The Use of Kefir Grains as an Anaerobic Method for Removal of β-Lactam Antibiotic Residue on Cows Milk

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Abstract. After mastitis therapy, using the right technology, it was necessary to break down the antibiotics in waste milk. Due to microbial activity, the microbiology approach as an anaerobic treatment presumably successfully removes antibiotic residues in milk. This study attempted to Eliminate Antibiotic Residue from Mastitis Treatment in Cow's Milk by Using Kefir Grain in an Anaerobic Method. Twelve samples of Holstein cows milk with 3% kefir grain were randomly assigned to one of four treatments: cows milk with 3% kefir grain and no antibiotics (control), cow milk with 3% kefir grain and six, eight, and ten micrograms per milliliter of Amoxycillin trihydrate, respectively. Completely Randomized Design (CRD) with four treatments and three replications was used to evaluate the collected data, followed by Duncan's Multiple Range Test (DMRT). This study demonstrates that adding 3% kefir grains eliminates antibiotics in milk, as indicated by a negative antibiotic residue test. On cow's milk containing 3% kefir grain and 6 μg/ml, a significantly different effect of pH (3.98 ± 0.2), syneresis (24.93 ± 0.7), and lactic acid bacteria viability (8.88 CFU/ml) were observed, except for dissolved protein (8.16 ± 1.7).

Keywords: antibiotic · milk · kefir grain

1 Introduction

Mastitis is one of the most severe illnesses that may strike the dairy business. Mastitis is a disease of dairy cows caused by pathogenic bacteria, including *Streptococcus agalactiae, S. disgalactiae, S. uberis, S. zooepidermicus, Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes* or fungal contamination [1]. These bacteria will cause damage to the alveoli cells in dairy cows, affecting a decrease in milk production and quality.

Antibiotics are often administered to dairy cows for clinical mastitis (CM) and local antibiotic therapy on the day of drying-off, around six weeks before the next calving. Significant success has been made in reducing mastitis, and antibiotics have enabled many dairy farms to eradicate certain infections from their herds. [2].

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Most chemical pollutants in milk and dairy products are veterinary pharmaceuticals, including antimicrobials (antibiotics and sulfamides), hormones, anthelmintic medications, and pesticides. Based on the Indonesian National Standard, Permissible tolerance limits for penicillin are 0.10 mg/kg in dairy products.

On the other hand, using antibiotics for extended periods will affect the residues in milk, which could cause antimicrobial resistance [3]. Antibiotics cause an increase in the antibiotic resistance of harmful bacteria, resulting in long-term health issues due to a shortage of suitable medications to treat the newly developed resistant bacteria [4]. The usage of antibiotics may result in antibiotic residues in milk, which provoke allergic responses in humans upon ingestion [5], to prevent the mentioned problems, antibiotics must be removed from milk for future consumption. Physical, biological, chemical, and physicochemical techniques have removed antibiotics from milk. Physical techniques like filtration, coagulation, flocculation and sedimentation have been demonstrated to be ineffective and provide unsatisfactory outcomes.

Anaerobic treatments are probably effective in treating antibiotic residues in milk because of microorganism activities. The fermentation process by lactic acid bacteria is considering improving food quality. Fermentation can simplify complicated chemicals so the human body can absorb fermented foods efficiently [6]. Antimicrobial compounds produced in the kefir fermentation could inhibit the growth of microbial pathogens. Therefore, it is necessary to develop appropriate technology for degrading antibiotics in waste milk. Due to microbial activity, the microbiology approach as an anaerobic treatment is likely suitable for eliminating antibiotic residues in milk. The present study aimed to eliminate the Antibiotic Residue of Mastitis treatment in Cow's Milk with an anaerobic method Using Kefir Grain.

2 Materials and Methods

2.1 Materials

The materials used were: 1) Cow's milk as raw material was obtained from a local farmer in Malang Sub-district. 2) Kefir Grain, Biomycin-M (Interchemie, Holland), deMan Rogosa Sharp Agar (MRS-A), Plate Count Agar (PCA) Oxoid CM0325B. The equipment used was: beta-lactam strip test (AuroFlowTM), pH meter (WalkLAB pH meter HP9010), Spectrophotometers (NanodropTM), sample container, petri dish, incubator, autoclave, micropipette, centrifugation.

