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Antimicrobial activity of goat milk yoghurt with addition of a probiotic *Lactobacillus acidophilus* IIA - 2B4 and roselle (*Hibiscus sabdariffa* L) extract

[1]Hanifah, R., [1*]Arief, I. I. and [1,2,3]Budiman, C.

[1]*Department of Animal Production and Technology, Faculty of Animal Science, Bogor Agricultural University, Jalan Agathis, Campus IPB Darmaga, Darmaga, Bogor 16680, West Java, Indonesia*
[2]*Okinawa Institute of Science and Technology, 1919-1 Tancha, Kunigami, Onna son, Okinawa, Japan, 904-0495*
[3]*Biotechnology Research Institute, Universiti Malaysia Sabah (UMS), Jl. UMS 88400, Kota Kinabalu, Sabah, Malaysia*

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Abstract

Despite its advantages, goat milk has limitations in term of short shelf life and goaty odour. To overcome these limitations, in this research, goat milk was processed into yoghurt. To improve its quality, a probiotic bacterium isolated from Indonesian cattle, *Lactobacillus acidophilus* IIA-2B4, and roselle extract were added to the yoghurt. Total lactic acid bacteria (LAB) in yoghurt with addition of *L. acidophilus* IIA-2B4 with or without combination of roselle extract is significantly higher compared to the control. The high population of LAB in yoghurt with addition of *L. acidophilus* IIA-2B4 with or without roselle extract is proportional to the acidity of the product that promotes higher viscosity compared to the control. Proximate analysis revealed that additions of *L. acidophilus* IIA-2B4 and/or roselle extract significantly reduce fat content, while ash content is significantly increased by the treatments. Antibacterial activity assay demonstrated that goat milk yoghurt is able to inhibit both of Gram positive and negative bacteria with high selectivity towards Gram positive bacteria. Addition of *L. acidophilus* IIA-2B4 with or without roselle extract increases the ability of yoghurt to inhibit Gram negative bacteria. This ability might be due to the presence of peptides exhibiting antimicrobial activity produced during fermentation by probiotic. SDS-Page revealed that addition of *L. acidophilus* IIA-2B4 with/without roselle extract produces <10 kDa peptides which display remarkable antimicrobial activity that might contribute to total antimicrobial properties of yoghurt. This indicated that indicating that increasing antimicrobial activity of yoghurt in the presence of *L. acidophilus* IIA-2B4 was also contributed by antimicrobial peptides produced during the fermentation.

Introduction

In developing countries, including Indonesia, milk of small ruminant, such as goat, has a high economic value. Despite goat milk production in the world is relatively minor compared to that of cow milk, goat milk has several advantages, such as brighter colour, and smaller fat globules, so it is easier to digest and can be consumed by people with lactose intolerance (Tziboula-Clarke, 2003). These advantages lead to increase demand and growing goat milk processing industry (Martin *et al*., 2003).

Yet, goat milk has limitations including relatively short shelf life and undesirable goaty odour. Processing of goat milk has been proved not only increase its shelf life, but also remarkably decrease goaty odour and promotes consumer acceptability. There are several processing methods converting goat milk to other derivative products, including yoghurt (Ribeiro and Ribeiro, 2010).

Yoghurt is a fermented dairy product, manufactured from cow milk that can be made from whole, low fat or skim milk, including reconstituted non-fat dry milk powder. Culture that commonly used as fermentation starters for yoghurt production is a mixture of lactic acid bacteria (LAB), *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. LAB have been regarded as probiotic due to their health benefits (Ljung and Wadstrom, 2006). The use of probiotics of LAB in yoghurt production hence may promote the health values.

Goat milk and yoghurt have been proved to be an excellent vehicle for probiotic bacteria, which might provide more health benefits for consumers. Arief *et*

*Corresponding author.
Email: *irma_isnafia@yahoo.com*
Tel: +62-251-8628379

al. (2015a) and Astawan *et al*. (2012) have isolated *Lactobacillus acidophilus* IIA-2B4 displaying probiotic properties. *L. acidophilus* IIA-2B4 has been shown as a promising fermentation starter in meat products (Afiyah *et al*., 2015; Arief *et al*., 2016), yet the study on this probiotic on yoghurt production has not been studied.

The use of probiotics in yoghurt fermentation contributes to prolonging product shelf life due to ability of probiotics to produce antimicrobial peptides. In addition to antimicrobial peptides, some organic compounds that exhibit antimicrobial properties produced by probiotics during fermentation including lactic acid and bacteriocin (Vesterlund and Ouwehand, 2004). Further, LAB also exhibit biochemical activities, including proteolysis that contribute to characteristic of final products. The activities produced peptide fragments that might exhibit antimicrobial activity.

