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## ORIGINAL PAPER

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# THE COMPARING OF ANTIMICROBIAL ACTIVITY OF CSN1S2 PROTEIN OF FRESH MILK AND YOGHURT GOAT BREED ETHAWAH INHIBITED THE PATHOGENIC BACTERIA

Lidwina Faraline Triprisila<sup>1</sup>, Suharjono Suharjono<sup>1</sup>, Antonius Christianto<sup>1</sup>, Fatchiyah Fatchiyah<sup>1\*</sup><sup>1</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java, Indonesia

Corresponding author: Fatchiyah Fatchiyah. Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java, Indonesia, Tel.: +62 341 578 248; fax: +62 341 578 248. E-mail address: fatchiya@ub.ac.id

**ABSTRACT**

**Background:** Goat milk is reported to have antimicrobial activity of several pathogen bacteria that contained on food materials. The research related with antimicrobial activity of Alpha-S2 casein from goat milk is relatively less than other casein components. Herein, we reported the antimicrobial activity of caprine Alpha-S2 Casein (CSN1S2) protein from Ethawah breed goat milk and yoghurt in Gram positive (*Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*) and negative pathogen bacteria (*Escherichia coli*, *Salmonella typhi* and *Shigella flexneri*). Those bacteria were known as pathogens that caused gastrointestinal infection. **Methods:** Serial dilution and agar diffusion analysis with three different concentrations of caprine CSN1S2, 1.25 mg/ml, 2.5 mg/ml, and 5 mg/ml were used to test the inhibition effect of protein on the viability of bacteria cells. The inhibitory activity of caprine CSN1S2 was based on dose dependent manner. Agar diffusion analysis was showed the larger diameter of clear zone at *B. cereus* and *S. flexneri*. **Results:** The serial dilution analysis was shown the inhibition of almost in all groups of bacteria with concentration 5 mg/ml higher by CSN1S2 protein of goat fresh milk than yogurt. The inhibitory activity caprine CSN1S2 protein of fresh milk was shown a vary inhibition clear zone with optimal concentration 5 mg/ml, however CSN1S2 protein of goat yogurt intermediate effectively was only in gram negative bacteria. The weakness bacteria against inhibition activity caprine CSN1S2 protein was *B. cereus* (Gram positive) and *S. flexneri* (Gram negative). Meanwhile the strongest bacteria against inhibition activity caprine CSN1S2 protein was *S. typhi* (Gram negative), may cause in this bacteria has lipopolysaccharide prevent to interact with that protein as proper. **Conclusion:** This study result concluded that the caprine CSN1S2 protein has inhibition activity in opposition to pathogenic bacteria by optimal concentration 5 mg/ml in all bacteria and indicated caprine CSN1S2 protein as anti-microbial agent.

**Key words:** Antimicrobial, CSN1S2, Ethawah goat, milk, pathogen bacteria.**1. INTRODUCTION**

Milk is a nutrient source with a complete and balance nutrition contents. One kind of milk from an animal with high nutrient contents is goat milk. Chemical contents of goat milk are different from cow milk, in which the total fat (3.8%), protein (3.4%), mineral and vitamin (vitamin A, B, B12 and D) is higher than cow milk (1, 2, 3, 4). Besides that, goat milk is easier to digest in human digestion systems than cow milk (2, 5).

Goat milk is the milk proteins that are the main source of bioactive peptides (6, 7). A bioactive peptide from milk protein was activated and released through the proteolysis

enzymatic process during food digestion and processing on gastrointestinal (8, 9). A protein on milk could divide into two different groups, soluble protein (whey protein) and insoluble protein (casein) at isoelectrical point (pI) and in milk caseins are soluble as micelles (10). Whey protein is contained of  $\alpha$ -Lactalbumin and  $\beta$ -Lactoglobulin, while casein are contains of  $\alpha$ S1-,  $\alpha$ S2-,  $\beta$ -, and  $\kappa$ -casein (11, 12).

