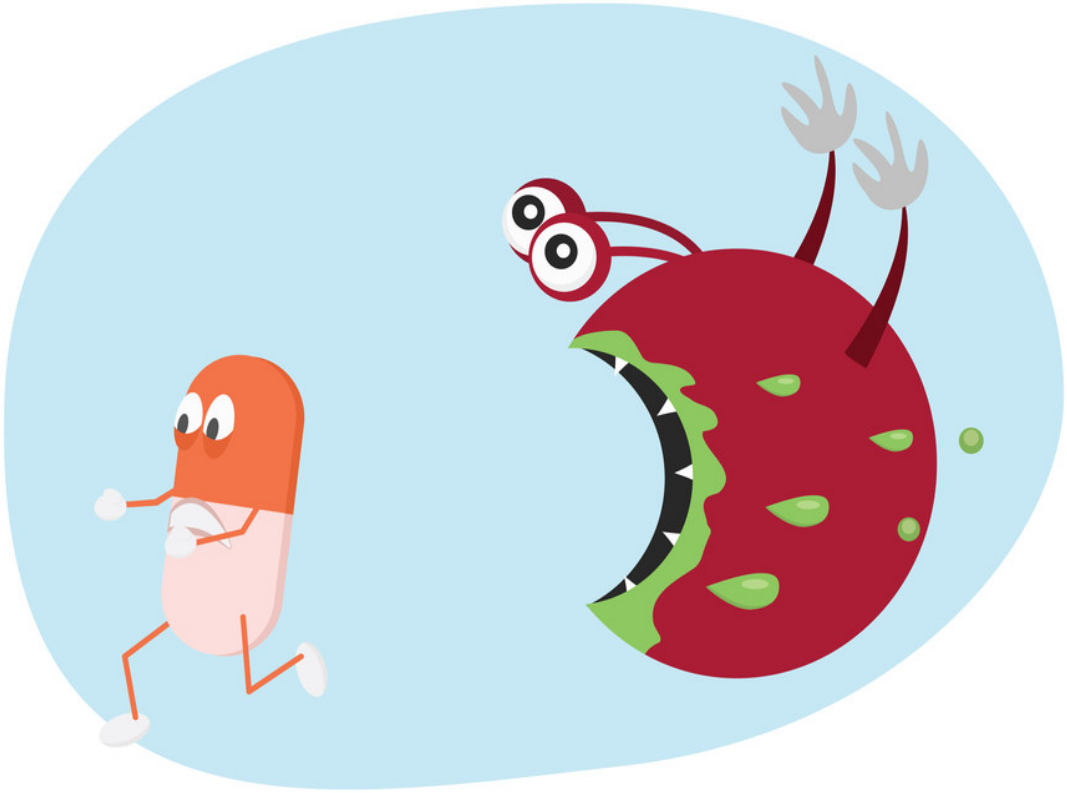
ANTIMICROBIAL ACTIVITY OF NISIN A ON *MICROCOCCUS LUTEUS* BACTERIA COMPARED TO ANTIOBIOTICS, TETRACYLINE



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Abstract

The discovery of Antibiotics in 1940 was a tremendous breakthrough in the fight against pathogenic bacteria and the spread of infectious diseases. However, the threat posed by resistant bacteria has increased the need for an alternative solution to fight bacteria resistance, which has been a global concern. Moreover, antimicrobial peptides (AMPs) such as Nisin has been widely used as a food preservative in the food industry and as an alternative approach to fighting the scourge against bacteria that are resistant to antibiotics. Bacteria resistance is a great concern to the health sector, as resistant bacteria prevent the effective treatment of diseases and infections.

The aim of this study was to analysed the effectiveness of the AMP activity of Nisin A on *Micrococcus luteus (M. luteus)* compared to the antibiotic, Tetracycline. The efficiency was tested by measuring the two antimicrobial agent's ability to inhibit the growth of *M. luteus* with inhibition zones assay. Therefore, the bactericidal or bacteriostatic effect of the antimicrobial agents was tested at different concentrations, ranging from 50 μg/ml to 125 μg/ml.

The treatment of Tetracycline against *M. luteus* showed a bigger zone of inhibition radii of 10.4 mm at the highest concentration compared to the Nisin A treatment which was 6.94 mm at the same concentration. Moreover, the inhibition zones for the Nisin A treatment was clearer and more visible compared to the inhibition zones from the Tetracycline treatment. However, this study showed that there was no significant difference between the two antimicrobial treatment against *M. luteus* with a p-value of 0.29.

Table of Contents

1.0 INTRODUCTION 4

2. PROBLEM FORMULATION 6

3.0 THEORY 7

3.1 BACTERIA 7

3.1.1 Bacteria Growth 7

3.1.2 Micrococcus Bacteria 9

3.2 BIOFILMS 9

3.3 ANTIBIOTICS 10

3.4 TETRACYCLINE 10

3.4.1 Tetracycline Mechanism of Action 11

3.4.2 Tetracycline Mechanism of Resistance 12

3.5 ANTIMICROBIAL PEPTIDES 12

3.5.1 AMPs Mechanism of Action 13

3.6 NISIN 14

3.6.1 Nisin Mechanism of Action 15

4.0 METHOD 16

4.1 MICROCOCCUS LUTEUS CULTURE 16

4.2 BACTERIA PLATING AND ANTIMICROBIAL TREATMENT 16

4.3 MEASUREMENT OF INHIBITION ZONE 17

4.4 DATA ANALYSIS 18

5.0 RESULT 19

6.0 DISCUSSION 21

6.1 EXPERIMENTAL METHOD 21

6.2 EXPERIMENTAL RESULT 23

6.3 AMPS RESISTANCE 24

7.0 CONCLUSION 26

8.0 PERSPECTIVE 27

9.0 APPENDIX 28

10.0 BIBLIOGRAPHY 30

1.0 Introduction

Bacteria are the smallest free living microorganisms, which was first observed under the microscope by a Dutch scientist called Anton van Leeuwenhoek in 1674 (Tan & Tatsumura, 2015). Bacteria are prokaryotes, and are characterized with a free DNA in the cytoplasm of its cell. They can be found in many forms and shapes, such as rod, spiral or cocci shape (Denyer, Hodges, & Gorman, 2004). Bacteria can thrive in different environment, such as in air, the upper soil layers, internal and external regions of the human body. However, bacteria can also survive in extreme and adverse conditions (Denyer e.a., 2004).

Bacteria are widely found in most environment, they are mainly beneficial and can play a vital role in the digestion of food, enhance the immune system and can be used for the production of food products such as cheese and yogurt. However, there are other opportunistic bacteria that can cause infection, which are referred to as pathogenic bacteria, these harmful bacteria produce chemical toxins that can cause disease and infections (Aparna, 2015).