In order to improve functional properties of yoghurt, additional materials are often used include some herbs that have demonstrated to exhibit desirable properties hence may improve characteristic of the final product. Desirable properties include antimicrobial, flavour enhancer, or others.

One of promising herbs for combination material for yoghurt production is roselle (*Hibiscus sabdariffa* L). Roselle is a species native to West Africa. Roselle flowers extract contain some compounds exhibiting antibacterial activity towards *Escherichia coli, Salmonella* Typhi, and *Staphylococcus aureus*. Furthermore, the extract can also inhibit the growth of *Streptococcus pyogenes, Staphylococcus* spp., and *Streptococcus sanguinis* (Olaleye, 2007). In some countries, roselle has been used as medicinal plant including antihypertensive, anticonvulsants, worm drugs, and intestine antiseptic. The use of roselle in yoghurt has been shown to maintain its physical and chemical qualities for 16 hours storage at room temperature (Adam and Andy, 2011). Antibacterial activity is speculated due to the presence of flavonoid and tannin (Olaleye, 2007). Due to the lack of information about the antimicrobial activity of peptides produced during fermentation of goat milk and effect of roselle on yoghurt properties, this research aims to characterize antimicrobial activity of yoghurt from goat milk with combination of additional probiotic, *L. acidophilus* IIA-2B4, and roselle extract. We found that additions of probiotic *L. acidophilus* IIA-2B4 and/or roselle extract significantly improved antibacterial activity. We also found that these additions widen the antimicrobial spectrum. Interestingly, addition of *L. acidophilus* IIA-2B4, regardless of the presence of roselle extract, yielded more <10 kD peptide as compared to the yoghurt without *L. acidophilus* IIA-2B4. This leads an assumption that these peptide might contribute to the antimicrobial properties of yoghurt with addition of *L. acidophilus* IIA-2B4.

Material and Methods

Materials

Goat milk was collected from university farm of Bogor Agricultural University. Starter for yoghurt production (*Streptococcus thermophilus* RRAM-01 and *Lactobacillus bulgaricus* RRAM-01 isolated from milk of local farm) was obtained from culture collection of Animal Product Technology Division, Faculty of Animal Science, Bogor Agricultural University. *Lactobacillus acidophilus* IIA-2B4 was previously isolated from local beef (Arief *et al*., 2015a). Roselle flower was obtained from local plantation, Bogor, Indonesia. Bacterial media were purchased from Oxoid, UK, while other analytical chemicals were obtained from Sigma, USA.

*Roselle flower extraction (*Hibiscus sabdariffa *Linn.)*

Extraction of Roselle flower extract was performed according to Tsai *et al.*, (2008). Briefly, dried roselle flower were grind until become a powder then sifted with 60-mesh screen. The sifted powder were dissolved in water at the level 20% (w/v) powder solution then pasteurized at 63–65°C for 30 minutes followed by cooling it down at 37°C.

Production of goat milk using L. acidophilus *IIA-2B4 and/or roselle extract*

Goat milk was heated at 75-80°C for 30 minutes, followed by cooling it down to 37°C. Yoghurt culture (*Streptococcus thermophilus* RRAM-01 and *Lactobacillus bulgaricus* RRAM-01) and probiotic starter (*Lactobacillus acidophilus* IIA-2B4) were inoculated to goat milk with population more than 10^7 cfu/mL for each. This population was reached by adding about 2% (v/v) of culture. The milk was then incubated at 37°C for 18 hours until it formed a coagulation (yoghurt plain) (Donkor *et al.*, 2006).

For yoghurt with addition of roselle extract, yoghurt with or without *L. acidophilus* IIA-2B4 was prepared as explained before. After incubation at 37°C for 18 hour, 1% (w/v) roselle extract was added.

pH and Titratable acidity

The pH of yoghurt was measured using pH meter (Hanna, Romania). Titratable acidity was measured by titration method (AOAC, 2005).

Proximate analysis

Proximate analysis was performed to determine ash, proteins, moisture, fat, and carbohydrates contents in yoghurts and the analysis were conducted according to AOAC (2005) procedures.

Water activity (a_w)

Water activity of yoghurt was determined using Novasiana aw meter (Pfaffikon, Switzerland) using 25 mL of the samples.

Viscosity

Viscosity of yoghurt was assessed by Rion VT-04F viscometer (Japan). Sample was placed in the chamber of viscometer and the temperature was set at room temperature (25-27°C).

Lactic acid bacteria population

The 1 mL amount of samples was diluted with buffer peptone water (BPW) to have stock sample solution with 10%. This solution was further diluted step wise yielding diluted solution with concentration of 10 to 10^8 times lower than stock solution, denoted as P1 to P8 solution. The inoculation were conducted by pour plate method, in which 1 mL of sample from P6 to P8 solution were poured into petri dish and mixed with 15 mL deMan, Rogosa and Sharp agar (MRSA) media. Petri dish was then incubated at 37ºC for 24–48 hour.

