Effects of tetM gene instability on resistance expression in *Lactobacillus casei*

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**Abstract.** *Lactobacillus casei* is one of the probiotic bacteria which has an antibiotic (tetracycline) resistance encoded by tetM gene. This gene can be unstable which leads to the changes of the DNA sequence (mutation) during the adaptation process under a sub-lethal dose of tetracycline antibiotic. The aim of the study is to observe the resistance level and genetic instability of tetM gene under a sub-lethal dose of tetracycline antibiotic and to examine the effect of gene instability on the antibiotic resistance level of *L. casei*. This study was conducted by 2 steps i.e. determination of sub-lethal dose of *L. casei* and isolation of *L. casei* with the highest resistance to tetracycline using adaptation process. This study confirmed that *L. casei* could survive with sub-lethal dose of 10-50 µg/mL tetracycline and has a lethal dose at 60 µg/mL. The concentration of more than 60 µg/mL tetracycline caused instability of tetM gene and lead to the survival of *L. casei* at 70 µg/mL tetracycline after adaptation process.

1. **Introduction**

Nowadays, bacteria evolution on antibiotic environment is rapid because of the massive usage of the antibiotic in the medical field. The use of antibiotics plays a crucial role in the evidence of antibiotic resistance amongst pathogenic bacteria, or also termed as superbug pathogenic bacteria. The term “Superbug” refers to microorganisms that enhanced morbidity and mortality due to the multiple mutations which make them resistant to the antibiotic [1]. Inappropriate use of antibiotics is considered to be one of the main factors that is responsible for the high prevalence of antibiotic-resistant pathogens. Increases of superbug pathogenic bacteria lead to increased disease in human life. Based on research, the evolution rate is affected by the gene expression through the antibiotic exposure which can induce the DNA damage or mutation that can cause gene instability [1].

A large number of bacteria colonize in the human gastrointestinal tract play an important role in nutrition and health by promoting nutrient supply, preventing pathogen colonization, shaping and maintaining the homeostasis of the intestinal immune system [2]. It has been observed that the use of antibiotics affects the composition of the gut microflora. Probiotic is microbes ingested with the aim of promoting good health, it can provide benefits to human health when administered in adequate amounts that confer a beneficial health effect on the host [3]. One advantage of consuming probiotics is that it can improve the intestinal microbial balance. The importance of keeping the microflora in balanced condition is to protect human from the intestinal disorder.
Keeping the balance of the microflora in the gut is become more challenging by the appearance of the superbug pathogenic bacteria. Therefore, a need better strain is needed for probiotic bacteria which have the same resistance to the antibiotic as the superbug pathogenic bacteria, to keep the balance of microflora in the body. One innovation that we can do is creating stronger probiotics by adaptation through antibiotic exposure environment. This can be conducted by adapting the probiotic bacteria on its sub-lethal dose of antibiotics [4]. The evolution of probiotic bacteria such as L. casei under the adaptation process is expected to induce antibiotic resistance expression. Higher antibiotic concentration could trigger the antibiotic resistance gene to be unstable and this condition may create higher possibility of the mutation to occur. The aim of this research was to identify the resistance level and instability of the antibiotic resistance gene in L. casei, and the effect of instability on the antibiotic resistance expression. L. casei produces from this research is expected to be as strong as the superbug bacteria, so that it will be one of the solutions to keep the balance of the microflora in the body.

2. Materials and Method
2.1. Bacteria growth condition and antibiotic preparation
Bacteria strain, Lactobacillus casei FNCC 0090 was collected from the culture collection of Gajah Mada University, Indonesia. Lactobacillus casei grows in anaerobic condition at 37 °C for 24-48 h. MRS (deMan, Rogosa and Sharpe) medium is suitable for the growth of Lactobacillus casei. Tetracycline antibiotic was diluted into 5 mg/mL stock solution by using 70% ethanol.

2.2. Isolation of Lactobacillus casei with high resistant level
L. casei culture was exposed to the tetracycline antibiotic from 10-100 µg/mL gradually using Up and Down method to determine lethal and sub-lethal dose. Up and Down method is carried out by exposing the antibiotic to the cells with gradually increasing concentration. The sub-lethal dose is a concentration below lethal dose which could not kill the bacteria. The aim of gradually exposing the L. casei in sub-lethal dose is to induce the adaptation process. The exposure method was conducted 3 times to go beyond its lethal dose [5].

2.3. Plasmid extraction
L. casei culture was harvested from MRS broth after 24 h incubation in 37 °C anaerobic condition. There are three methods used in L. casei plasmid extraction considering of difficult to lyse bacteria. L. casei is one of the lactic acid bacteria which can produce high exopolysaccharide [6]. The methods consist of alkaline lysis, Anderson and Mckay and Klaenhammer. Gel electrophoresis used to visualize the plasmid extract using 1% of agarose (Bio-Rad) and 10 kb DNA marker [7, 8].