Milk protein and whey from goat milk is a precursor of bioactive components that contribute to antimicrobial activity with a wide spectrum of bacterial pathogen types of food materials (13, 14, 15). As many studies was made in order to analyze the ability of bioactive peptide from milk

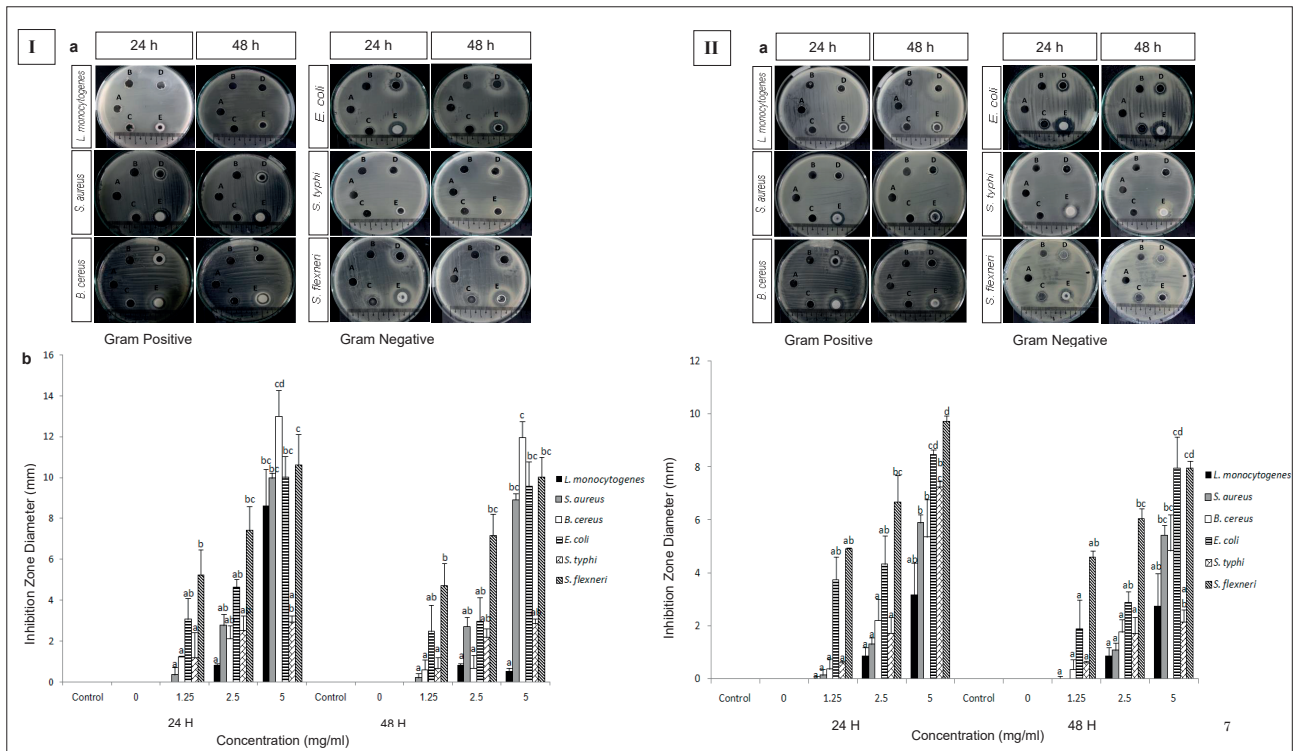


Figure 1. Inhibitory Growth Activity of Goat And Yoghurt Milk CSN1S2. I (a) Inhibitory growth activity of Goat milk CSN1S2 against pathogen bacteria. (b) inhibition zone diameter of goat milk csn1s2 against pathogen bacteria ( $p < 0.05$ ). II (a) Inhibitory growth activity of yoghurt milk CSN1S2 against pathogen bacteria. (b) inhibition zone diameter of yoghurt milk CSN1S2 against pathogen bacteria. a,b,c,d the mean value is indicate a significant difference ( $p < 0.05$ ).

protein on inhibition or destruction of pathogen (16, 17, 18, 19). Previous study, reported that fresh milk and yoghurt from goat Ethawah crossbreed had specific protein with molecular weight 36kDa, in which this specific protein did not find in fresh cow milk (20). This protein was identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis (21) as protein  $\alpha$ -S2 casein (CSN1S2) contents eight bioactive peptides that each peptide has specific biological function in difference mechanism as anti-oxidant (22) anti-osteoporosis, and anti-inflammation (23, 24). We predict that some peptides of goat CSN1S2 protein may able to inhibit the pathogen bacteria growth as antimicrobial agent.