Total antimicrobial activity of yoghurts

Total antimicrobial activity of yoghurts was performed using well diffusion method (Bintang, 1993). *Bacillus cereus, Escherichia coli, Salmonella* Typhi and *Staphylococcus aureus* were used as representative of pathogenic bacteria. In addition, *Saccharomyces cerevisiae* and *Aspergilus niger* were also used as representative of yeast and fungi groups. The addition of these groups were aimed to determine whether or no the treated yogurt exhibit antimicrobial fungal properties. This assumption is based on the finding that some LAB were reported to produce some anti fungal substances. These bacteria, fungi and yeast were prepared to obtain 10^7 cells population in BPW, denoted as ready-to-use cultures. 1 mL of each culture was poured into the petri dish followed by adding 15 mL of Muller Hinton Agar (MHA). After solidification of the medium, 6 wells were created in the middle of each petri dishes by using Durham tube with 6 mm diameters. Every petri dish consists of 3 repetitions in duplo. Approximately 50 µL of the samples (roselle, yoghurt, yoghurt roselle, yoghurt probiotic, and yoghurt probiotic roselle) either full or separated, were poured into the well by using pipette and cooled down at 7°C for ± 2 hours.

After incubation at 37°C for 24 hours, inhibition zone that appeared as transparent zone around the well were measured by using vernier calipers. Measurements were taken in three different spots and inhibition activity was determined from the averaged diameter of inhibition zones.

Analysis of peptide fragments from proteolysis

Analysis of peptide fragments produced by proteolysis during fermentation of all tested samples. All samples were centrifuged at 5,000 g for 15 min and non-coagulated fractions were separated for further analysis and observation. The fragments from non-coagulated fractions was performed using sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) method (Lowry *et al*., 1951).

Antimicrobial activity of peptide fragments

Non-coagulated fractions from yoghurts were separated as explained before. The fractions were further filtered using centrifugal filter units (Milipore, USA) with molecular weight cutting-off is 10 kDa. The fractions below 10 kDa were collected for antimicrobial activity. Antimicrobial activity was examined not only by using diffusion method as explained before (Bintang, 1993) but also the direct contact method (AOAC, 2005). For direct contact method, *E. coli* was used as antagonist bacteria. One mL of 10 kDa fractions of yoghurts were mixed with 1 mL of *E. coli* bacteria population 10^6 cfu / mL. The solution was then diluted with dilution of 10^4 to 10^6 times (for observation 0 hours). Further, series dilutions from 10^4 to 10^8 were prepared for 24 hours of observation. Dilution was transferred into a sterile petri dish in duplicate then added as much as 15 mL Eosin Methyline Blue (EMB) agar by the method of casting and homogenized. Petri dishes were incubated upside down at 37°C for 24 hours.

Statistical analysis

The experimental design used in this research is completely randomized design (CRD) and the data was analysed using analysis of variance with Tukey`s post hoc test (Steel and Torrie, 1995). The data were presented as mean with standard deviation from at least three independent experiments.

Result and Discussion

Phytochemicals roselle extract

Some phytochemicals components of roselle extract were qualitatively detected. The compounds

Table 1. Some microbiological, chemical and physical qualities of yoghurts

Parameters	Y*	YR*	YP*	YPR*	Codex**
Total LAB (cfu/mL)	9.13 ± 0.06b	9.10 ± 0.03b	9.42 ±0.02a	9.43 ±0.02a	Min 7.00
Proximat (%)					
- moisture	72.34 ±1.37b	78.07 ±1.11a	77.89 ±0.22a	79.66±2.47a	
- ash	0.80 ±0.06b	0.91 ±0.03a	0.88 ±0.02b	0.96 ±0.02a	
- fat	15.73 ±2.95a	10.38 ±1.30b	13.79 ± 1.28ab	10.86 ± 2.55b	Less than 15%
- protein	3.41 ±0.22a	3.63 ±0.05a	3.72 ±0.11a	3.75 ±0.34a	Min 2.7%
Physical and Chemical Quality					
- pH	4.20 ±0.03a	4.12 ±0.02b	3.72 ±0.01c	3.68 ±0.02c	
- lactic acid percentage	1.08 ±0.01c	1.27 ±0.06b	2.15 ±0.02a	2.08 ±0.11a	Min 0.6%
- viscosity (dpa)	3.76 ±0.15a	3.36 ±0.11a	2.43 ±0.15b	2.30 ±0.20b	
Parameters	Y	YP	YR	YPR	Codex
Total LAB (cfu/mL)	9.13 ± 0.06b	9.42 ±0.02a	9.10 ± 0.03b	9.43 ±0.02a	Min 7.00
Proximat (%)					
- Moisture	72.34 ±1.37b	77.89 ±0.22a	78.07 ±1.11a	79.66±2.47a	
- Ash	0.80 ±0.06b	0.88 ±0.02b	0.91 ±0.03a	0.96 ±0.02a	
- Fat	15.73 ±2.95a	13.79 ± 1.28ab	10.38 ±1.30b	10.86 ± 2.55b	Less than 15%
- Protein	3.41 ±0.22a	3.72 ±0.11a	3.63 ±0.05a	3.75 ±0.34a	Min 2.7%
Physical and Chemical Quality					
- pH	4.20 ±0.03a	3.72 ±0.01c	4.12 ±0.02b	3.68 ±0.02c	
- Lactic acid percentage	1.08 ±0.01c	2.15 ±0.02a	1.27 ±0.06b	2.08 ±0.11a	Min 0.6%
- Viscosity (dpa)	3.76 ±0.15a	2.43 ±0.15b	3.36 ±0.11a	2.30 ±0.20b	