The discovery of antibiotics in 1928, has saved millions of lives and prevented the spread of infectious diseases caused by bacteria (Tan & Tatsumura, 2015). Antibiotics are chemical substances which are used to inhibit the growth of bacteria and treat diseases caused by pathogenic bacteria. Antibiotics can either be bacteriostatic, stop the rapid replication of bacteria or bactericidal, kill the bacteria. However, the misuse of antibiotic when it is not needed can cause bacteria resistance, a situation where antibiotics have no effect on certain bacteria which used to be susceptible to antibiotics (Davies & Davies, 2010). Bacteria can develop resistance to antibiotics through the modification of the antibiotics target site, preventing the antibiotics from entering the cell or pumped out quickly before it can act on the cell. Furthermore, the bacteria can produce an alternative target site like an enzyme while it continues to produce the original sensitive target site or acquire resistance from other bacterium (Hawkey, 1998). Moreover, these may lead to new strain of bacteria which can be resistant to several type of antibiotics, such as methicillin resistant *Staphylococcus aureus* (MRSA) (Davies & Davies, 2010).

The emergence of disease caused by resistant bacteria is a globally issue, because it affects the prevention and treatment of the rapidly increasing disease and infections caused by harmful bacteria

(WHO, 2016). Moreover, antibiotic resistant bacteria are also known to be one of the world's greatest threat to human health (World Economic Forum, 2013).

With the increasing concern on the resistance of bacteria to antibiotics and the ineffectiveness of antibiotic to kill resistance bacteria, also known as superbugs, an alternative use of antimicrobial peptides (AMPs) has been adopted. AMPs are used as a substitute approach to fight against the scourge of bacteria resistance (Bahar & Ren, 2013; Hancock & Sahl, 2006). AMPs are natural peptides that are produced by many prokaryote and eukaryote. However, there are also synthetic AMPs that are produce by chemical synthesis (Wade, Lin, Hossain, & Dawson, 2012). They can regulate the immune system and kill other bacteria (Bahar & Ren, 2013; Delves-Broughton, 1996). An example of an AMP is Nisin, which will be used to analyze the antimicrobial effect on the bacteria *M. luteus* in this study.
This project will investigate the effectiveness of AMP as an alternative to antibiotic in the prevention and treatment of bacterial resistance.

2. Problem Formulation

How efficient is the antimicrobial peptide activity of Nisin A on *Micrococcus luteus* bacteria compared to antibiotics, Tetracycline?

Hypothesis:
We expect that the antimicrobial peptide Nisin A *will have a better effect on the bacteria than Tetracycline*

3.0 Theory

3.1 Bacteria

Bacteria can be classified based on forms, shapes and cell wall structure. Bacteria are categorized by their different cell wall structure into two major groups, Gram-positive and Gram-negative bacteria. The Gram-positive bacteria have a cell wall that are thicker and consist primarily of a single lipid membrane whereas the Gram-negative bacteria have a thin cell wall and consist of two layers of lipid membrane (Silhavy, Kahne, & Walker, 2010). An example of Gram-negative bacteria is *Escherica coli* and for Gram-positive is *Micrococcus luteus* bacteria (Fair & Tor, 2014).

3.1.1 Bacteria Growth

Growth can be characterized as an increase in the cell size or cell mass during the development of an organism. Most bacteria reproduce by binary fission, a cell division of a parent bacterium cell into two daughter cells (Gerardi, 2006). Bacteria growth can be influenced by environmental and nutritional factors. The environmental factors include; temperature, pH, oxygen level, pressure, and the moisture content of growth medium of the bacteria. The nutritional factor includes the amount of carbon, nitrogen, phosphorus, nitrogen, sulphur and other trace elements provided by the growth medium (Denyer e.a., 2004). However, to study the growth population of bacteria, a viable bacterium cell is inoculated in a sterile broth under an optimal growth conditions. The increase in the cell mass of the bacteria cells is measured with the aid of a spectrophotometer. The spectrophotometer measures the optical density (OD), which is a measure of the amount of light absorbed by the bacterial cells. The dynamics in the growth pattern of the bacteria can be studied by a growth curve, a plot showing the logarithm of the number of the viable cells as a function of the incubation time. The curve has four different phases as seen in figure 1, which include; the lag phase, exponential phase, stationary phase and death phase (Willey, M., & J., 2014).

During the lag phase, there is no instant increase in the number of cells when the bacteria are introduced into the growth medium. This may be a result of the cells trying to adapt to the new environment. At this phase, the cells are synthesizing new components such as ATP, essential co-factor and ribosome that are necessary for growth to begin. Moreover, a delay in the lagging phase,

can also be associate with the nutritional difference in the previous and the present growth medium (Madar e.a., 2013).

In the exponential phase, the bacteria cells are rapidly growing and dividing at the maximum rate. During this phase, the metabolic process of the bacteria increases and the DNA replicates exponentially by binary fission at a constant rate. Then, the cells begin to replicate into four, eight, sixteen and so on, with a doubling factor of 2^n; where n is the number of generation. Therefore, the time taken for bacteria to double in number during a specific time is referred to as generation time, which may differ in respect to different organism (Gerardi, 2006).

In the stationary phase, the total number of the viable bacteria cells remain constants which is represented by the horizontal line in the growth curve as seen in figure 1, this may result from a balance between cell division and cell death, or the bacteria cell may stop replication but remain metabolically active. However, the availability of nutrient, accumulation of waste material, toxic metabolite in the medium can result in a shift in the environmental factors such as oxygen availability, pH of the medium and temperature. This may prevent the replication of the bacteria and cause death. Therefore, resulting in the stationary phase (Denyer e.a., 2004).

During the death phase, there is an exponential death of bacteria cells, as a result of the depletion of nutrient, the continuous accumulation of metabolic waste and toxic material in the growth medium. In the death phase, the bacteria losses its ability to produce and begin to die at a constant rate due to the unfavorable condition (Willey e.a., 2014).

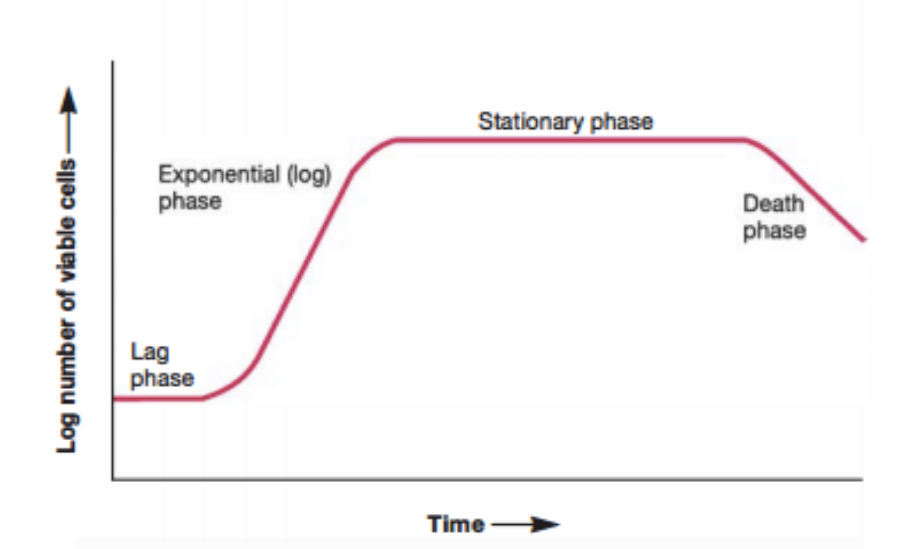

Figure 1. Bacteria growth curve.
Showing the lag, exponential (log), stationary and death phase (Willey e.a., 2014).

