

POTENTIAL ANTIMICROBIAL IN WHEY AND LACTIC ACID BACTERIA IN SUMBA MARE'S MILK

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ABSTRACT

Sumba mare's milk is rich in whey protein, fat, and lactic acid bacteria known to boost antimicrobial activities required in the prevention of diarrhea and inflammation. This study is, therefore, aimed at determining the antibacterial properties of whey and lactic acid bacteria as therapeutic compounds against pathogenic *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Salmonella* Enteritidis, respectively. The investigations were conducted at the Veterinary Public Health Laboratory of the Faculty of Veterinary Medicine, Nusa Cendana University. Whey antimicrobial test was performed using *Salmonella* Typhimurium ATCC® 19585™ and *Staphylococcus aureus*, while for lactic acid bacteria, *Salmonella* Enteritidis ATCC® 13076™ obtained from the Veterinary Public Health Section, Faculty of Veterinary Medicine, Bogor Agricultural University, was applied. However, mare colostrum was acquired from East Sumba, East Indonesia. The results showed the average penicillin inhibitory zones of whey protein were estimated at 20 mm and 17 mm against *Staphylococcus aureus*. This also revealed the average diameter of the penicillin inhibitory zone and whey protein were equally evaluated at 2.1 mm, although the latter demonstrated sufficient antimicrobial activity against *Salmonella* Typhimurium. Furthermore, the lactic acid bacteria inhibitory zone in filtrate and non-filtrates are strongly characterized using the agar method against *Salmonella* Enteritidis. In conclusion, whey protein and lactic acid bacteria from Sumba mare's milk have significant antimicrobial potentials during the treatment of Salmonellosis and *Staphylococcus aureus* infections.

Keywords: antimicrobial, mare's milk, whey

INTRODUCTION

Mare's milk as a source of food from animals contains complete and balanced nutritional components (Kilara & Vaghela, 2018), including sufficient essential amino acids, lactose, fat, vitamins, and minerals (Khan *et al.*, 2019, Pessione & Cirrincione, 2016, Zulueta *et al.*,

2009). In certain areas, mare's milk is consumed as a therapeutic supplement for most diseases, due to their important dietary content (Charu & Dhan, 2017, Coutinho da Silva *et al.*, 2017).

There are two primary classes of milk proteins, termed whey and

casein known to form colloidal substances (Minjigdorj *et al.*, 2012). Whey protein comprises a number of important compounds to support antimicrobial properties (Detha *et al.*, 2013; Salami *et al.*, 2010), including α lactoalbumin, β lactoglobulin, lactoperoxidase, lactoferrin and serum albumin. These substances are significant in the prevention of diseases caused by antimicrobials, antioxidants, anticancer, and immunomodulators (Minjigdorj *et al.*, 2012, Patel, 2015, Pessione & Cirrincione, 2016, Ramos *et al.*, 2015). Furthermore, the antimicrobial ability of mare's milk is also enhanced by the presence of vital lactic acid bacteria isolated from natural ingredients, and are widely spread (Detha *et al.*, 2019). Previous studies have suggested these bacteria possess the capability to prevent diarrhea and inflammation (Mazahreh & Ershidat, 2009) as a result of the initialized antimicrobial properties (Abushelaibi *et al.* 2017, Volzing *et al.* 2013).

Inflammation and diarrhea are triggered by several factors, including pathogenic infections of *Salmonella* Enteritidis, *Salmonella* Typimurium, and *Staphylococcus aureus*. The current pressing challenge describes the resistance of these bacteria to antibiotics, and therefore created a more complex situation in terms of treatment (Fečkaninová *et al.* 2017). *Staphylococcus aureus* is a normal flora in the human body, but with a