Different letters following values on the same line indicate statistically significant differences ($P < 0.05$).

*Y corresponds to control yoghurt which is made without addition of *L. acidophilus* IIA-2B4 and/or roselle extract; YP and YR refer to yoghurt with addition of *L. acidophilus* IIA-2B4 an roselle extract, respectively; YPR refers to yoghurt with addition of both *L. acidophilus* IIA-2B4 and roselle extract is designated as YPR.

**Standard value from Codex (STAN 243-2003) as a benchmark for each parameter.

include flavonoid, phenol hydroquinone, steroid, triterpenoid and tannin. Total phenol of the extract was quantified up to 0.11%. Meanwhile, alfanoid and saponin were not detected in the extract. The compounds have been known to exhibit antimicrobial activities. Tannins can cause damage to the bacterial cell wall polypeptides (Cowan, 1999) and phenol can interfere with the activity of the cytoplasmic membrane and interfere with active transport in bacteria (Michalowicz and Duda,2007). The presence of these compounds promotes roselle extract to exhibit antimicrobial activity. Olaleye (2007) reported that 1 g/mL of roselle extract is sufficient to inhibit *E. coli, S.* Typhi, *S. aureus*, and *Streptococcus sanguinis.*

Analysis quality of yoghurt probiotic with roselle extract

Probiotic *L. acidophilus* IIA-2B4 was isolated from Indonesian local beef and have shown to have antibacterial activity against pathogenic bacteria group (Ariel *et al*., 2015a). Another strain was isolated from Indonesian beef, *Lactobacillus plantarum* IIA-1A5, also has antimicrobial peptides called plantaricin as bacteriocin, effectively killed pathogenic bacteria (Arief *et al*., 2015b), which could be used as biopreservative on fresh beef (Sihombing *et al.*, 2015) Utilization of probiotics from lactic acid bacteria (LAB) are added to goat milk yoghurt will have a positive impacts on health because the bacteria are able to provide acidic conditions which inhibit disease-causing bacteria (Vesterlund and Ouwehand, 2004; Arief *et al*., 2010). As a minimum requirement of microbial populations in yoghurt starter cultures based on Codex (2003) amounted 10^7 cfu / mL. Yoghurt quality analysis result is displayed in Table 1.

Table 1 showed that the addition of *L. acidophilus* IIA-2B4 and/or roselle extracts significantly change some compounds in proximate analysis, especially for ash and fat contents. While fat content is slightly reduced by the addition of *L. acidophilus* IIA-2B4 and/or roselle extract, ash content has been slight increased. This might be due to dynamic of metabolism of *L. acidophilus* IIA-2B4 and other LAB in yoghurt in the absence or in the presence of roselle extract. The dynamic promotes the changes in chemical and physical properties of yoghurt by the addition probiotics and/or the extract (Table 1). This implied that roselle extract has no significant contribution to final LAB population in the product. pH value, lactic acid content and viscosity of the yoghurts are changed by the treatment that is certainly due to the metabolism activity of *L. acidophilus* IIA-2B4 or other LAB used in the fermentation. Table 1 also indicated that the quality of the tested samples had met Codex standard for yoghurt (2003). Roselle

Table 2. Inhibition zone of the tested yoghurts against some pathogenic bacteria

Bacteria	Yoghurts (mm)				R (mm)**	C (mm)**
	Y*	YR*	YP*	YPR*		
B.cereus	9.94±0.40fg	12.01±1.53ef	13.67±0.21de	14.75±1.19cd	6.82±0.40	28.76±0.03
E.coli	3.17±0.94ij	8.09±1.13gh	5.88±0.49h	11.88±0.48ef	4.84±0.03	31.34±0.13
*S.*Typhi	2.99±0.45j	11.78±0.10ef	5.64±0.55hi	18.15±1.09ab	2.18±0.04	32.06±0.02
S.aureus	7.45±0.15gh	18.03±0.6ab	16.28±0.393bc	19.98±0.19a	10.65±0.27	31.63±0.24
S.cerevisiae	0.00	0.00	0.00	0.00	0.00	0.00
A.niger	0.00	0.00	0.00	0.00	0.00	0.00