3.1.2 Micrococcus Bacteria

Bacteria that belongs to the genus *Micrococcus* are mainly Gram-positive cocci bacteria, which are categorized in the family *Micrococcaceae. Micrococcus* bacteria are spherical in shape, and occur in pair, tetrad or irregular clusters. *Micrococcus* are non-spore forming and aerobic bacteria, with an optimal growth temperature ranging from 25 to 37 °C. *Micrococcus* bacteria are commonly found on the human skin (Seifert, Kaltheuner, & Perdreau-Remington, 1995; Skerman, Mcgowan, & Sneath, 1997). However, *Micrococcus* is not regarded as pathogenic organism, but there have been recent studies that show that some infectious disease such as septic arthritis, meningitis and endocarditis were attributed to *Micrococcus* bacteria (Fosse e.a., 1985; Seifert e.a., 1995).

There are nine different species of *Micrococcus* bacteria which include, *Micrococcus agilis*, *Micrococcus halobius*, *Micrococcus kristinae*, *Micrococcus lylae*, *Micrococcus nishinomiyaensis, Micrococcus roseus, Micrococcus sedentarius, Micrococcus varians* and *Micrococcus luteus* (Young e.a., 2010). The specie *Micrococcus luteus* will be used to analyse the effect of AMP Nisin A compare to the antibiotic Tetracycline.

3.2 Biofilms

The ability of microorganism to form biofilm is a universal characteristic of bacteria. Bacteria can adhere to almost every surface and form an architectural complex structure known as biofilm. This commonly consist of more than one species of bacteria, which collectively exist together as a functional group. However, all biofilms contain an extracellular matrix that holds the cell together, which is composed of a polysaccharide biopolymer along with other component such as protein or DNA (Branda, Vik, Friedman, & Kolter, 2005). The formation of biofilm usually starts with the adhesion of the pioneer bacteria cells to a surface with the aid of a fimbriae or non-specifically by extracellular polysaccharide (EPS) and then the encased cells grow and divide to produce micro-colonies. Furthermore, biofilm help bacteria to trap nutrient for the growth of the enclosed cells and provide support for the attachment to a surface in a flowing system (Denyer e.a., 2004).

Biofilms are of beneficial importance to bacteria, because it gives bacteria resistance to the effect of antimicrobial agents, protection from predators and prevent the immune response in host organism (Mah & O'Toole, 2001). Moreover, in humans the encased bacteria are not easily exposed to the

effect of the immune system response and bacteria biofilms are less susceptible to antimicrobial agents compared to the free living bacteria (Jamal, Tasneem, Hussain, & Andleeb, 2015).

3.3 Antibiotics

Alexander Fleming discovered that a glass with *Staphylococcus* culture in his laboratory was contaminated with mold, which inhibited the growth of the bacteria around the infected area and these mold that killed the bacteria were discovered to be Penicillin (Tan & Tatsumura, 2015). The mechanism of action of antibiotics can either have a bacteriostatic or bactericidal effect on bacteria. These can be categorized under the four main modes of action, which include; the inhibition of DNA, cell wall, RNA and protein synthesis of the bacteria cell.

Firstly, antibiotics such as Quinolones can inhibit the synthesis of DNA in bacteria, by binding to topoisomerases, an enzyme that catalyze the under winding and relaxation of DNA. The antibiotics forms a complex with the topoisomerase II (DNA gyrase) of the bacteria, blocking the replication fork and prevent the synthesis of DNA. Resulting in the death of the bacteria cell (Drlica, Malik, Kerns, & Zhao, 2008; Kohanski, Dwyer, & Collins, 2010).

Secondly, beta Lactams is an antibiotic, that can cause lysis of bacteria cell wall. The antibiotic interfere with the cross linking of the peptidoglycan by inhibiting the peptides bonds formation reaction, which is catalyzed by trans-peptidases and this prevent the biosynthesis of the bacteria cell wall (Rice & Bayles, 2008).

Thirdly, the mode of action of antibiotics that target RNA such as Rifampicin inhibits the initiation stage of transcription. This kind of antibiotics binds with RNA polymerase with high affinity and prevent transcription in bacteria (Floss & Yu, 2005) .

Lastly, some antibiotics can inhibit protein synthesis by targeting the ribosome of bacteria cells. These can either inhibit the 50S or 30S ribosomal subunit (Joseph, 2003). Macrolides and Oxazolidinone are examples of 50S inhibitors while Tetracycline is an example of 30S inhibitor. These type of antibiotics can cause cell death by binding to the ribosomal subunit and prevent the synthesis of protein (Kohanski e.a., 2010).

3.4 Tetracycline

The first Tetracycline, Chloretetracycline was discovered in 1945 by Dr. Benjamin Dugger.

Tetracycline belong to the family of antibiotics that are characterized by four cyclic rings, as seen in figure 1 (Wright, Seiple, & Myers, 2015).

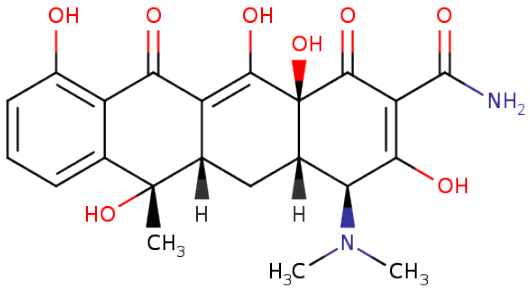

Figure 2: Chemical structure of Tetracycline.
(https://www.drugbank.ca/structures/DB00759/image.png)

Tetracycline is produced as a by-product from the metabolism of *Streptomyces* bacteria, such as *Streptomyces aureofaciens, Streptomyces rimosus* and *Streptomyces viridofaciens.* Chlortetracycline molecules are produced from *Streptomyces aureofaciens* and oxytetracycline is produced from *S. rimosus*. However, Tetracycline has broad spectrum, with the ability to inhibit the growth of aerobic Gram-positive and Gram-negative bacteria (Chopra & Roberts, 2001).

3.4.1 Tetracycline Mechanism of Action

Tetracycline is a bacteriostatic antibiotic that block the binding to the 30S of aminoacyl tRNA to the A site of the ribosome and stop protein synthesis (as seen in Figure 2) (Chopra & Roberts, 2001).

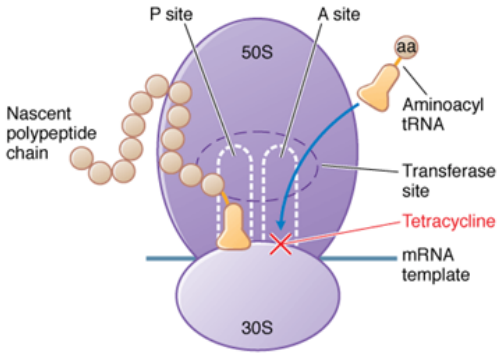

Figure 3: ***Tetracycline's Mechanism of action***.
Showing the binding of Tetracycline to the A- site of 30S sub unit.
(http://www.antibiotics-info.org/tetracycline.html)

The ribosome is composed of two sub units; 30S which is the small part and 50S which is the large part. The small part of the ribosome binds to the mRNA from the 5'- end. The ribosome contains three tRNA binding sites, the exit (E) site, the peptidyl (P) site and the aminoacyl (A) site. The anticodon on the tRNA binds to the complementary codon on the mRNA template. The process starts when the tRNA binds to the ribosome on the P site with the anticodon AUG (the start codon), which encodes the amino acid methionine and binds to the complementary codon. The A site binds the specific tRNA following anticodon to its complementary code in the mRNA and a new amino acid is translated which are linked together by peptide bonds. The A site moves into the P site and the empty P site moves over to E site. The process continues until the stop codon moves into the P site. The ribosome's subunits split apart from each other. Then, Tetracycline binds to the A site and prevent binding of the amino acyl, which inhibit the synthesis of protein (Joseph, 2003). However, the use of Tetracycline are reduced due to the increased bacteria resistance (Chopra & Roberts, 2001).