Research related with antimicrobial activity for alpha casein from goat milk is relatively less than other casein components. Herein, we reported the effect of antimicrobial activity from CSN1S2 protein as a member of casein protein from Ethawah breed goat milk and yoghurt against pathogen bacteria in some gram negative and positive.

## 2. MATERIALS AND METHODS

### 2.1. Bacteria culture

Gram positive bacteria (*Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*) and Gram negative bacteria (*Escherichia coli*, *Salmonella typhi* and *Shigella flexneri*) was obtained from the Medical Faculty, Brawijaya University Type Culture Collection. All bacteria were maintained on Luria Bertani Broth agar plates (Sigma-Aldrich, USA).

### 2.2 Isolation of CSN1S2 protein of Ethawah goat milk and yoghurt

A CSN1S2 protein with molecular weight 36kDa was isolated from Ethawah goat milk and yoghurt using Sodium

Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the previous study (20).

### 2.3. Diffusion agar analysis

Diffusion agar analysis for antimicrobial activity was carried out with some modification (25). Antimicrobial action of CSN1S2 protein and yoghurt were determined using an agar well assay. The isolated pathogenic bacteria were grown in Luria Berthani (LB) Broth at 37° C for 24 hours and adjusted approximately to a density of  $1.5 \times 10^8$  CFU/ml and then each pathogen bacteria were then transferred to Luria Berthani Agar with cotton swab. Well was made on agar for seeded 200  $\mu$ L of sample CSN1S2 protein and yoghurt was added to each well. The plates were then incubated aerobically at 37° C for 24 h and 48 h. The diameters of the clear zones (in the top agar layer) were measured to the nearest mm. Each assay was done in triplicate.

### 2.4. Serial dilution analysis

The determination of effect of the antimicrobial peptide CSN1S2 protein from goat milk and yoghurt Ethawah on microbial cells were performed with minor modification (25). Luria Berthani (LB) broth (containing 10 g tryptone, 5 g yeast extract, 10 g sodium chloride per liter, pH 7.2 at 37° C) was carried out in serial dilution test tube preparation. Serial dilutions of the two CSN1S2 protein samples were made in Luria Berthani (LB) Broth medium containing 1 ml to give a final concentration of 1.25, 2.5 and 5 mg/ml. Briefly, tubes were seeded 20  $\mu$ L of the test organisms with approximately  $1 \times 10^7$  CFU/ml using early log-phase culture,  $1.5 \times 10^8$  CFU/ml using middle log-phase culture, and  $3 \times 10^8$  CFU/ml using end log-phase culture. Positive controls were containing Luria Berthani (LB) Broth medium and bacterial inoculum. Negative controls tubes contained CSN1S2

