Total Bacteria and pH of Dangke

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Total Bacteria and pH of Dangke Preserved Using Natural Antimicrobial Lactoferrin and Lactoperoxidase from Bovine Whey

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ABSTRACT

Dangke is the Indonesian cheese produced from bovine milk using latex from young papaya to coagulate casein. Dangke is generally consumed by Indonesian people located in South Sulawesi Province. In line with demand of Dangke, the preservation is needed. Since, there was no literature which was found about preservation of Dangke, this study is aimed at knowing the quality of Dangke based on total bacteria and pH value stored in antimicrobial agent of lactoferrin and lactoperoxidase system from bovine whey, aquadest and phospate buffer at ambient temperature for 12 h. The lactoferrin, lactoperoxidase and whey were obtained from bovine milk and purified using ion exchange chromatography method. The result of the study showed that lactoperoxidase system provide remarkable effect of decreasing total bacteria from 8 log CFU mL^{-1} to 5 log CFU mL^{-1} while other storage solutions have no antimicrobial activity against bacteria in Dangke. The pH value of Dangke was stable when stored in lactoferrin and lactoperoxidase system. Since, both of these preservatives could be categorized as safe, the application in Dangke may open the alternative method to store Dangke.

Key words: Dangke, lactoferrin, lactoperoxidase, whey, total bacteria

INTRODUCTION

Dangke is a traditional cheese from South Sulawesi Province in Indonesia. Dangke is mostly made from cow's milk but buffalo's milk or their mixture can also be used. Dangke is a semi solid and salty cheese that available in the traditional market and traditionally manufactured by local people. A small amount of papain has been used to coagulate casein from whey. After whey removal, the mild pressure is usually applied to produce semi solid cheese. The compositions of Dangke are 47.75% of water, 2.32% of ash, 33.89% of fat and 17.01% protein (Marzoeki *et al*., 1978). The process of making Dangke initially is started by heating in low temperature for long time (65°C, 30 min) and for casein coagulation, subsequently 5 g of papain is added into milk. The addition of papain exerts bitter taste since the papain may promote the hydrophobic groups generation (Amri and Mamboya, 2012). The bitterness taste of Dangke may be neutralized by the addition of salt. It has been understood that salt may also inhibit the spoilage of bacteria (Beresford *et al*., 2001). Native people commonly consume Dangke for the complimentary of their food, so the salt may promote the better taste in food (Sirajuddin *et al*., 2013).

Dangke manufacturing is mostly made from cow's milk but sheep's and goat's milk or a mixture of them. Since, the local people consume Dangke daily, they did not pay high attention for the preservation because local people will consume it immediately after manufacturing. However, since the number of local people is travelling from and to this province, the demand has increase resulting in the need for preservation. Natively, Dangke's shelf live is relatively short (about six hours), this is because Dangke is made from fresh milk that contains various elements and mostly consists of food substance that is also needed for bacteria growth. One of methods to extend the storage period of food product is the preservation by using antimicrobial substances or compounds.

The preservative for prolonging the shelf live of Dangke may be obtained from chemicals however, since the people may pay much more concern on their health, the chemicals based preservation may be avoided. In line with this demand, researchers pay much more attention for the utilization of the Generally Recognize As Safe (GRAS)'s preservatives. Lactoferrin or most commonly called lactotransferrin is transferrin that is isolated from milk. Lactoferrin is antimicrobial agent because it contains glycoprotein-703 amino acid that has extremely high ability to bind Fe from microbe, so that it significantly inhibits microbe growth (Conneely, 2001). Lactoperoxidase system is widely known as a system that naturally exists in fresh milk as antimicrobial. Lactoperoxidase system has been proven for being active to positive and negative gram microorganism (Naidu, 2000; Marks *et al.*, 2001). Lactoperoxidase system catalyses reaction of hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-) that occur naturally in milk to become a compound named hypothiocyanite ($OSCN^-$) (Barrett *et al.*, 1999; Kussendrager and van Hooijdonk, 2000; Seifu *et al.*, 2007). The $OSCN^-$ is a compound that takes responsibility for killing bacteria, fungi and virus by breaking down sulfhydryls groups (S-H group) from cell membrane causing vital impairment of cell membrane finally leading to the death of the cell (Al-Baarri *et al.*, 2011a; Borch *et al.*, 1989; Dajanta *et al.*, 2008; Touch *et al.*, 2004).

Based on the remarkable antimicrobial activity of lactoferrin and lactoperoxidase system and there is no study that was found in the preservation of both compound in Dangke, this study was aimed at analysis of total bacterial growth and pH value of Dangke stored at ambient temperature. The result of this study may provide an alternative way for Dangke's storage.