3.4.2 Tetracycline Mechanism of Resistance

The mechanisms for resistance of Tetracycline are mainly efflux and ribosomal. The efflux proteins transfers Tetracycline and reduces the intracellular concentration of the antimicrobial agent, which protects the ribosome in the cell. The proteins exchange a proton for a Tetracycline cat-ion complex and this process reduces the intracellular concentration of Tetracycline and protect the ribosomes in the bacteria cell. Moreover, the ribosomal protein from the bacteria can prevent Tetracycline's mode of action in both *in vivo* and *in vitro*. The bacteria ribosomal proteins compete for the binding on the ribosomes with a higher affinity and reduce the binding of Tetracycline's to the ribosomes (Chopra & Roberts, 2001).

3.5 Antimicrobial Peptides

AMPs are peptides with a varying number of amino acids from five to over a hundred and are arranged in different groups based on amino acid composition, size and conformation. AMPs can be found in both prokaryotes and eukaryotes, which play a vital role in the innate immune system against viruses, bacteria and fungi. AMPs can also reduce inflammatory response and have a broad spectrum of targeted organisms ranging from viruses to parasites (Bahar & Ren, 2013; Guilhelmelli e.a., 2013). AMPs can be divided into two groups, cationic peptides and non- cationic peptides. Cationic peptides are the largest group, which is divided into three classes based on their structure. The first class is

linear peptides composed of α-helical structures. The second class contains cysteine and have a single or some disulphide bonds, the third class of cationic peptide group consist of molecules that have specific amino acids such as proline, glycine or histidine. The non-cationic peptides group include anionic peptides, aromatic dipeptides and peptides derived from oxygen-binding proteins. These molecules are weak in bactericidal activity compared with cationic peptides, but can improve the activity of cationic peptides (Vizioli & Salzet, 2002)

3.5.1 AMPs Mechanism of Action

AMP can be divided into four categories based on their target and mechanism of action, which include; antiviral, antifungal, antiparasitic and antibacterial activities. Moreover, most antibacterial peptides belong to the cationic peptides group. The activity of these peptides is dependent upon interaction with the bacterial cell membrane. The interaction takes place through attraction between the cationic peptide and anionic components on the outer bacterial envelope such as lipoteichoic acid on Gram-positive bacterial or phosphate group on the lipopolysaccharides on Gram-negative bacteria and form transmembrane pores (Jenssen, Hamill, & Hancock, 2006). The AMP mediated killing mechanisms can either be transmembrane pore formation mechanism or intercellular killing (Bahar & Ren, 2013).

In the transmembrane pore formation mechanism, the AMPs are amphipathic having both hydrophobic regions, which can interact with the lipid components of the bacteria membrane, and hydrophilic regions interact with the phospholipid head group of the bacteria. In addition, there are several models that has been recommended to describe how the antibacterial peptides interact with the bacteria membrane to form pore. These models include, aggregate model, toroidal pore model, carpet model and barrel-stave model (Jenssen e.a., 2006).

However, in the intercellular target mechanism, the AMPs have the ability to penetrate the bacterial cell membrane to inhibit the cell wall synthesis, enzymatic activity, DNA, RNA and protein synthesis can also inhibit protein folding and cause cell death (Guilhelmelli e.a., 2013; Jenssen e.a., 2006).

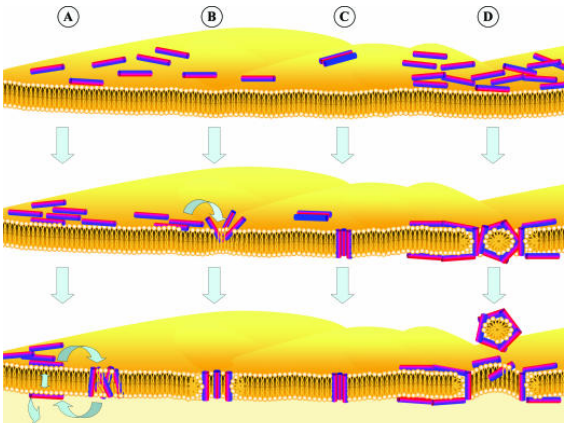

Figure 4*: Antibacterial peptides' Mechanism of action.*
(cylinder, red is the hydrophilic regions, blue is the hydrophobic regions) interact with bacteria membrane (yellow is the lipid bilayer) in four models: A (Aggregate model), B (Toroidal pore model), C (Barrel-stave model) and D (Carpet model) (Jenssen e.a., 2006).

3.6 Nisin

Nisin is a AMP made up of 34 amino acids, as shown in figure 4. It is produced from the fermentation of *Lactococcus lactis* and widely used as a food preservative (Delves-Broughton, 1996). There are five known variants of Nisin, which are Nisin A, Z, Q, U and F (De Kwaadsteniet, Ten Doeschate, & Dicks, 2008). The two naturally occurring Nisin variant A and Z have been found to have similar activities. However, Nisin A shows a difference in a single amino acid residue at position 27 having a histidine while in Nisin Z is replaced by an asparagine. The structural substitution between these two amino acid, histidine and asparagine has no influence on the antimicrobial effect of the Nisin variants, but it gives the Nisin Z a higher solubility and diffusion characteristics in contrast to Nisin A (De Vos, Mulders, Siezen, Hugenholtz, & Kuipers, 1993).

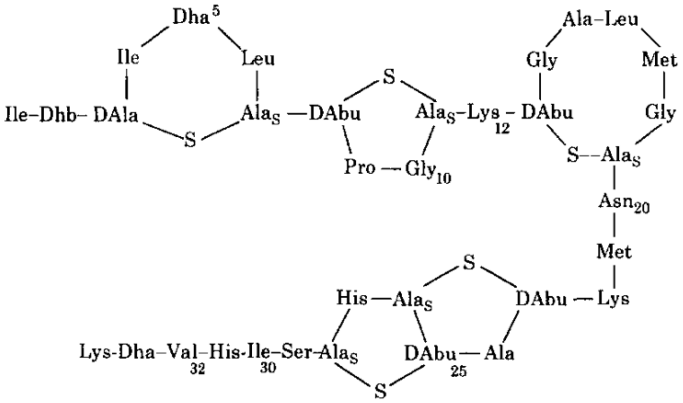

Figure 5: *Structure of Nisin*, modified (Chan e.a., 1996)

Nisin belongs to class of bacteriocin called lantibiotics, a group of ribosomally synthesized peptides that is made up of cyclic structure formed lanthionine and dehydrated amino acid residue (De Kwaadsteniet e.a., 2008).