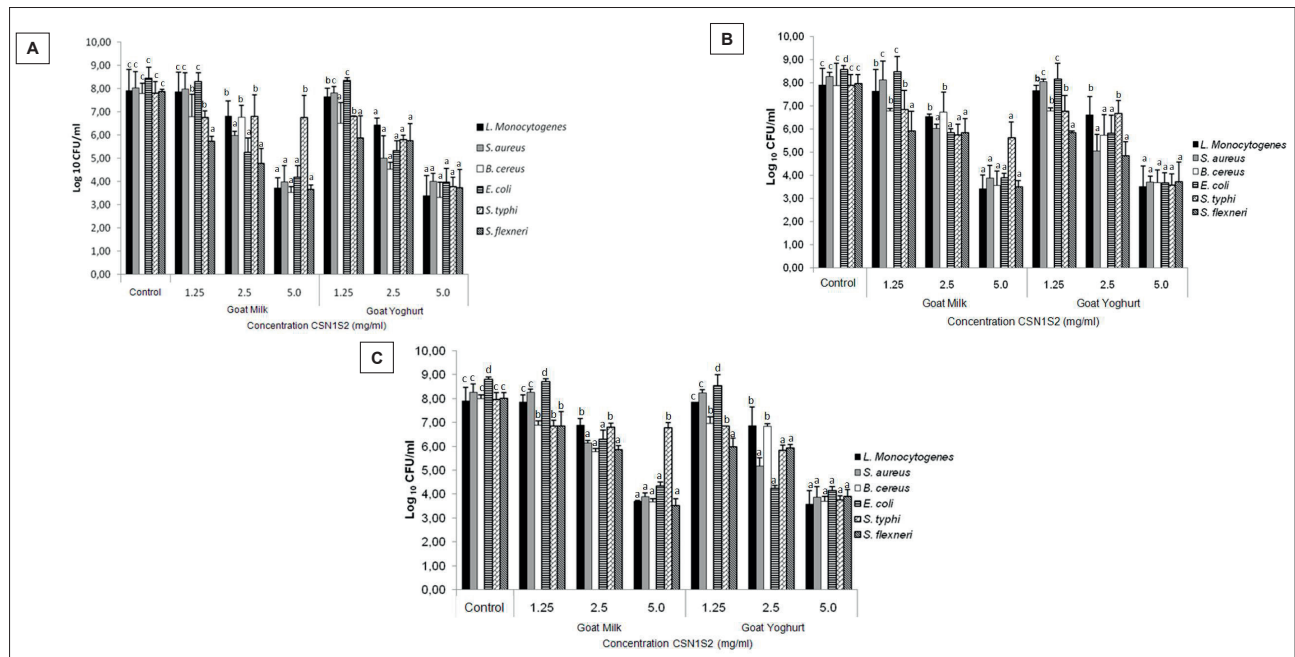


Figure 2. Antimicrobial activity of CSN1S2 Goat milk and Goat yoghurt against microbial cells of *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi* and *Shigella flexneri* at (A) Early exponential growth phase, (B). Middle exponential growth phase, (C). Late exponential growth phase. a,b,c,d the mean value is indicate a significant difference ( $p < 0.05$ ).

protein milk or yoghurt. The determination microbial count was employed in triplicate samples plated onto LB agar and incubated at 37°C for 24 h.

2.5. Statistical analysis

Data analysis results were expressed as means. The data was variance analyzed (ANOVA) statistically with SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). The differences effect and interaction among treatments were considered significant when  $P \leq 0.05$  and then followed up by turkey test.

3. RESULTS

Effect of antimicrobial peptides on the inhibition of bacterial growth base of clear zone analysis

Qualitative antimicrobial activity analysis was done using the agar diffusion methods of measuring the diameter of the clear zone (Figure 1). Treatment uses CSN1S2 from milk, both in 24 and 48 hours incubation, was showed highest inhibition at concentration 5 mg/ml in almost all bacteria comparable to the other concentrations. *B. cereus* was obtained the highest inhibition with clear zone diameter  $13.01 \pm 1.25$  mm and  $11.96 \pm 0.81$  mm in 24 and 48 hours incubation, respectively. The lowest inhibition at concentration 5 mg/ml in 24 and 48 hours incubation was obtained in two different bacteria, *S. typhi* ( $2.91 \pm 0.35$  mm) and *L. monocytogenes* ( $0.51 \pm 0.16$  mm), respectively.

Although the highest inhibition was obtain by different bacteria compare to the CSN1S2 from milk, but in term of concentration, similar result was obtained by treatment using CSN1S2 from yoghurt. The highest inhibition was resulted at concentration 5 mg/ml. Incubation for 24 hours at concentration 5 mg/ml was resulted the highest inhibition in *S. flexneri* with clear zone diameter  $9.73 \pm 0.22$  mm. There were no significant different between *S. flexneri* and *E. coli* in 48 hours incubation, both were resulted the highest inhibition. In contrast with result from treatment milk

CSN1S2, although the lowest inhibition was obtained also from *S. typhi* and *L. monocytogenes*, but with different incubation time, 48 and 24 hours respectively.

Effect of antimicrobial peptides on the microbial cell pathogen numbers, during exponential growth phase: early, middle, and late.

Antimicrobial activity of CSN1S2 from goat Ethawah breed milk and yoghurt in all concentrations to Gram positive and negative bacteria at three different growth phases (early, middle, and late exponential) were shown various results (Figure 2, Table 1). In all phases, for treatment using milk, most of the bacteria were showed significant decreasing, especially at concentration 5 mg/ml compared to control and other treatments, except for *S. typhi*. Although *S. typhi* was decreased significantly at concentration 1.25 mg/ml, but the number was almost did not. Values are presented as mean amount of microbial cells, \*indicated a significant different ( $p < 0.05$ ) compared with control group all bacterial species.