2.4. Oligonucleotide primer
The tetM gene primer was designed by using SnapGene based on Lactobacillus species. L. plantarum is chosen as one of the sequence references based on its closest tetM sequence similarity to L. casei [9, 10]. The primer used in tetM isolation is 5’-GAC ACG CCA GGA CAT ATG GAT TT-3’ for forward primer which complements with tetM gene between base 220-242 and 5’-CAC CGA GCA GGG ATT TCT CCA C-3’ for reverse primer which complements between base 1733-1754 of tetM gene. Both primers produces 1534 bp of tetM gene fragment as an amplicon.

2.5. Polymerase chain reaction (PCR)
PCR mix consisted of some component such as 20 µL PCR-grade water, 5 µL Go Taq Green Master Mix (Promega), 0.4 µL primer forward and reverse (10 µM final concentration), and 1 µL DNA template. The amplification process was carried out by using PCR GeneAmp 9700. The PCR reaction was performed with a 25 µL reaction mix containing 100 ng of plasmid DNA. The reaction mixture was subjected to initial heating at 94 °C for 5 min. The temperature was cycled at 94 °C for 1 min, at 55 °C for 1 min, at 72 °C for 2 min and a final extension at 72 °C for 10 min. The cycle was repeated 30 times according to the laboratory manual for PCR [11].
3. Results and Discussion

3.1 Isolation of Lactobacillus casei with high resistant level

The isolation of Lactobacillus casei which has high resistant level was conducted by using Up and Down method to determine lethal and sub-lethal dose [5]. The method was performed by applying some tetracycline antibiotics with gradually increasing concentration, range from 10-100 µg/ml into diluted overnight with 9 x 10^7 CFU/ml of initial culture concentration. L. casei culture was then plated in MRS agar and incubated in 37 °C for 24-48 h. Data on determining lethal and sub-lethal dose is described in Table 1.

| Tetracycline Dose (µg/ml) | Number of colonies (CFU/ml) | Description |
|---------------------------|----------------------------|-------------|
| 0                         | 9 x 10^7                   | Initial cell concentration |
| 10                        | 2.204 x 10^2               | Sub-lethal dose |
| 20                        | 1.072 x 10^2               | Sub-lethal dose |
| 30                        | 0.736 x 10^2               | Sub-lethal dose |
| 40                        | 0.364 x 10^2               | Sub-lethal dose |
| 50                        | 0.334 x 10^2               | Sub-lethal dose |
| 60                        | 0                         | Lethal dose |
| 70                        | 0                         | |
| 80                        | 0                         | |
| 90                        | 0                         | |
| 100                       | 0                         | |

Based on Table 1, L. casei has a lethal dose in a concentration of 60 µg/ml tetracycline, as there was no colony growth on the plate which indicates 100% colony death. Meanwhile, lower dose below the lethal dose called as sub-lethal dose, a dose that still can suppress the growth of L. casei colony. The suppression of colony growth caused by the sub-lethal dose can induce the existence of mutation which leads to the new phenotypic changes [4, 12]. The reduction of colony growth in every plate and concentration is also considered to determine the sub-lethal dose. The isolation of highest resistance level of L. casei is using adaptation process. This process aims to get adapted L. casei in high concentration of antibiotics over its lethal dose. The result of adaptation process is described in Table 2. First adaptation was carried out by applying some several concentration (10-45 µg/mL) of antibiotics to the culture. It was observed that there was a reduction of colony number at 45 µg/ml antibiotic concentration. The second stage of the adaptation process began from 45-55 µg/mL. L. casei colony was isolated from first stage of 45 µg/ml concentration for the second adaptation process. In the second process of adaptation, the growth of the L. casei did not show development in every increasing concentration. In the third stage, the colony was exposed to concentration of 50-70 µg/mL to obtain highly tolerant of tetracycline antibiotic colony. In the concentration of 65-70 µg/mL, L. casei has surpassed its lethal dose and it can survive in higher antibiotic concentration.

L. casei ability to survive over its lethal dose is caused by the adaptation process carry out by using its sub-lethal dose. According to Shapiro et al. [4], exposing microorganisms to sub-lethal dose or concentration of antibiotics can trigger mutation which affects the instability of genes. Tetracycline antibiotic can be a mutagen to a gene that encodes ribosomal protection protein to tetracycline antibiotic or tetM gene. Direct or indirect damage to DNA could be the result of antibiotic exposure [13]. There are a lot of mechanisms which described how mutation occurred after the exposure process of antibiotics. First possible mechanism is that antibiotic molecule will directly target the ribosome that will affect the protein synthesis [14]. Another mechanism is by increasing cell stress which triggered the ROS or Reactive Oxygen Species mechanism as one of the cell metabolic anomaly [15-
Due to the high cell stress level, extreme environment triggered the production of abundant free radical hydrogen peroxide inside the cell. Hydrogen peroxide is one the strongest oxidizing agent which can directly oxidize or mutate the DNA. Guanine is one nitrogen base which is very easy to oxidize, its residues will change into another type of nitrogen base which caused changes in DNA structure [17]. Meanwhile, the tendency of a gene to change its nucleotide sequence or mutation called a instability of gene [18]. The tetM gene or other genes encode antibiotic-resistant in L. casei has a tendency to be unstable. This instability of genes can be observed in molecular level by the changes of its nucleotide sequence.