multi-drug resistant phenotype known to complicate possible treatments (Gardete & Tomasz, 2014, A. S. Lee *et al.*, 2018). The strains of these bacteria are highly resistant to vancomycin, a glycopeptide antibiotic responsible for the inhibition of cell wall biosynthesis, and serves as a choice drug for treating *Staphylococcus aureus* infections (McGuinness *et al.*, 2017). However, microbial resistance to antibiotics also occurs in pathogenic *Salmonella* sp. where appropriate and effective antibiotic therapy is required (Eng *et al.*, 2015). Specifically, multi-drug-resistant (MDR) *Salmonella* serotypes have also emerged with the potential to increase the mortality rate of *Salmonella* infections due to enhanced virulence and longer-lasting disease (Grant *et al.*, 2016, Nair *et al.*, 2018). A nutraceutical reference supports that whey protein and lactic acid in mare's milk possess potentially therapeutic properties to improve health (Corrochano *et al.*, 2019, Markiewicz-Keszycka *et al.*, 2013, Sandi & Salasia, 2016). Therefore, it is important to study the antimicrobial property of whey as a therapeutic material against pathogenic *Staphylococcus aureus*, and *Salmonella* Typimurium, and also the need to analyze the antimicrobial activity of the from Sumba mare's milk against *Staphylococcus aureus*, and *Salmonella* Enteritidis.

METHODS

Whey antimicrobial test was conducted using pathogenic bacteria *Salmonella* Typhimurium ATCC® 19585™ and *Staphylococcus aureus*, while for lactic acid bacteria, *Salmonella* Enteritidis ATCC® 13076™ was employed. Preparation of whey proteins was performed by previous method (Detha *et al.*, 2013). The resulting product was then added to HCL 2 N in order to achieve a pH of 4.6, where casein and whey are easily separated. Furthermore, whey was neutralized by introducing 2N NaOH and centrifuged in 10000 × g, for 30 minutes at 4 °C temperature (Detha *et al.*, 2013; Yoshida *et al.*, 2000).

In detecting the antibacterial activity of whey against *Salmonella* Typhimurium and *Staphylococcus aureus*, sterile round discs (6 mm) soaked in 100µL of each whey was applied, where the bacteria were extracted and placed on a plate repeatedly at four intervals. In addition, the antibiotics and sterile deionized water were used as the positive and negative terminal, respectively. Therefore, the inhibitory zone diameter was then measured and the antimicrobial activity was further expressed in terms of mm and the results as the mean.

The analysis of the activity of the Sumba mare's milk bacteria against *Salmonella enteritidis*

The isolation, as well as the determination of the *Lactobacillus*

spp. were performed in accordance with the LVS ISO 15214: 1998 guidelines, by using MRS media (de Man Rogosa and Sharpe with Tween, OXOID, UK). Also, the culture media were prepared in agreement with the LVS guidelines CEN ISO / TS 111331: 2009, and the sample dilutions involved salt-peptone solution, according to ISO 6887- 5: 2010. The selected parameters for cultivating these bacteria in MRS include a duration of 72 hours at 37 °C, based on the published scientific literature (Coeuret *et al.*, 2004; Kasi *et al.*, 2017). Therefore, confirmation tests for lactic acid bacterial isolates were performed through the application of the Gram stain, cell shape, catalase, alongside motility tests (Ismail & Yulvizar, 2017).

A volume of 1 mL extracted from the viable bacterial suspension of the mare's milk in the MRS Broth was withdrawn with the use of a micropipette and transferred into a test tube. Subsequently, centrifugation of the tube was performed at 3000 rpm for 30 minutes to separate the liquid from the filtrate, followed by filtration through a 0.45 micrometer millipore membrane. The evaluation of the efficacy of the *Lactobacillus spp.* against pathogenic bacteria was based on earlier studies (Ismail & Yulvizar, 2017; Kasi *et al.*, 2017; Schved *et al.*, 1993), and achieved by filtrate and non-filtrate analysis. This

investigation was performed by agar diffusion (disc paper), along with well methods (agar diffusion well), and the positive control examination of the pathogenic bacteria conducted using chloramphenicol antibiotics. The results obtained were adjusted to correspond with literature on the

categories of the zones of bacterial, composed of 4 groups namely, low (<5mm), average (5-10mm), high (> 10-20mm), and very high (> 20-30mm) (Detha *et al.* 2018; Morales *et al.* 2003). The experiment was repeated four times.

RESULTS AND DISCUSSION

The activity of mare's milk whey versus *Salmonella typhimurium* and *Staphylococcus aureus*

The analysis results revealed the average zones of penicillin inhibition of the whey protein against *Staphylococcus aureus* to be 20 mm and 17 mm. Subsequently, the existence of antimicrobial proteins in whey purported by various studies was corroborated by these results. Furthermore, the outcomes of this study functioned as a foundation for

researches promoting the advancement of lactoferrin obtained from the horse milk as a potential antibiotic agent against *Staphylococcus aureus*. The assessment results of the average inhibitory zone diameters were 2.1 mm each the pathogenic *Salmonella typhimurium* and the whey protein. Therefore, the susceptibility of this organism to antibacterial agents present in whey was indicated by these results.