3.6.1 Nisin Mechanism of Action

Nisin is one of the natural AMP that is effective against Gram-positive bacteria (Chandrasekar, Knabel, & Anantheswaran, 2015). It has been recorded to have an efficient bactericidal effect on Gram-positive bacteria, such as strains of *Lactococcus, Streptococcus, Staphylococcus, Micrococcus, Pediococcus, Lactobacillus, Listeria* and *Mycobacterium* (Sahl, Jack, & Bierbaum, 1995). Moreover, spores of Gram-positive bacteria strains like *Bacillus* and *Clostridium* are more susceptible to Nisin, which means that spores are more sensitive than vegetative cells, a bacteria cell that is actively growing (Delves-Broughton, 1996). However, some Gram-positive bacteria have shown resistance to Nisin by inhibiting the effect of Nisin, due to the ability to produce Nisinase, an enzyme that could stop the antimicrobial action of Nisin (Abee, Krockel, & Hill, 1995).

The bactericidal effect of Nisin on bacteria cells depends on the concentration of the antimicrobial agent. At a higher concentration, the antimicrobial action of Nisin on the target bacteria in the vegetative cells is exercised on the cytoplasmic membrane. Moreover, Nisin disrupts the proton motive force and the pH equilibrium, which results in ion leakage and the hydrolysis of ATP, through the formation of pores resulting to the death of the bacteria cell (Deegan, Cotter, Hill, & Ross, 2006; Delves-Broughton, 1996).

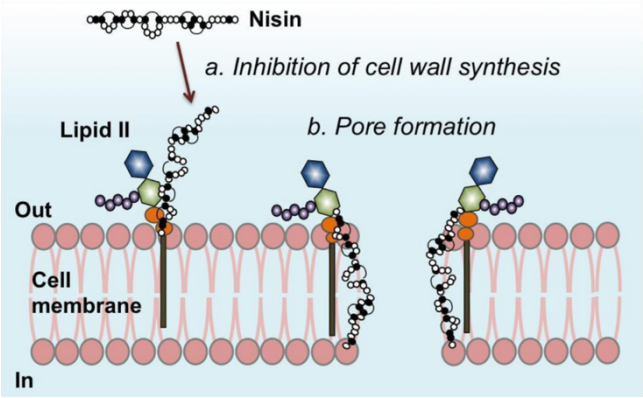

Figure 6: ***Mechanism of Action of Nisin.***
A. inhibition of cell wall synthesis,b. pore formation (Perez, Perez, & B., 2015).

However, at a lower concentration it has been observed that Nisin can have effect on bacteria by inhibiting the cell wall biosynthesis. In this condition, Nisin binds to lipid II, which is the main

transporter of peptidoglycan subunit from the cytoplasm to the cell wall and prevent the synthesis of the cell wall and resulting to the bacteria cell death (Breukink, Wiedemann, & Kuipers, 1999).

4.0 Method

The scope of this project was to investigate how efficient the use of AMP, Nisin A on the bacteria *Micrococcus luteus* in comparison to antibiotic, Tetracycline. The efficiency was tested by measuring the two antimicrobial agent's ability to inhibit the growth of *Micrococcus luteus* with inhibition zones assay.

4.1 Micrococcus luteus Culture

To prepare the overnight culture, a single colony of the bacteria, *M. luteus* was transferred from an agar plate into a test tube with 5 ml Lysogeny broth (LB) medium. The test tube was placed into a shaking water bath at an optimal temperature of 37 ^{o}C for 24 hours.

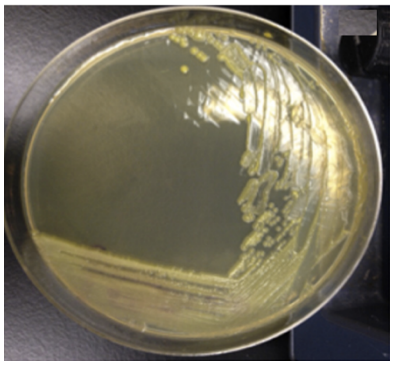

Figure 7: ***Colonies of Micrococcus luteus on an Agar plate*** (Hanafy e.a., 2016)

4.2 Bacteria Plating and Antimicrobial Treatment

For the bacteria plating, new agar plates were taken from the refrigerator and placed into the incubator at a temperature of 37 ^{o}C for about 10 minutes to dry. To maintain a sterile environment, the Bunsen burner was turned. The test tube containing the bacteria was vortex to have an even distribution of the bacteria in the LB medium. The tip of the test tube was heated to avoid bacteria contamination, then 100 µl of the *M. luteus* sample and 900 µl of the LB medium were transferred into a cuvette for

spectrophotometric measuring to determine the optical density (OD) of the bacteria. These was to ascertain the increase in the cell mass of the bacteria. The OD was measured several times at a wave length 600 nm. Furthermore, in order to derive a dilution for the agar plating, the bacteria sample was dilute to an OD of 1 which accounted for 400 µl of the bacteria sample which was plated on each agar plate using a drigalskispatel.

For the antimicrobial treatment, a stock solution of Tetracycline with a concentration of 1 mg/ml and Nisin A with a concentration of 250 µg/ml was made available by Roskilde University. The ability of the antimicrobial agents to inhibit the *M. luteus* was tested at certain of range between 50 µg/ml to 125 µg/ml. The stock solutions were both diluted using the standard curve formula:

$$C_1V_1 = C_2V_2$$

The plated agar plates with the bacteria sample were allowed to dry and then treated with 5 µl of the antimicrobial agents at the different concentration range from 50 µg/ml to 125 µg/ml and with two experimental control, one with water and the other with LB medium. This treatment procedure was repeated twice, two independent times. After the treatment, the agar plates were placed in the incubator overnight to determine the inhibition zones of the bacteria, *M. luteus*.

4.3 Measurement of Inhibition Zone

The treated bacteria agar plates with the antimicrobial agent were taken out from the incubator after 24 hours and the inhibition zone was measured using an automated inhibition zone analysis system called ProtoCol 3 Zone system (ProtoCol 3 count system) which had an embedded software.

Figure 8: ProtoCol 3 Zone system

4.4 Data Analysis

The student t-test was used to analyze the difference between the effect of Nisin A and Tetracycline on *M. luteus*. T-test is a statistical method used to compare, if the means of two data sets are equal, which can be used to compare either paired or unpaired data set. The unpaired t-test was used in this study to analyze if there was a difference in the inhibition zones from Nisin A and Tetracycline treatment of *M. luteus*, as the antimicrobial agents were tested independently. The formula for calculating t-test:

$$t = \frac{x - \mu_0}{\frac{SD}{\sqrt{n}}}$$

Based on the t-value, the p-value can be calculated, the P value is a probability used in determining the difference between the two variables (Nisin A and Tetracycline). Therefore, 5% is taken as the significance level, which implies that if the p-value is below 5 %, the chance of reaching the coincidence is less than 5%. However, the stated hypothesis can either be accepted or rejected based on the p-values.

H_0: $\mu = \mu_0$
H_A: $\mu \neq \mu_0$

Where $H_{0:}$ is the null hypothesis and H_A: is the alternative hypothesis.

P > 0,05 H_0: Accepted
P < 0,05 H_0: Rejected

The t-test was calculated using excel program, where the means and standard deviations of Nisin A and Tetracycline were calculated and then the excel function for a two sample t-test was used to get the find the p-value (Winters, Winters, & Amedee, 2010).