Different letters following values on the same line indicate statistically significant differences (P <0.05).
*Y corresponds to control yoghurt which is made without addition of *L. acidophilus* IIA-2B4 and/or roselle extract; YP and YR refer to yoghurt with addition of *L. acidophilus* IIA-2B4 an roselle extract, respectively; YPR refers to yoghurt with addition of both *L. acidophilus* IIA-2B4 and roselle extract is designated as YPR.
**Inhibition of roselle extract (R) or chloramphenicol (C) towards the tested pathogenic bacteria are measured for comparison.

extract also was used in koumiss, a fermented milk using yeast *Saccharomyces cerevisiae*, and give good quality (Nuraeni *et al*., 2014).

Antimicrobial activity of yoghurts

One of functional properties of yoghurt is antibacterial activity that produced from fermentation process during its production. The use of *L. acidophilus* IIA-2B4 and/or roselle extract, which originally exhibited their own antimicrobial activities (Chuayana *et al*., 2003; Olaleye, 2007), would possibly contribute to the total antimicrobial activities of the yoghurt (Suarsana, 2011). The existence of antimicrobial activity using the well indicated the presence of a clear zone indicates that the test bacteria are unable to grow around the wells filled with yoghurt.

The results in Table 2 showed that the antimicrobial of all yoghurts remarkably inhibited the growth of bacteria (*Bacillus cereus, Escherichia coli, Staphylococcus aureus* and *Salmonella* Typhi). Noteworthy, yoghurt with addition of *L. acidophilus* IIA-2B4 and/or roselle extract displayed wider clear zone indicating that they have higher antimicrobial activity compared to that of yoghurt without addition of *L. acidophilus* IIA-2B4 and/or roselle extract. It is interesting that combination of *L. acidophilus* IIA-2B4 and roselle extract display strongest antimicrobial activity among all treatments and control. The activity is significantly higher than that of roselle extract only, yet lower than commercially available antimicrobial compound, chloramphenicol. Comparison with chloramphenicol is aimed to provide benchmark for antimicrobial activity of yoghurt produced in this study. These differences might be due to concentration issue, since it is difficult to identify and calculate the exact concentration of antimicrobial compounds in yoghurt and/or roselle extract. Nevertheless, this result clearly showed that the antimicrobial activity of *L. acidophilus* IIA-2B4 and/or roselle extract have(s) accumulative effect on the total antimicrobial properties of the yoghurt product(s). The accumulative effect refers to the total antimicrobial properties of yoghurt is due to the sum of antimicrobial properties of *L. acidophilus* IIA-2B4 and/or roselle extract with indigenous antimicrobial properties of yoghurt itself.

Yoghurt displays antimicrobial activity due to the present of some antimicrobial compounds, including lactic acid and bacteriocins (Vesterlund and Ouwehand, 2004). These compounds are produced during fermentation either by indigenous bacteria (including LAB) in goat milk or additional probiotic added. Also, source for the compounds are non-bacterial material externally added into the yoghurt (roselle extract). This might explain high antimicrobial activity of yoghurt with addition of *L. acidophilus* IIA-2B4 and/or roselle extract since it might provide more antimicrobial compounds in the products. Other possible antimicrobial compounds are peptides derived from parent proteins through proteolysis during fermentation (Muralidhara *et al.*, 2007). These peptides are different compared to bacteriocin since they are not ribosomally synthesized. Addition of *L. acidophilus* IIA-2B4 provides more chances for the product to have antimicrobial peptides due to additional proteolytic activity of *L. acidophilus* IIA-2B4. This may explains the antimicrobial activity of yoghurt with addition of *L. acidophilus* IIA-2B4, which is higher than the control. The results also showed that the antimicrobial activity increased by addition of roselle extract.

Table 2 also showed that antimicrobial activity of yoghurt without addition of *L. acidophilus* IIA-2B4 and/or roselle extract are bigger in Gram positive bacteria (*B. cereus* and *S. aureus*) than that of in Gram negative bacteria. This might be due to the simplicity of cell walls, which is easily biodegradable. The cell wall of Gram negative bacteria composed by more complex components. Peptidoglycan in the cell

Table 3. Inhibition zone of <10 kDa peptide fragments from the tested yoghurts against some pathogenic bacteria

Microorganism	Fraction <10 kDa (mm)			
	Y*	YR*	YP*	YPR*
B.cereus	0.00	0.00	2.70±0.17a	2.91±0.46a
E.coli	0.00	0.00	0.00	0.00
S.Typhi	0.00	0.00	2.98±0.54a	2.99±0.42a
S.aureus	0.00	0.00	0.00	0.00
S.cerevisiae	0.00	0.00	0.00	0.00
A.niger	0.00	0.00	0.00	0.00

Same letters following values on the same line and coloumn indicate statistically not significant differences (P >0.05).