2.2 Methods

Analysis procedure of antibiotic residue in milk using AuroFlow™ Beta-Lactam Strip Test. The Physicochemical and Microbiological quality was evaluated: (1) Syneresis analysis was performed by centrifugation method followed [7] (2) pH analysis was done with pH meters (3) Dissolved protein analysis was carried out using the Nanodrop tool, and Total Lactic Acid Bacteria (LAB) was enumerated according to microbiological assay by [8].

This study used a completely random design with four treatments and three replications. Twelve samples of Holstein cows' milk with 3% kefir grain were randomly assigned to one of four treatments: cow's milk with 3% kefir grain and no anti-biotics (control) or cow's milk with 3% kefir grain with six, eight, and ten micrograms per milliliter of Amoxycillin trihydrate, respectively. ANOVA (Analysis of Variance) was used to evaluate the data, and any significant difference effect of the variables was determined, followed by Duncan's Multiple Range Test (DMRT).

3 Results and Discussion

3.1 Antibiotic Residue

Twelve milk samples were obtained from this research according to β-lactam Antibiotic Residue Of Mastitis Treatment. The effect of grain kefir against the antibiotic residue in waste milk revealed Negative results. The negative results are due to the yeast and bacterial activity, including *Lactobacillus Plantarum, Lactobacillus kefir, and Lactobacillus parakefir* combined with *Saccharomyces sp* as microorganisms that effective in the treatment of antibiotic residues in milk.

Some bacteria can produce beta-lactamase enzymes called Extended-spectrum beta-lactamase (ESBL) bacteria. ESBL is an enzyme that can hydrolyze the cyclic amide bond of the beta-lactam ring so that the activity of β -Lactam antibiotics such as penicillin becomes inactive. However, ESBL can harm health, so it must be combined with probiotics. After ESBL deactivating antibiotics, it can be continued with the fermentation

Table 1. Microbiological and Physicochemical Properties of Kefir

Sample	Variable				
	LAB (Log CFU/mL)	**Dissolved Protein**	**Syneresis (%)**	**pH**	**Antibiotic Residue**
Milk + 3% Kefir	8.68 ± 0.2^{ab}	6.49 ± 2.3^{a}	22.11 ± 0.4^{a}	4.20 ± 0.2^{c}	Negative
Milk + 3% Kefir + 6 µg/ml of Amoxycillin trihydrate	8.44 ± 0.1^{a}	8.16 ± 1.7^{bc}	24.93 ± 0.7^{bc}	4.13 ± 0.2^{b}	Negative
Milk + 3% Kefir + 8 µg/ml of Amoxycillin trihydrate	8.86 ± 0.1^{b}	7.49 ± 1.2^{b}	24.16 ± 0.9^{b}	3.94 ± 0.2^{a}	Negative
Milk + 3% Kefir + 10 µg/ml of Amoxycillin trihydrate	8.88 ± 0.1^{bc}	7.06 ± 0.5^{c}	29.06 ± 0.3^{c}	3.98 ± 0.2^{a}	Negative

process using probiotic bacteria. The fermentation process with lactic acid bacteria can produce bacteriocins [9].

In this approach, bacteriocins and antimicrobial peptides have recently received interest. Bacteriocins from lactic acid bacteria (LAB) are antimicrobial peptides or proteins that inhibit the growth of several pathogens [10]. Lactobacillus, Enterococcus, Pediococcus, and Bifidobacterium are nonpathogenic probiotic microbes found in natural flora that positively affect host physiology [11].

3.2 pH

As a result of pH value, we found that the value of pH declined from 4.13 to 3.94 ($p < 0.05$). The level of Amoxycillin was not affected by the viability of Lactic Acid Bacteria. LAB can still produce significant products formed during fermentation: lactic acid, CO2, and alcohol. Due to the metabolic activity of yeast, the alcohol concentration has risen after post-acidification. When the pH falls below 4, bacterial activity will decrease significantly. This environment is utilized by yeast to proliferate and oxidize glucose, resulting in a rapid increase in the alcohol level of kefir. LAB and yeast had improved growth conditions and maximized metabolic activity at the optimal temperature [12]. Depending on the production parameters and environment, kefir fermentation produces varying quantities of ethanol and carbon dioxide [13].