5.0 Result

The inhibitory effect of Nisin A and Tetracycline on *M. luteus* bacteria can be seen in table 1. These were tested using an agar plate with *M. luteus*, which was treated with Nisin A and Tetracycline with a concentration ranging from 50 µg/ml to 125 µg/ml with two controls, H_2O (Mili Q) and LB medium.

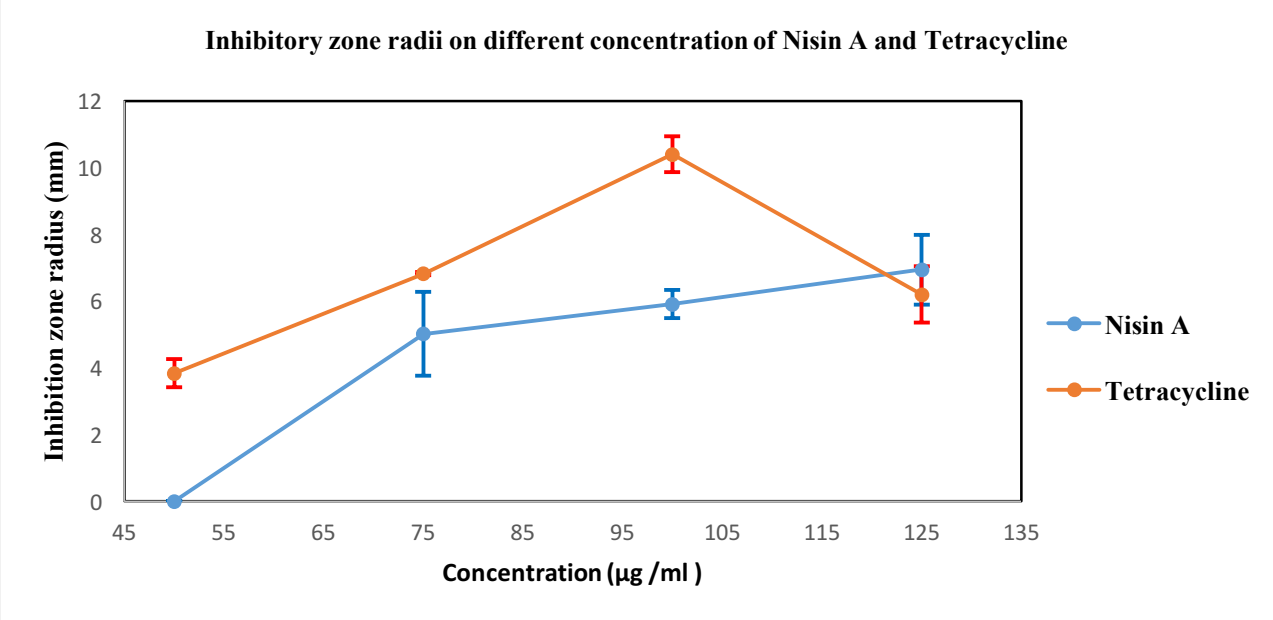

Figure 9: Inhibition zone radii on different concentration of Nisin A and Tetracycline.
Showing the average inhibition zones for the two antimicrobial agents with standard deviation error bars, n=2.

It was observed that the lowest inhibitory concentration from the Nisin A treatment was at 75 µg/ml, which gave a radius of 5.02mm. Moreover, there was no inhibition effect of Nisin A on *M. luteus* at a concentration of 50 µg/ml.

However, the effect of the antibiotic, Tetracycline on *M. luteus* at various concentrations indicated that the minimum inhibition concentration was at a concentration of 50 µg/ml, which had an inhibition zone radius of 3.84 mm. There were more visible inhibition zones radii from the agar plates treated with Nisin A compared to that of Tetracycline, as seen in figure 10 and 11,

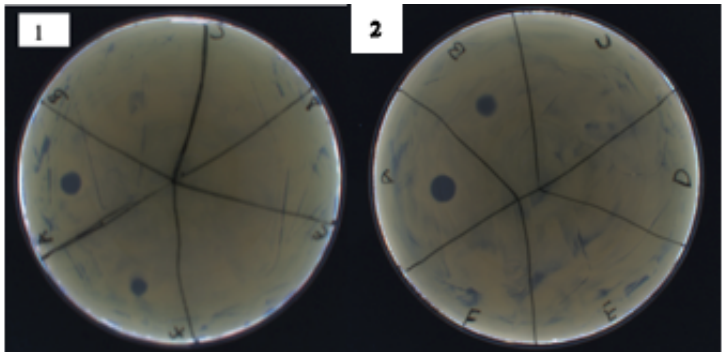

Figure 10: Inhibition zone of Nisin A on M. Luteus,

Showing the replicate, A:125 µg/ml, B**:** 75 µg/ml .*C: 50* µg/ml D: H_2O (Control), E: LB medium, F:100 µg/ml

The inhibition zones from the Nisin A treatment were round and clear with no trace of bacteria colonies. However, there was a difference from the two independent treatment with Nisin A, one of the agar plate showed a clearer inhibition zone at 100 µg/ml (F) and 125 µg/ml (A) while 75 µg/ml (B) was relatively clear as seen in figure 10.1. In contrast to the other replicate, the inhibition zones were seen at A and B while F was relatively clear as seen in figure 10.2.

However, the inhibition zone from the Tetracycline treatment were not clear, the zones were not round and had some trace of bacteria colonies. Moreover, the agar plate with the Tetracycline treatment had areas with uneven growth of bacteria. However, A and F showed irregular shades of inhibition zone as seen in figure 11.1, which gave different values of inhibition zone radius. The size of the inhibition zone from A is smaller than F and B as seen in figure 11 compared to the other replicate with the Tetracycline treatment.

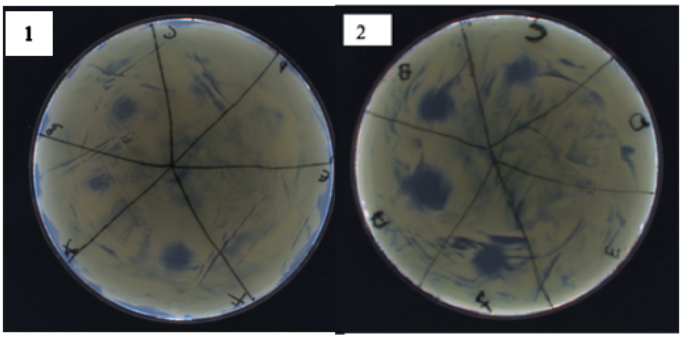

Figure 11: Inhibition zone of Tetracycline on M. Luteus,

Showing the two replicate, A:125 µg/ml, B**:** 75 µg/ml .*C: 50* µg/ml D: H_2O (Control), E: LB medium, F:100 µg/ml

Furthermore, from the data analysis the t-test gave a p-value of 0.26.

6.0 DISCUSSION

The aim of this project was to investigate the effectiveness of AMPs as an alternative approach in the prevention and treatment of bacteria resistance.

6.1 Experimental Method

In this study, inhibition zone assay was used to ascertain the efficiency of the two antimicrobial agent, Tetracycline and Nisin A. However, there are other studies which have also analyzed the effect of this antimicrobial agents against *M. luteus.* We would want to compare this study with three other studies done by Chandrasekar et al., Lalpuria M. et al. and Fazlani et al.