*The fragments were isolated from control yoghurt (Y), which is made without addition of *L. acidophilus* IIA-2B4 and/or roselle extract, and yoghurts with addition of *L. acidophilus* IIA-2B4 and/or roselle extract. Yoghurt with addition of *L. acidophilus* IIA-2B4 or roselle extract is denoted as YP or YR, respectively. Meanwhile, yoghurt with addition of both *L. acidophilus* IIA-2B4 and roselle extract is designated as YPR.

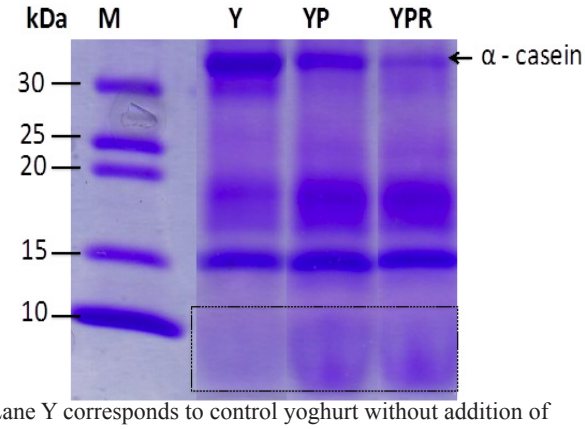
wall of Gram negative bacteria groups contained only about 10%. The remaining compounds include protein, lipoprotein and lipopolysaccharide. Another difference is the presence of outer membrane in Gram negative bacteria. The outer membrane is composed of phospholipids and lipopolysaccharide. Outer membrane of Gram negative bacteria can act as a barrier to compounds that are harm full for the cells (Jay *et al*., 2005).

Interestingly this selectivity is abolished when *L. acidophilus* IIA-2B4 and/or roselle extract are used for yoghurt production. The antimicrobial activity has wider selectivity for both Gram positive and negative bacteria. This wide selectivity is similar to Chloramphenicol, yet different to that of roselle extract that display selectivity to Gram positive bacteria.

It is also clear that yoghurt with or without addition of *L. acidophilus* IIA-2B4 and/or roselle extract are unable to inhibit fungi and yeast (*Aspergillus niger* and *Saccharomyces cerevisiae*). It indicates that compounds resulted during fermentation had no antifungal activity or the concentration of these compounds are too low to display remarkable antifungal activity. Some LAB have been reported to display antifungal activity (Schnurer and Jesper, 2005), thus there is possibility as well for the used probiotic, or indigenous LAB in goat milk, also have antifungal activity. Another possibility for survival of fungi and yeast are their ability to survive under acidic conditions (Jay *et al*., 2005).

Peptide fragments from yoghurts

As we explained before that antimicrobial activity of yoghurts is possibly originated from either ribosomally synthesized peptides (bacteriocin) or peptides produced from proteolysis of parent proteins during fermentation. To confirm the peptide



*Lane Y corresponds to control yoghurt without addition of *L. acidophilus* IIA-2B4, while yoghurt with addition of *L. acidophilus* IIA-2B4is shown in lane YP. Yoghurt with combination of *L. acidophilus* IIA-2B4 and roselle extract is shown in lane YPR. Low molecular weight protein markers (Thermo Scientific, Singapore) is shown (lane M) for size reference.

fragments among the sample, SDS-PAGE was used (Figure 1). In this experiment, we focus on the fragments lower than 10 kDa as common size for peptides. Yoghurt with addition of roselle extract is not examined in this experiment since we focused on proteolysis rate of LAB and *L. acidophilus* IIA-2B4 added in the yoghurt.