MATERIAL AND METHODS

Materials: Fresh bovine milk was provided by Campus Farm in Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang-Indonesia. Papain enzyme was obtained from 3-4 month old fresh papaya fruits. Commercial microbial rennet was obtained from Singapore. The spectrophotometer (Mini UV-1800, Schimadzu, Japan) was used for analysis of LPO activity and detection of protein concentration. The H_2O_2, KSCN, 2, 2-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS) were purchased from Sigma. Unless other specified compound were reagent grade.

Whey preparation: The whey was prepared as method conducted by Al-Baarri *et al.* (2011b) without any modification.

LPO production from whey: Whey was used for production of lactoperoxidase and lactoferrin through ion exchange method using SP Sepharose Fast Flow Column (GE Healthcare Bio-Science AB, Sweeden, Lot. No. 10081054). Subsequently, 0.4 M NaCl in 300 mL of 0.1 M PB (pH 7.0) was flowed into SP Sepharose® Fast Flow in order to generate lactoperoxidase solution. Three hundred millilitres of 1 M NaCl in 0.1 M PB (pH 7.0) was then poured to produce lactoferrin solution. Each

eluate obtained from above mentioned method was analyzed for approximate protein concentration in each tubes (10 mL tube^{-1}) using spectrophotometer and its absorbance was measured at 280 nm. Top ten highest absorbance of tubes after peak were collected to determine the LPO enzyme activity using ABTS at 412 nm (Al-Baarri *et al*., 2011b). To check the purity of lactoperoxidase and lactoferrin, the SDS PAGE was applied.

Manufacture of Dangke: Procedure of Dangke's making was adapted from method of JICA (2009). It was started by a heating of 3 L of fresh bovine milk at 60°C for 30 min. The next step was the addition of 0.03% (v/v) papain enzyme. After aglutination occurred, the whey was drained by using sterile filter cloth. The curd was then stored in ambient temperature and gently pressed for 3 h to produce the Dangke.

Microbial count: Petrifilm Aerobic Count Plates (3 M Microbiology, St. Paul, Minn., U.S.A.) was used to count the microbial appeared in Dangke. After manufacture, Dangke was cut into cube with the approx. of weight 1 g. The number of total bacteria in Dangke in the presence of lactoperoxidase system was determined as follows: 1 g Dangke was stored at 1000 μL hyphothiocyanite-rich-solution and incubated for 6 h at 30°C. Hyphothiocyanate-rich-solution was made from the addition of 250 μL of 1.0 mM H_2O_2 and 250 μL of 1.0 mM KSCN into 500 μL of LPO solution (35 U mL^{-1}). After incubation at 30°C for 10 min, hyphothiocyanite-rich-solution should be generated. Enumeration of bacteria was done by counting the solution that was obtained from serial dilutions of the assay mixture with a sterile 0.88% NaCl solution. The diluted mixture (1000 μL) was spread onto plates. The plate were incubated at 37°C for 48 h. The CFU of microbes in the sample solution were counted on the plates.

Statistical analysis: Total bacteria of Dangke stored in various storage solutions for 12 h were analyzed statistically using one-way analysis of variance (ANOVA) and the means were compared by the Duncan test at a significant level of 0.05 (Free Statistical Software Package R for Macintosh, U.S.A).

RESULT AND DISCUSSION

Purification of lactoperoxide and lactoferrin: Lactoperoxidase and lactoferrin was obtained from whey using ion exchange chromatography method. Both components were collected from top ten highest absorbance of tubes after peak at 280 nm (10 mL per tube). A high peak of absorbance at 280 was detected from fraction number 17 (for lactoperoxidase) and fraction number 11 (for lactoferrin) (Fig. 1). The fraction number 17-26 (for lactoperoxidase) and 11-20 (for lactoferrin) were collected and checked the protein profile using SDS-PAGE (Fig. 2). Lactoperoxidase activity from the collected eluate was analyzed resulting the value of 45 U mL^{-1}. The protein concentration of the collected eluates containing high concentration of lactoferrin was analyzed using Lowry method resulting value of 8.1 mg mL^{-1}.

Total microbe: The manufacture of Dangke consumes 3-6 h, so, these long time of treatments may sometimes have a negative effect on bacterial count of Dangke. Furthermore, the high temperature at local area may promote the growth of bacteria resulting in the upturning the elevation of bacteria. This study used phosphate buffer, lactoferrin and lactoperoxidase system for

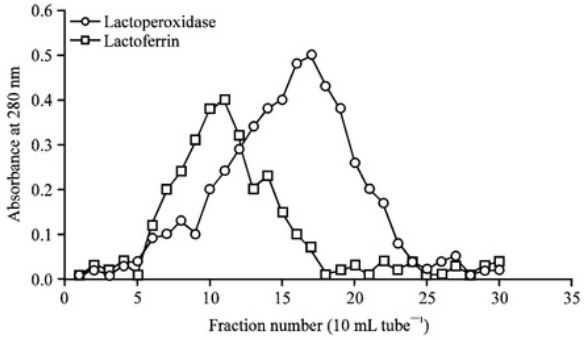

Fig. 1: Absorbance at 280 nm of the eluate from SP sepharose fast flow column (10 mL tube^{-1}) containing high concentration of lactoperoxidase and lactoferrin. The ten tube after peak was collected to analyze its protein profile using SDS PAGE