Firstly, according to the study by Chandrasekar et al. in 2015, on the modeling development of inhibition diffusion bioassay which is similar to this study, because it also analyzed the effect of Nisin on *M. luteus.* The compared study focused on developing a finite element method for predicting the diffusion of Nisin in an agar plate over a period of time for two temperature diffusion assay, an experimental determination of the minimum inhibitory concentration (MIC) and critical time for the growth of *M. luteus*. Furthermore, the study also analyzed how to theoretically determine inhibition zone radius for Nisin concentration. The MIC of Nisin (variant was not stated) from the compared study was experimentally determined to be 0.156 µg/ml. The MIC was determined using two temperature agar diffusion assay, the agar medium used contained 0.8% nutrient broth, 0.75% Bacto agar, and 1% Tween 20 and the agar medium was inoculated with a strain of *M. luteus ATCC 10240* for 24 hours, then the concentration of the culture was maintained until they achieve an OD between 1.6 to 1.7, which was measured at 600 nm. Then the liquid agar was solidified at 4^{o}C for 30 minutes. Thereafter, holes were bored on the seeded agar plates and filled with 15 µl of Nisin with concentrations ranging from 0.625 µg/ml to 0.009 µg/ml and the plates were incubated first for 48 hours at 4°C followed by incubation for 48 hours at 30°C. Furthermore, the lowest concentration that had effect on *M. luteus* was at 0.156 µg/ml. Therefore, comparing the method used by the compared study with the method used in this study, the growth medium used was LB medium which contained peptone, yeast extract, NaCl and agar. However, it was not ascertained the strain of *M. luteus* used in this study. In this study, the agar plates with the antimicrobial treatment were incubated for 24 hours at 37^{o}C. The difference in the method used in the compare study could have also influenced the difference in the outcome of our result in this study.

Secondly, in another study by Lalpuria M. et al. in 2012, which focused on the effect of the different well size and pre-diffusion times at 4°C, on the sensitivity of a solution which contained Nisin A using agar diffusion bioassay. In the study, the effect of Nisin A was examined on *M. luteus,* strain ATCC 10240 using the two temperature agar bio assay. The Nisin A in the compared study was from a solution called Nisaplin, which contained about 2.5% of Nisin A. The Nisin A solution was filled into wells of 3.5 mm and 7 mm with a concentration ranging from 0.625 to 125 µg/ml (Lalpuria, Karwa, Anantheswaran, & Floros, 2013). However, the growth medium was the same, as used in Chandrasekar et al., 2015, but *M. luteus* was grown in a different medium, Difco nutrient broth. Thereafter, the agar plates containing the Nisin A solution were stored at 4°C for 0, 24, 48 and 72 hours for pre-diffusion, which was followed by incubation for 48 hours at 30°C. Furthermore, the inhibition zones were measured from the edge of the well with a digital Vernier caliper.

Thirdly, in another study by Fazlani et al. in 2011, which also analyzed the effect Tetracycline on *M. luteus*. The study focused on comparing the susceptibility of 12 antimicrobial agents (including Tetracycline) on bacteria species (including *M. luteus)* identified from mastitic milk of camel. According to the method used in the study, Fazlani et, al. inoculated the mastitic milk samples with a growth media, which contained nutrient broth, blood and MacConkey's agar. This was done to identify the different bacteria species. Thereafter, disc diffusion method was used to analyze the MIC and determine the sensitivity of the 12 antimicrobial agent to the identified bacteria species from the mastitic milk, which contained *M. luteus*.

Moreover, based on the three studies examined, with two of the studies focusing on MIC determination while the third study focused on measuring inhibition zone, to ascertain the effect of antimicrobial agent after pre-diffusion at different time period (0, 24, 48 and 72 hours). Therefore, these could give a relationship in the use of MIC and size of inhibition zone radius to determine the effect of an antimicrobial agent. Furthermore, the MIC and the diameter of inhibition zones are inversely correlated, which means that the zone of inhibition determines the sensitivity of a bacteria to an antimicrobial agent. The more a microorganism is susceptible to an antimicrobial agent, the lower the MIC and the larger the zone of inhibition. Conversely, the more resistant the microorganism, the higher the MIC and the smaller the zone of inhibition. Deducing from the results of this study we could assume that *M. luteus* was more susceptible to Tetracycline based on the inhibition size radii as it shows a higher inhibition size at the same concentration than Nisin A in this study.

6.2 Experimental Result

Based on the results from our experiment and data analysis using a two-way T-test, which gave a p-value of 0.296. It can be established that there was no significant difference in the inhibition zones radii of the *M. luteus* treated with Nisin A in comparison to Tetracycline. However, it was observed that Nisin A had no antimicrobial effect at the lowest chosen concentration range for this study in contrast to Tetracycline, which had an antimicrobial effect on the concentration at 50 µg/ml. Moreover, Nisin A inhibition zones were more clear and visible compared to that of Tetracycline which had bigger zones that were not clear. These probably could have resulted from the susceptibility effect of Tetracycline on the *M. luteus* bacteria or the response of the bacteria strain to the antimicrobial agent. Although, the source of the bacteria strain used in this study was not specified, if it was clinical or from a sick patient, which probably might be developing resistance to antimicrobial effect, as this could also influence the response effect of the antimicrobial agent. Furthermore, it can be observed that there was a relationship between the concentration and inhibition zone radii for *M. luteus* treated with Nisin A. The inhibition zones from the Nisin A treatment increased with increasing concentration of Nisin A. However, for Tetracycline there was no correlation between the concentration and the inhibition zone radii, as the result from this experiment showed that at the highest concentration of 125 µg/ml, the inhibition zone was lower than the inhibition zone radius of the other concentrations at 100 µg/ml and 75 µg/ml. Moreover, this discrepancy in the zone of inhibition from the Tetracycline treatment, may had resulted from laboratory error during the application of the antimicrobial agents to the agar plate with the bacteria. Another reason, may had been as a result of the wrong labeling of the sectioned agar plates, which were labelled from A to F. All these could have summed up to the reason why there was a bigger inhibition zone at the concentrations of 100 µg/ml and 75 µg/ml than the inhibition zone at the concentration of 125 µg/ml.

It was also observed that the optical density of the *M. luteus* at 0.2 resulted in few bacteria colony after incubation, which means the growth of the bacteria was slow and probably the bacteria were still in the lag phase. The low increase in the cell mass could had been associated with environmental or nutritional factors, such temperature, pH, availability of nutrient in the growth medium or oxygen (O_2) as *M. luteus* is an aerobic bacterium. In order to achieve an even distribution of the bacteria colony, an OD of 1 was used in this study, which may have also influenced the concentration of the used antimicrobial agent. In comparison to the other two studies by Lalpuria M. et, al. 2012 and Chandrasekar et. al that we examined, which used an OD of between 1.6 and 1.7. However, the

difference in the OD can also be attributed to the different growth mediums used in this study and the compared studies.
The concentration of Nisin A used in this study was relatively high compare to the concentration used in other studies, the lowest concentration of Nisin A that had an inhibitory effect on *M. luteus* was at 75 µg/ml where there was no effect at a concentration of 50 µg/ml compare to the study done by Chandrasekar et al. in 2015, where the MIC was at 0.156 µg/ml.