Figure 1 indicated that despite the fragments <10 kDa are not clearly seen in the gel (indicated by broken square in Figure 1), yet the intensity of blue colour, which is originated from chomassie blue staining, in lane 2 and 3 are significantly higher than that of control in lane 1. It might be caused by the concentration of <10 kDa fragments are higher compared to that of control. The gel resolution is not sufficient to provide clear separation for <10 kDa fragments in the gel. Yet, the thickness band corresponds to α-casein (Nitsche, 2011), getting thinner from lane 2 to 3 (indicated by arrow in Figure

Table 4. Direct confrontation <10 kDa peptide fragments from yoghurts against *Escherichia coli*

Variables	0 hour (log cfu / mL)	24 hour (log cfu / mL)
E. coli	8.26±0.06a	10.09±0.01a
E. coli + YP-10*	8.16±0.03a	6.26±0.07b
E. coli + YPR-10*	8.16±0.03a	6.07±0.08b

**E.coli* + YP-10 corresponds to growth of *E. coli* in the presence of <10 kDa peptide fragments isolated from yoghurt with addition of *L. acidophilus* IIA-2B4, meanwhile *E.coli* + YPR-10 corresponds to growth of *E. coli* in the *presence of <10 kDa peptide fragments isolated from yoghurt with addition of L. acidophilus* IIA-2B4 and roselle extract (YP-10).

1). It indicates that proteolysis in the yoghurt with addition of probiotics with or without roselle extract is indeed occurs and is higher compared to that of control. These events promoted higher production of peptide fragments including the fragments with lower than <10 kDa in their sizes.

To confirm whether <10 kDa fragments display antibacterial activity, the antimicrobial activity of these fragments from the yoghurts was tested. Table 3 showed that remarkable antimicrobial activity was not shown in yoghurt without probiotics. This might be due to the concentration of <10 kDa fragments in these yoghurts are relatively low to exhibit detectable antimicrobial activity. The addition of roselle extract did not significantly improve the antimicrobial activity caused by <10 kDa fragments. It indicates that the extract perhaps did not significantly affect the proteolysis rate of probiotics or LAB in yoghurts. In the yoghurt with probiotic in the presence or in the absence of roselle extract, antimicrobial activities toward *B. cereus* and *S.* Typhi were detected, but not for *E. coli* and *S. aureus*. This result indicates that that 10 kDa fragments in these yoghurts indeed contribute to antimicrobial activity of the products and the concentrations are sufficient to display detectable antimicrobial activity for some bacteria. Undetectable antimicrobial activity of these fragments towards *E. coli, S. aureus, S. cerevisiae* or *A. niger* is probably due to insufficient concentrations of these fragments to display detectable activity. Alternatively, this is also due to weak antimicrobial activity of the peptides. It also speculates that each microorganism might need different concentration of antimicrobial peptides to be inhibited.

To confirm that the absence of antimicrobial activity of <10 kDa fragments towards *E. coli* and *S. aureus* is due to concentration issue, we performed direct confrontation towards *E. coli* as a representative bacteria. The result shown in Table 4 indicated that <10 kDa fragments indeed inhibited *E. coli* during 24 hrs growing at 37°C. This result showed that inhibition of yoghurts to Gram positive and negative bacteria are indeed contributed by <10 kDa peptide fragments produced during proteolysis.

Conclusions

Addition of *L. acidophilus* IIA-2B4 and roselle extract for production of goat milk based-yoghurt significantly increased antimicrobial activity with wider selectivity towards Gram positive and negative bacteria (*Bacillus cereus, Salmonella* Typhi, *Escherichia coli* and *Staphylococcus aureus*). This activity is might be due to production of higher antimicrobial compounds, including organic acid and antimicrobial peptides with <10 kDa in the sizes.

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References

Adam, M. A. and Andy. 2011. Adding of roselle (*Hibiscus sabdariffa* Linn) flower extract for improvement quality of yoghurt. Journal Agrisystem 7: 1858-4330.

Afiyah, D. N., Arief, I. I. and Budiman, C. 2015. Proteolytic characterization of trimmed beef fermented sausages inoculated by Indonesian probiotics: *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4. Advance Journal of Food Science and Technology 8(1): 27-35.

Arief, I. I., Jenie B.S.L., Astawan M, and Witarto, A.B. 2010. The effectiveness of probiotic *Lactobacillus plantarum* 2C12 and *Lactobacillus acidophilus* 2B4 as antidiarrhea on rats. Media Peternakan 43:137-143. *http://dx.doi.org/10.5398/medpet.2010.33.3.137.*

Arief, I. I., Jenie, B. S. L., Astawan, M., Fujiyama, K. and Witarto, A. B. 2015a. Identification and probiotics characteristics of lactic acid bacteria isolated from Indonesian local beef. Asian Journal of Animal Science 9(1): 25 – 36.

Arief, I. I., Budiman, C., Jenie, B. S. L., Andreas, E. and Yuneni, A. 2015b. Plantaricin IIA-1A5 from *Lactobacillus plantarum* IIA-1A5 forms pores in the membrane of *Staphylococcus aureus*. Beneficial Microbes 6(4): 603-613.

Arief, I. I, Afiyah, D.A., Wulandari , and Budiman, C.