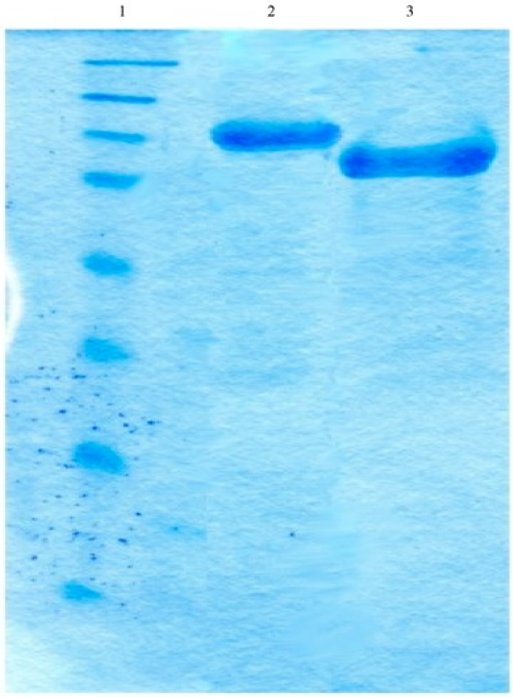

Fig. 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of eluate containing high concentration of lactoferrin, lactoperoxidase and purified from bovine milk using SP Sepharose Fast Flow. Lane 1: Standard protein from 16.5-120 kDa, Lane 2: Lactoferrin, Lane 3: Lactoperoxidase

the storage solution of Dangke. The 1 h of dipping in the storage solutions were applied then the total bacteria was calculated based on the bacteria growth in the surface area of Dangke (Fig. 3).

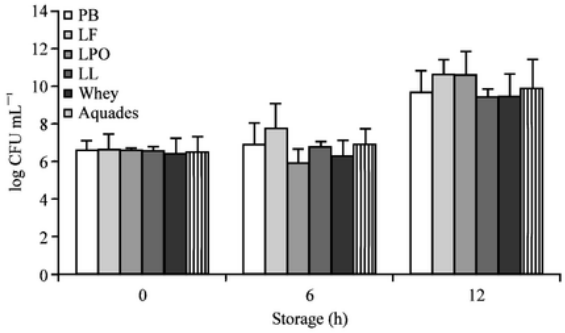

Fig. 3: Dangke total microbe with soaking treatment in solution of phosphate buffer, lactoferrin, lactoperoxidase system, lactoferrin+lactoperoxidase system, whey and pure water during the storage

Table 1: pH value of Dangke soaked in phosphate buffer, lactoferrin, lactoperoxidase system, lactoferrin+lactoperoxidase system, whey and pure water/aquades at ambient temperature

	Dangke pH value after treatment					
Storage period (h)	PB	LF	LPOS	LF+LPOS	Whey	Aquades/pure water
0	6.72±0.19	6.53±0.01	6.52±0.08	6.47±0.06	6.20±0.01	7.17±0.03
6	7.07±0.06	6.58±0.02	6.87±0.06	6.53±0.06	6.63±0.06	7.18±0.01
12	6.64±0.01	6.50±0.00	6.50±0.00	6.38±0.08	6.10±0.10	6.66±0.01
Mean	6.81^{b}	6.54^{d}	6.63^{c}	6.46^{e}	6.31^{f}	7.00^{a}

a,b,c,d,e,f value with superscript letter behind number that is different on mean line shows real difference. (x,y,z) value with superscript letter behind different number on mean column shows real difference ($p<0.05$)

Based on the figure, initial total bacteria in Dangke was detected from a range of 6.46±0.78 up to 6.64±0.80 CFU mL^{-1}. If compare to the maximum limit of total bacteria in soft cheese, i.e., 6 log CFU mL^{-1} (Indonesian National Standard, 2000), the number of total bacteria just on the limit. The amount of total bacteria on the standard limit indicating probable contamination of the milk as a result of poor hygiene and the contamination at the processing plant may increase the number of total bacteria in Dangke.

The increase of total bacteria was detected on the Dangke stored in phosphate buffer from 6.65±0.5-6.95±1.1 CFU mL^{-1}. The prolongation of incubation into 12 h resulting in the remarkable increase of total bacteria to 9.70±1.12 CFU mL^{-1}. The remarkable amount of total bacteria on Dangke stored for 12 h was detected on all treatments ranged from 9.46±0.4-10.61±0.8 CFU mL^{-1}.