Change significantly in other following concentrations, especially in the early and late phase. The highest decreasing for Gram positive and negative bacteria at early, middle and late phase were obtained from *B. cereus* ( $3.4E+03$ ) and *S. flexneri* ( $4.57E+03$ ); *L. monocytogenes* ( $2.55E+03$ ) and *S. flexneri* ( $3.35E+03$ ); and *B. cereus* ( $4.78E+03$ ) and *S. flexneri* ( $3.13E+03$ ), respectively.

Similar with milk, in all phases, the highest decreasing for treatment using yoghurt were also resulted at concentration 5 mg/ml. Although, almost all bacteria were decreased significantly at concentration 5 mg/ml compare to control and other treatments, but except for *E. coli* in the late phase. There was no significant difference on *E. coli* between concentration 2.5 and 5 mg/ml treatment. The highest decreasing for Gram positive and negative bacteria at early, middle and late phase for treatment using yoghurt were obtained from *B. cereus* ( $2.02E+03$ ) and *S. flexneri* ( $5.35E+03$ );



Bacterial Species		Control	CSN1S2 Goat Milk (mg/ml)			CSN1S2 Goat yoghurt (mg/ml)		
			1.25	2.5	5	1.25	2.5	5
<i>L. monocytogenes</i>	Early	8.17E+07	7.07E+07	6.37E+06	5.12E+03	4.43E+07	2.62E+06	2.42E+03
	Middle	8.00E+07	4.20E+07	3.35E+06	2.55E+03*	4.57E+07	4.15E+06	3.32E+03
	End	8.12E+07	7.00E+07	7.45E+06	4.92E+03	7.22E+07	7.27E+06	3.82E+03
<i>S. aureus</i>	Early	1.08E+08	9.60E+07	9.58E+05	9.60E+03	6.58E+07	1.00E+05	9.83E+03
	Middle	1.81E+08	1.32E+08	1.09E+06	7.58E+03	1.15E+08	1.15E+05	5.20E+03
	End	1.84E+08	1.79E+08	1.40E+06	7.90E+03	1.75E+08	1.50E+05	7.38E+03
<i>B. cereus</i>	Early	6.15E+07	6.15E+06	5.87E+06	3.40E+03	3.25E+06	3.27E+04	2.02E+03*
	Middle	7.65E+07	6.08E+06	5.25E+06	3.63E+03	6.10E+06	5.38E+05	4.87E+03
	End	1.01E+08	7.87E+06	5.90E+05	4.78E+03	9.25E+06	6.85E+06	4.92E+03
<i>E. coli</i>	Early	2.81E+08	2.03E+08	1.77E+05	1.51E+04	2.32E+08	2.13E+05	9.55E+03
	Middle	3.77E+08	3.13E+08	7.20E+05	8.08E+03	1.51E+08	6.75E+05	4.70E+03
	End	6.05E+08	4.88E+08	1.97E+06	2.14E+04	3.29E+08	1.66E+04	1.34E+04
<i>S. typhi</i>	Early	6.28E+07	5.72E+06	6.47E+06	5.70E+06	6.32E+06	6.43E+05	5.95E+03
	Middle	8.15E+07	7.23E+06	5.70E+05	4.33E+05	5.93E+06	4.77E+06	3.88E+03
	End	8.75E+07	6.93E+06	6.05E+06	5.93E+06	6.73E+06	6.73E+05	5.73E+03
<i>S. flexneri</i>	Early	7.67E+07	5.57E+05	5.97E+04	4.57E+03	7.70E+05	5.85E+05	5.27E+03
	Middle	9.43E+07	8.58E+05	7.00E+05	3.35E+03	7.32E+05	7.00E+04	5.35E+03
	End	9.70E+07	7.00E+06	6.87E+05	3.13E+03*	9.43E+05	8.52E+05	8.00E+03

Table 1. Antimicrobial activity of CSN1S2 Goat milk and Goat yoghurt against microbial pathogen cells at early, middle and end exponential growth phase

*L. monocytogenes* (3.32E+03) and *S. typhi* (3.88E+03); and *L. monocytogenes* (3.82E+03) and *S. typhi* (5.73E+03), respectively.