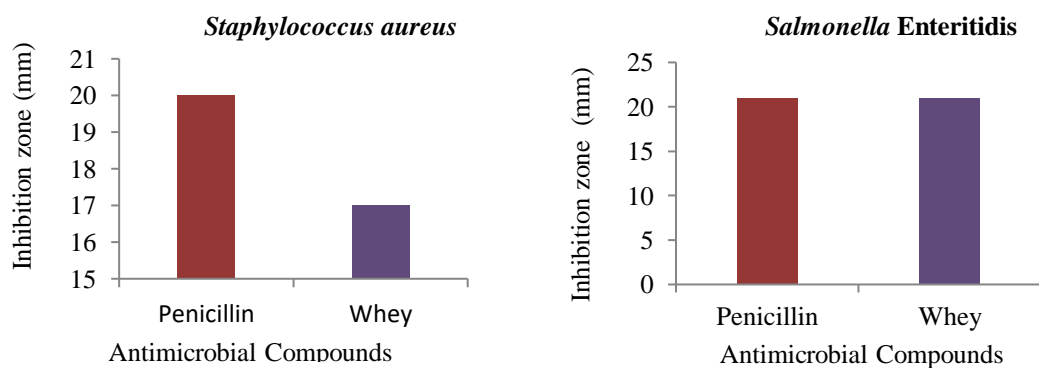


Figure 1: Inhibition zone diameter of whey from Sumba mares milk isolates against *Staphylococcus aureus* and *Salmonella Enteritidis*

The interaction between antimicrobial ingredients in mare's milk whey and the specified pathogenic bacteria were evidently in vitro, and the inhibition mechanisms numerous. This activity was

consistent with the presence of antibacterial peptides as constituents of whey proteins (Patel, 2015), including α lactalbumin, β lactoglobulin, blood serum albumin immunoglobulin, lysozyme, and

lactoferrin, known to play important roles in this process (Atanasova *et al.*, 2014; Markiewicz-Keszycka *et al.*, 2013; Ramos *et al.*, 2015). Furthermore, Lactoferrin interacted with target bacterial cells by the absorption of iron, and also facilitates the permeability of bacterial cell walls by binding to lipopolysaccharides on specific receptors to inhibit bacterial cell growth (Scala *et al.*, 2017). Moreover, Lysozyme and Lactoferrin are synergistically capable of effectively inactivating Gram negative and positive organisms, including *Salmonella typhimurium* and *Staphylococcus aureus* (A. Detha *et al.*, 2019; Wiesner & Vilcinskas, 2010). The antimicrobial activity of mare's milk whey adds to the data regarding the antimicrobial activity of natural ingredients against pathogenic

agents that cause infection (Detha *et al.* 2015, Detha and Wuri 2016). Moreover, the peptides in α -lactoalbumin exhibit immunomodulatory effects, including the stimulation of phagocytosis from macrophages, and therefore, α -la is regarded as a compound with essential antimicrobial abilities (Marshall, 2004; Ramos *et al.*, 2015). In addition, immunoglobulin, lactoferrin and lysozyme strengthen the body's immunity, and also protect against infections (Ng *et al.*, 2015; Pessione & Cirrincione, 2016). Therefore, this research was regarded as the basis for the production of probable antimicrobial agents from the whey extracted sumba mare's milk in the therapy against Salmonellosis and *Staphylococcus aureus* infections.

Antimicrobial activity of lactic acid bacteria isolates of mare's milk against *Salmonella Enteritidis*.