The storage of Dangke in phosphate buffer, lactoferrin, lactoferrin+lactoperoxidase system, whey and pure water for 6 h slightly increased the total bacteria to the amount of total bacteria ranged from 6.36±0.7-7.70±1.3 CFU mL^{-1}. Amazingly, the lactoperoxidase system storage remarkable decreased the total bacteria from 6.59±0.1-5.95±0.7 CFU mL^{-1}.

The occurrence of the decrease of total microbe at the sixth hour using lactoperoxidase system as soaking media at ambient temperature is shown in Fig. 3. Dangke that was soaked in lactoperoxidase system had 5.95 log CFU mL^{-1} of total microbe. The result of Touch *et al.* (2004) study could reduce the amount of *S. enteretidis* in vegetable product as much as 5.4 log unit and could inhibit the organism growth for 4 h at 30°C incubation with lactoperoxidase system

treatment. Lactoperoxidase catalysed thiocyanate oxidation by hydrogen peroxide and resulted in product with antimicrobial characteristic (Seifu *et al.*, 2005) especially hypothiocyanate ion, this ion will react with membrane of bacteria cytoplasm and interrupt metabolic enzyme function and produce antimicrobial effect (Jooyandeh *et al.*, 2011). Hypothiocyanate is bacteriostatic and tends to have main part in lactoperoxidase system (Aune and Thomas, 1977).

Treatment with lactoferrin soaking at the sixth hour could not reduce total microbe, this was suggested that lactoferrin activity decreased, so that the holding capacity to iron weakened. Adlerova *et al.* (2008) reported that though lactoferrin had the ability to hold free iron, that is one of essential elements for the growth of bacteria and responsible for bacteriostatic effect. However, some bacteria can adapt with new condition and release siderophores (Iron chelat compound that is derived from bacteria) that compete with lactoferrin for Fe^{3+} ion (Crosa, 1989; Ratledge and Dover, 2000). Some types of bacteria that include in Neisseriaceae family adapt with new condition by expressing specific receptor that can hold lactoferrin and cause the change of lactoferrin molecule tertiary structure that caused iron dissociation (Ekins *et al.*, 2004).

Storage for 12 h in all treatments cannot reduce the total microbe, it was suggested that the longer the storage at ambient temperature, the higher the amount of total microbe of milk product. This is along the lines with Buckle *et al.* (1987) study stated that condition of storage temperature has effect on the amount of total microbe, it is caused by the storage temperature influences metabolism and the growth of microbe. The higher the temperature (ambient temperature 20-30°C), the faster the speed of microbe metabolism and growth, in reversed, the lower temperature (cold temperature 4°C), the slower the speed of bacteria metabolism and growth. Dangke storage in this study was stored at ambient temperature (30°C) so that the increase of the amount of total microbe on the treatment at the 12th h was occurred. The antibacterial activity of lactoperoxidase system depends on bacteria species or strain used, temperature of incubation, type of media used in activation and concentration of lactoperoxidase system components (Sarkar and Misra, 1992; Fuglsang *et al.*, 1995).

pH value: The pH value of Dangke stored in various medium at ambient temperature is presented in Table 1. It is showed that the pH of Dangke was significantly affected by medium ($p<0.05$). Dangke stored in lactoperoxidase sistem and lactoferrin were more stable in pH value if compare to other medium (the decrease were 0.3-0.4%). The less change of pH of Dangke stored in lactoperoxidase system and lactoferrin indicated less of microbial activity since the pH value may indicated the microbial activity. The remarkable decrease in pH value (1.2-7.6%) was found in Dangke stored in PB, LL, whey and aquadest. The lowest pH value was found in the whey medium since there was no buffer applied in whey. This study was used PB pH 7.0 as solvent in all applied enzymes, therefore, the minimum achieved pH of danke stored in enzymes was stable (Stoll and Blanchard, 1990). The range of pH of Dangke in all treatments were at a range 6.10±0.1-7.18±0.01, however, the sampel with enzyme treatment achieved pH at range 6.10±0.1-6.87±0.06 indicating inline the requirement of pH in milk derived product in Indonesia (from pH 6.0-7.0) (Indonesia National Standard).

Lactoperoxidase system and lactoferrin inhibited the reduction of pH value, however the combination both of these enzymes were unable to inhibit the reduction resulting in the decrease of pH from 6.47±0.06-6.38±0.08. Synergistic effect of two enzyme on antibacterial activity was found in many investigation (Murdock *et al.*, 2007; Chung and Hancock, 2000), however, as described previously LPOS and LF were unable to inhibit the decrease of pH.

CONCLUSION

It can be concluded from the result of this study that lactoperoxidase system can be used as antimicrobial agent that can reduce Dangke total microbe with 6 h incubation period at ambient temperature. The soaking using lactoferrin and lactoperoxidase system can maintain Dangke pH value.

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