2016. Physicochemical properties, fatty acid profiles, and sensory characteristics of fermented beef sausage by probiotics *Lactobacillus plantarum* IIA-2C12 or *Lactobacillus acidophilus* IIA-2B4. Journal of Food Science 81 (11) : M2761-M2769. doi:10.1111/1750-3841.13509

Association Official Analytical Chemistry. 2005. Official Methods of Analysis. 18th edition. Maryland: AOAC International.

Astawan, M., Wresdiyati T, Suliantari, Arief, I.I and Septiawan, R. 2012. Production of symbiotic yogurt-like using indigenous lactic acid bacteria as functional food. Media Peternakan 35: 9-14. *http://dx.doi.org/10.5398/medpet.2012.35.1.9*

Bintang, M. 1993. Study antimicrobial from *Streptococcus lactis* BCC2259. Bandung: Institut Teknologi Bandung. PhD Dissertation.

Chuayana, E. L. J., Ponce, C .V., Rivera, M.R.B. and Cabrera, E.C. 2003. Antimicrobial activity of probiotic from milk product. Philippine Journal Microbiology Infectious Diseases 32: 71-74.

CODEX. 2003. Codex standard for fermented milk. Codex STAN 243. FAO/WHO Food Standars: Codex Alimentarius Commission.

Cowan, M. 1999. Plants Products as Antimicrobial Agents. Clinical Microbiology 4: 564-582.

Donkor, O. N., Henriksson, A., Vasiljevic, T. and Shah, N. P. 2006. Effect of acidification on the activity of probiotics in yogurt during cold storage. International Dairy Journal 16: 1181–1189.

Jay, J. M., Loessner, M. J. and Golden, G.A. 2005. Modern Food Microbiology. 7th edition. New York: Springer.

Just, M.J., Recsio, M.G., Gner, R.M., Cuellar, M.J., Marez, S., Bilia, A. R. and Rios, J. 1998. Anti-inflammatory activity of unusual lupane saponins from *Bupleurum fruiticescens*. Planta Medica 64 (5): 404-407.

Ljung A. and Wadström, T. 2006. Lactic acid bacteria as probiotics. Current Issues In testinal Microbiology 7: 73-89.

Lowry, O. H., Roserbrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. Journal Biology Chemistry 193: 265-275

Martin, D. A. B., Janer, C., Pelaez, C. and Requena, T. 2003. Development of a fermented goat's milk containing probiotic bacteria. International Dairy Journal 13: 827-833.

Michalowicz, J. and Duda, W. 2007. Phenol sources and toxicity. Polish Journal of Environment Study 16: 347-362.

Muralidhara, C., Haque, E. and Chand, R. 2007. Antimicrobial peptides purified from bovine milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus*. Milk Science International 62: 62-65.

Nitsche, R. 2011. Milk protein analysis with the Agilent 2100 Bioanalyzer and the Agilent Protein 80 kit. Germany: Agilent Technologies, Inc.

Nuraeni, E., Arief, I. I. and Soenarno, M. S. 2014. Characteristics of probiotic koumiss from goat mil with addition of roselle extract (*Hibiscus Sabdariffa* Linn). Journal Indonesian Tropical Animal Agriculture 39: 117–125.

Olaleye, M.T. 2007. Cytotoxicity and antibacterial activity of Methanol extract of *Hibiscus sabdariffa.* Journal of Medicinal Plants Research 1: 9-013.

Ribeiro, A. C. and Ribeiro, S. D. A. 2010. Specialty products made from goat milk. Small Ruminant Research 89: 2-3.

Schnurer, J. and Jesper, M. 2005. Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science and Technology 16: 70–78.

Sihombing, D. E., Arief, I. I. and Budiarti, S. 2015. Application of antimicrobial agents produced by *Lactobacillus plantarum* IIA-1A5 as natural preservative on beef during room temperature storage. Advance Journal of Food Science and Technology 8(4): 251-255.

Steel, R. G. D. and Torrie, J. H. 1995. Principles and procedures of statistica biomedical approach. 3rd edition. Singapore: McGraw Hill Inc.

Suarsana, I. N. 2011. Physicochemical characterization of bacteriocin extracted from yogurt. Buletin Veteriner Udayana 1: 1-8.

Tsai, P. J, Melntosh, J., Pearce, P., Camden, B., and Jordan, B. R. 2002. Anthocyanin and antioxidant capacity in Roselle (*Hibiscus sabdariffa* L) extract. Food Research International 35: 351-356.

Tziboula-Clarke A. 2003. Encyclopedia of Dairy Science. 2nd edition. Academics Press. USA: California

Vesterlund, S. and Ouwehand, A.C. 2004. Antimicrobial components from lactic acid bacteria. In: Salminen, S. and Von Wright, A. Eds. Lactic Acid Bacteria, Microbiology and Functional Aspect. London: Marcel Dekker, Inc.