After one day, lactose was hydrolyzed more slowly, the rate of pH drop was delayed, the concentration of L(+)-lactic acid fell slightly, and the concentration of D()-lactic acid increased [14]. [15] analyzed the qualities of kefir generated with different milk samples (bovine, ovine, and caprine) and culture types (kefir grain and commercial starter culture). They found that the starting culture, duration of storage, and species of mammals greatly impacted pH changes.

3.3 Syneresis

Syneresis, also known as serum release, is one of the most significant quality indicators for kefir. Table 1 shows that adding Amoxycillin increased the syneresis rate of kefir compared with the control ($p < 0.05$). The average syneresis were 22.11%, 24.93%, 24.16%, and 29.06% respectively. The increase in the syneresis rate was proportional to the increase in Amoxycillin concentration, so the serum separation was higher. Increased concentrations of sour cherry as the polyphenol source in yogurts led to increased serum separation [16]. On the other hand, according to [17], Varying levels of Canna starch inhibited the syneresis of yogurt drinks, which caused the amylopectin in Canna starch to be capable of binding water for suppressed the serum separation.

Amoxycillin, as the β-lactam Antibiotic, could not increase water holding capacity. Water holding capacity represented the protein's ability to retain moisture inside the yogurt structure. The ability to keep water is associated with minimizing the risk of syneresis [18]. The potential to show syneresis also depends on the pH adjustment, which influences the gel structure, which consists of a casein micelle network with heat-denatured whey proteins linked to the surface of the casein micelles [19].

3.4 Dissolved Protein Content

The average results in Table 1 with the addition of kefir grains to milk containing antibiotic residues, there was an increase in the average dissolved protein content from P0 (6.49 mg/ml) to P1 (8.16 mg/ml) and a decrease in P1 by 8.16 mg/ml to P3 of 7.06 mg/ml. The highest value of dissolved protein content was at P1, 8.16 mg/ml, while the lowest average value of soluble protein content was P0, 6.49 mg/ml. The research data showed no significant difference ($p < 0.05$) in the dissolved protein content. The dissolved protein content in whey only ranges from 0.5–0.7%.

The average value of dissolved protein content shows in Table 1. The results showed that more levels of Amoxycillin added would decrease the dissolved protein content compared with the control ($p < 0.05$). Dissolved protein content decreased from the T1 treatment was 8.16 mg/ml, to the T3 treatment was 7.06 mg/ml. In the presence of proteolytic enzyme activity, microbe-hydrolyzed complex proteins degrade into free amino acids or smaller peptides, reducing the concentration of dissolved protein. The rising dissolved protein level is due to the activity of Lactic Acid Bacteria hydrolyzing proteins during fermentation [20]. Kefir grains include bacteria and yeasts that can create protease enzymes, which disintegrate proteins. Starter bacteria can produce protease enzymes, which activate proteins to hydrolyze the simplest components of soluble proteins, including peptides and amino acids.

3.5 Microbiological Characteristic

The microbiological characteristic of milk is given in Table 1. The Viability of LAB differed between 8.68 and 8.88 Log CFU/mL. The results showed a significant difference between the control and treatment ($P < 0.05$). The high LAB Viability is due to the resistance of the LAB to Amoxycillin as an antibiotic. Amoxicillin is a type of penicillin, which is a drug used to treat bacterial infections. LAB are generally sensitive to beta-lactam antibiotics, except penicillin [21]. Antibiotic resistance occurs through gene mutation or the transfer of resistant genes between bacteria. Probiotic bacteria contain genetic elements that can provide resistance to antibiotics [22].

LAB native to traditionally fermented foods were isolated and analyzed for their ability to create bacteriocins that inhibit the growth of -lactamase-producing clinical isolates of gram-negative bacilli. L. Plantarum can create antibacterial compounds, which can serve as a viable alternative to antibiotics in treating pan-drug-resistant pathogens [23]. Antibiotics and bacteriocins have been offered as a unique treatment option for food-producing animals.

4 Conclusions

Adding 3% kefir grain can potentially eliminate up to 10 μg/ml of Amoxycillin as a β-lactam antibiotic residue of mastitis treatment. Further research is needed to know the precise role between LAB and Yeast in eliminating antibiotics.

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