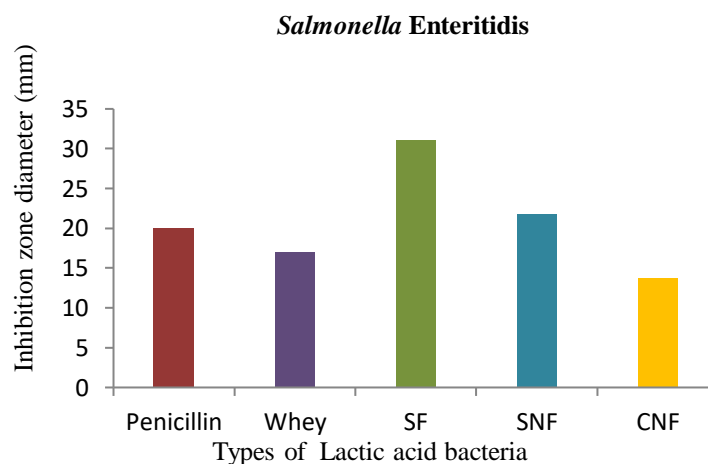


Figure 2: Inhibition zone diameter of lactic acid bacteria of mare's milk isolates against *Salmonella Enteritidis* (CF indicates Lactic acid bacteria in the form of disc filtrate, CNF denotes non lactic acid filtrate bacteria, SNF represents Non lactic acid bacteria wells filtrate and SF symbolizes Lactic acid bacteria wells filtrate)

These results showed the ability for lactic acid bacteria to inhibit *Salmonella* Enteritidis growth. Therefore, filtrate and non-filtrate test using the agar diffusion process (disc) observed at 48 hours were known to have an average inhibition zone of 23.8 and 21.3 mm respectively. Meanwhile, the well method had 21.1 mm and 26.4 mm respectively. The inhibition zone of 32 mm was recorded for the control antibiotics. According to the results, lactic acid bacteria inhibitory zone for the filtrate and non-filtrate form with the agar well method against *Salmonella* Enteritidis was included in a very strong category, while the non-filtrate test with the diffusion process (discs) was in a strong class. Moreover, there are four groups of inhibitory zone activities, including weak (<5mm), moderate (5–10mm), strong (> 10–20mm), and very strong (> 20–30mm) activities (Morales *et al.*, 2003).

Furthermore, several previous studies on lactic acid bacteria discovered an antimicrobial ability because of various microbicide compounds produced (Atanasova *et al.* 2014). These products include organic acids, diacetyl, hydrogen peroxide, bacteriocin, and reuterin with certain characteristics. Also, each product has a different microbicide level (Stoyanova *et al.*, 2012). The organic acid compounds have the broadest level of antibacterial activity. This broad spectrum is known to be as a result of the synergistic collaboration between

lactic and acetic acid and therefore induce the killing power of bacteria, including the pathogenic bacteria *Salmonella* Typhimurium (Suryani *et al.*, 2014; Topisirovic *et al.*, 2006; Volzing *et al.*, 2013). The lactic acid produces pH change and results in low acidity level, therefore antimicrobial properties are added to pathogens (Stoyanova *et al.* 2012).

Also, another lactic acid bacterial compound includes Diacetyl. This has proven effective in the inhibition of pathogenic bacteria, including the genera *Salmonella*, *Yersinia*, *Escherichia*, *Aeromonas*, and *Bacillus* (Mays & Nair, 2018; Pessione & Cirrincione, 2016; Stoyanova *et al.*, 2012). The bacteriocin is also excreted by lactic acid bacteria and has the ability to destroy bacterial cells, therefore inhibiting the growth of pathogens and possibly induce death (Urnemi *et al.*, 2016). In addition, reuterin binds with enzymes, SH, including ribonucleotide reductase, during anaerobic processes by *Lactobacillus reuteri*, *L. brevis*, *L. buchneri*, *L. collinoides*, and *L. corniformis* (Hernández-Aquino *et al.*, 2019; Šušković *et al.*, 2010). Reuterin is known to inhibit pathogenic enterobacteria including *Salmonella* and *Shigella*, genera *Clostridium*, *Staphylococcus*, and *Listeria* (Schaefer *et al.*, 2010). Therefore, lactic acid bacteria against are an anticipated cure for salmonellosis due to the antibiotic properties exhibited towards *Salmonella* Enteritidis. Furthermore, the need for these

beneficial micro-organism increases with the rising threat of bacterial resistance.

CONCLUSION

The results obtained are a sufficient basis to develop whey from sumba mare's milk into a treatment

for Salmonellosis and *Staphylococcus aureus* infections.

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CONFLICT OF INTERESTS

There were no competing interests at any stage in this study.

AUTHORS' CONTRIBUTION

All the authors contributed to this study equally. AD participated in the design of the research thought-flow, data acquisition and analysis, as well as drafting and reviewing the

paper. Meanwhile, AS, AO and HD were responsible for the adaptation of analytical techniques, plus acquisition and analysis of data.

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