Table 2. Isolation of high tolerant L. casei

| Tetracycline Dose (µg/ml) | 1st Adaptation | 2nd Adaptation | 3rd Adaptation |
|--------------------------|----------------|----------------|----------------|
| 10                       | +++            |                |                |
| 15                       | +++            |                |                |
| 30                       | +++            |                |                |
| 45                       | ++             | ++             |                |
| 50                       | -              | +              | +++            |
| 55                       | -              | +              | +++            |
| 60                       | -              | -              | +++            |
| 65                       | -              | -              | ++             |
| 70                       | -              | -              | +              |

Notes: (+++) indicated high colony growth; (+++) indicates medium colony growth; (+) indicates low colony growth; and (-) indicates no colony

3.2. Plasmid extraction

The change of tetM gene nucleotides arrangement could be known by molecular observation. Lactobacillus has plasmid that encodes many resistant genes to antibiotics [19] and the location of tetM gene is suspected to be in the plasmid of the bacteria. Plasmid isolation was carried out to observe the instability or the change of the nucleotide arrangement of tetM gene caused by the adaptation process in different concentrations of the antibiotic tetracycline. Plasmid isolation was carried out by using alkaline lysis method and obtained results as in Figure 1 [11]. From Figure 1A. It is known that plasmid extraction by using alkaline lysis did not form a band in the electrophoresis gel, it was because the alkaline lysis was a method that used to extract the plasmid of gram-negative bacteria which has thinner cell wall, and Lactobacillus casei was classified into gram-positive bacteria. The cell wall was composed of peptidoglycan as a layer. Besides, the method does not have lysozyme as an enzyme to degrade the bacterial cell wall and it makes the plasmid could not be extracted maximally [20].

The second isolation method used to maximize the plasmid extraction was carry out by using a modified method of Anderson and McKay [7]. Both methods have been completed with the addition of lysozyme. By using this method, we could know that the band did not form yet because in Anderson and McKay's method the incubation time was only 5 minutes when the time was still not enough for the enzyme to lyse the bacterial cell wall. The minimum time for the enzyme to lyse the cell wall was about 30 minutes at 37°C [21]. The next method used to optimize the plasmid extraction was the Klaenhammer method modified by prolonging the incubation time. In this method, lysozyme was added with concentration of 30 mg/mL and then incubated within 60 minutes at 37°C. Another factor that influenced the extraction process was because of exopolysaccharide produced by the L. casei which made the lysozyme could not work optimally. The result of plasmid isolation using this method...
could be seen in Figure 1C. In the electrophoresis gel, there formed bands in wells number 5 and 6. The plasmid size was estimated to be >10,000 bp or 10 Kb. According to the literature, the length of the L.casei plasmid was 26,706 bp [22]. Based on Figure 1C the band was smear due to some reasons, such as the purity of the isolate that is still contaminated by protein or other residues like ethanol. Purification could be carried out by using chloroform and isoamyl alcohol to get a clearer band [23].

Figure 1. Result of agarose gel electrophoresis of plasmid isolation; (A) Alkaline lysis; (B) Anderson and McKay Method; (C) Klaenhammer Method; and amplification of tetM gene using PCR (D-E)

3.3. Polymerase chain reaction (PCR)
Extracted plasmid provided from the modified Klaenhammer protocol were amplified using specific primers to isolate the tetM gene. The PCR will amplify 1534 bp of specific DNA fragments. The PCR products was then visualized by using gel electrophoresis with 1% of agarose and 10 kb of DNA marker. From the experiment, the gel produced a smeared band. The smear appearance caused by the high concentration of DNA template used in PCR. The minimum DNA template for PCR is between 1 ng to 1 µg for genomic and 1 pg to 1 ng for the plasmid DNA template. The DNA template has to be pure before it is used in PCR process [23]. Other factors that affected the smear result on lanes were not only the presence of contaminant, but also the exact location of tetM gene. It might not present in extrachromosomal DNA (plasmid), but it exists in chromosomal DNA or transposon. Another possibility is L.casei might not have any tetM gene but other tetracycline resistance genes like tetS, tetW, tetO, or another antibiotic resistance gene [24].

4. Conclusions
This study confirmed that L. casei could survive on its sub-lethal dose (10-50 µg/ml). Continuous exposure to a sub-lethal dose of tetracycline helps the L. casei culture to adapt to the higher antibiotic concentration and survive beyond its lethal-dose (70 µg/ml). It also triggers the mechanism of mutation inside the cell in order to overexpress the tetM gene as a gene which regulates the tetracycline-resistant mechanism. Gene instability is a result of mutation mechanism triggered by the adaptation process during the environment changes. Klaenhammer method with modification by prolonging the incubation time and adding higher concentration of lysozyme have been proved to be the best method for extracting the bacterial plasmid compared to other methods. The isolation of tetM gene by using Polymerase Chain Reaction needs further studies in PCR system optimization, gene location, sequence and mechanism to gain clear band of targeted gene.
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