However, in comparison of this study to the study by Lalpuria M. et al. 2012, it was observed that the pre-diffusion of the solution with Nisin A for 24 hours, gave an inhibition zone radius of 8.91 ±0.06 at a concentration of 125 µg/ml compared to this study which gave 6.9±1.03 at the same concentration. Moreover, the predicted inhibitory zone radii of the compared study (Lalpuria M. et, al. 2012) at the concentration between 50 µg/ml to 125 µg/ml was more than 10 mm which was twice the inhibition zone radii in the Nisin A treatment from this study. Furthermore, it is important to note that, the difference in the effect of Nisin A in these two studies could have been as a result of the two different methods used in this study and the compared studies.

According to Fazlani et al., 2011, *M. luteus* was found to have a higher sensitivity to Tetracycline in comparison to the other 11 antibiotics analyzed, in the compared study Tetracycline had an inhibition zone radius of 11mm, which was relatively close to the inhibition zone radius of 10.4 mm in this study. However, the difference in the zones of inhibition could be due to the different method and concentrations used in this study and the compared study.

6.3 AMPs Resistance

The new approach to the use of AMPs to treat resistance bacteria has been a tremendous development as the use of both natural and synthetic AMPs has helped in the fight against the increased occurrence of resistance to conventional antibiotics (Bahar & Ren, 2013; Hancock & Sahl, 2006). Moreover, from the experimental analysis of this study, it can be observed that the area of inhibition with the Nisin A treatment was round and clear with no trace of any bacteria colonies compared to that with the Tetracycline treatment that had trace of some *M. luteus* colonies, which make AMP a more effective agent against the scourge of bacteria resistance. However, some studies have showed that

bacteria are beginning to develop resistance to AMPs. According to Andersson et al. in 2016, resistance mechanism such as membrane modification and other intrinsic mechanism has been identified amongst some gram-positive and Gram-negative bacteria. Moreover, in another study by Guilhelmelli et al. in 2013, it was also stated that AMP resistant bacteria are developing different mechanism of resistance to AMPs. This resistance mechanism is often controlled by coordinated stress regulated response of the bacteria operons, a three adjacent structural genes which comprises of a promoter, terminator and an operator. One of the common mode of resistance mechanism by the bacteria is by changing the cell surface and preventing the AMP from its target, which is done by inhibiting the binding or penetration of the AMP into their cells. Furthermore, other mechanism of bacteria resistance to AMP include; the formation of biofilms, modulation of AMP gene expressions, efflux and proteolytic degradation (Guilhelmelli e.a., 2013).

Furthermore, based on the effect of Nisin against *M. luteus* in this study, we could assume that *M. letus* was more susceptible to Nisin A.

7.0 Conclusion

Based on the statistical analysis, which showed that there was no significant difference between the effect of the two antimicrobial agents against *M. luteus.* It could be concluded that Nisin A did not show considerable difference in its antimicrobial effect than Tetracycline against *M. luteus.*
However, the treatment with Nisin A had a better bactericidal effect on the *M. luteus* bacteria, having round and clearer zones of inhibition. Moreover, the zones from the Tetracycline treatment was not clear and visible. Furthermore, on the bases of MIC and inhibition zones we can not conclusively state that either Nisin or Tetracycline was more effective or susceptible against *M. luteus* as MIC was not investigated in this study.

8.0 Perspective

There are many ways we think this study could have been improved if we had more time at our disposal.

Firstly, we could improve more on the experimental method, by having more replicates to compare with and validate the outcome of our experimental results. Moreover, this would help prevent the kind of laboratory errors that was associated with this study.

Secondly, we could have also tried a different growth medium, to determine if the LB medium was not enrich with available nutrient for *M. luteus* growth. As compare to other studies which used different growth medium.

Thirdly, we could have use the two temperature diffusion assay to compare our result, to determine if there would have been a difference in the outcome of our result at difference temperatures.

Fourthly, we could have also analyzed MIC using the disc diffusion method. To give us a suitable comparison between the MIC and inhibition zone radii. These would make it easy to compare the sensitivity and susceptibility of the bacteria to the antimicrobial agent.

Lastly, we would have examined the effect of Nisin on biofilms and the immune-regulatory effect of Nisin A.

9.0 APPENDIX

***Table 1**: Dilutions for Nisin A (Nis A) and Tetracycline (Tet) with a Stock solution of 250 µg/ml*

concentration (µg/ml)	H_2O (µl)	Nis A / Tet (µl)
6.25	24.38	0.63
12	23.75	1.25
25	22.50	2.50
50	20.00	5.0
75	17.50	7.5
100	15.00	10
125	12.5	12.5

***Table 2**: Inhibition zones of Nisin A on M. Luteus, showing the two replicate with the average inhibition zones radii and standard deviations*

concentration (µg/ml)	Inhibition zone radii (mm)		Mean (mm)	Standard Deviation
	Replicate 1	Replicate 2		
6.25	0	0	0	0
12	0	0	0	0
25	0	0	0	0
50	0	0	0	0
75	5.9	4.13	5.02	1.25
100	6.2	5.61	5.91	0.417
125	7.67	6.2	6.94	1.039

***Table 3**: Inhibition zones of Tetracycline on M. Luteus, showing the two replicate with the average inhibition zones radii and standard deviations*

concentration (µg/ml)	Inhibition Zones Radii (mm)		Mean (mm)	Standard Deviation
	Replicate 1	Replicate 2		
6.25	0	0	0	
12	0	0	0	
25	0	0	0	
50	3.54	4.13	3.84	0.417
75	6.86	6.78	6.82	0.05
100	10.03	9.74/ 11.8	10.4	0.532
125	6.79	5.31/ 5.9	6.2	0.838

9.1 Bacteria Growth with different OD Measurement

Figure 1: M. luteus with an OD of 0.2

Figure 1: M. luteus with an OD measurement of 1 and 2

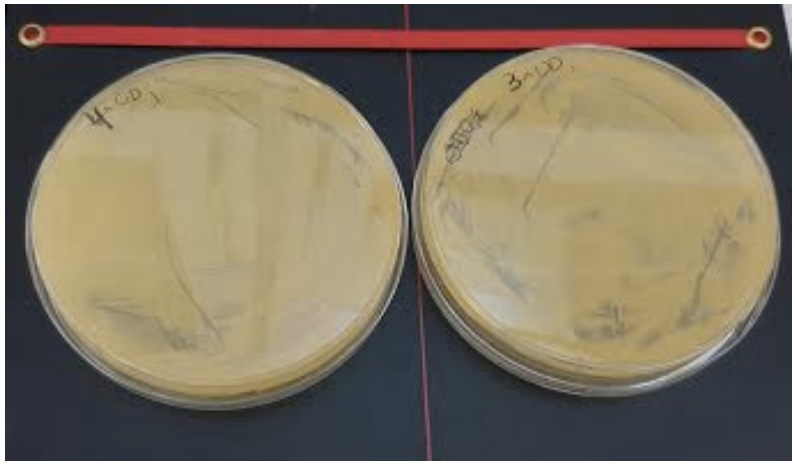

Figure 1: M. luteus with an OD measurement of 3 and 4

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