#### 4. DISCUSSION

The clear zone diameter was measured as a representation of inhibitory activity by CSN1S2. Inhibition zone category is divided into five levels. There are very weak for diameter < 5 mm; weak for diameter >5 mm; intermediate for diameter 5-10 mm; strong for diameter >10-20 mm; and very strong or the most sensitive for diameter >20-30 mm (26). Based on those criteria, the strong inhibition level was obtained in treatment of CSN1S2 from milk at concentration 5 mg/ml on *B. cereus* and *S. flexneri*. Vice versa, in treatment of CSN1S2 from yoghurt at same concentration was resulted only a intermediate inhibition level. The antimicrobial activity of CSN1S2 from milk and yoghurt in this study could be categorized as bacteriostatic, because at least in part, the growth inhibition was observed in all pathogen bacteria. Bacteriostatic activity can be shown by the slow growth activity when the treatment was applied. In contrast, bactericidal activity can be shown by no growth activity after treatment (4, 27, 28).

Effects of milk protein to the bacteria in middle exponential phase having antimicrobial activity with high sensitivity when compared to the right when approaching or on the stationary phase (29, 30, 31). Milk protein effect toward bacteria on middle exponential phase has a high sensitivity of antimicrobial activity when compared to early or exact stationary phase (31).

Based on those two analyses, antimicrobial activity of CSN1S2 from milk and yoghurt was showing a higher activity on Gram positive bacteria comparable to Gram negative bacteria. The outer membrane of Gram negative bacteria was consist of lipopolysaccharides, in which this molecule when binding to the peptide could decrease the peptide affinity and in turn will decrease the antimicrobial activity toward this bacteria (32, 33). Basically, our study was showing a higher potential inhibition by CSN1S2 from milk than yoghurt in all pathogenic bacteria. This result was almost

similar to previous studies, (34, 35) it reported that whey protein from goat and cow milk was showed antimicrobial activity on several pathogen bacteria.

#### 5. CONCLUSION

At least in part, from our study we could conclude that antimicrobial activity of CSN1S2 from milk and yoghurt was based on dose dependent manner, higher concentration will resulted higher antimicrobial activity. In our study, the optimum concentration was obtained at 5 mg/ml. Compare to CSN1S2 from yoghurt, milk had a higher antimicrobial activity. And overall, our result was showed the higher inhibition on Gram positive bacteria than Gram negative bacteria. Further research related the mechanism of inhibition still need to observe.

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#### REFERENCES

1. Lopez-Aliaga I, Alferez MJM, Barrionuevo M. et al. Study of Nutritive Utilization of Protein and Magnesium in Rats with Resection of the Distal Small Intestine. Beneficial Effect of Goat Milk. *J Dairy Sci.* 2003; 86 (9): 2958-66.
2. Haenlein GEW. Goat milk in human nutrition. *Small Ruminant Res.* 2004; 51: 155-63.
3. Pereira PC Milk Nutritional Composition and Its Role in Human Health. *Nutrition.* 2014; 30(6): 619-27.
4. da Costa, WKA, de Souza EL, Filho, EMB et al. Comparative Protein Composition Analysis of Goat Milk Produced by the Alpine and Saanen Breeds in North-

- eastern Brazil and Related Antibacterial Activities. *PLoS One*. 2014; 9(3): e93361.
5. Ceballosa LS, Morales ER, Adarvea GT. et al. Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. *J Food Compost Anal*. 2009; 22(4): 322-9.
  6. Slacanac V, Bozanic R, Hardi J. et al. Nutritional and therapeutic value of fermented caprine milk. *Int J Dairy Technol*. 2010; 63: 171-89.
  7. Selvaggi M, Laudadio V, Dario C. et al. Major Proteins in Goat Milk: An Updated Overview on Genetic Variability. *Mol Biol Rep*. 2014; 41(2): 1035-48.
  8. Meisel H. Biochemical Properties of Peptides Encrypted in Bovine Milk Proteins. *Curr Med Chem*. 2005; 12(16): 1905-19.
  9. El-Agamy EI. The challenge of cow milk protein allergy. *Small Ruminant Res*. 2007; 68: 64-72.
  10. St-Gelais D, Turcot S, Ould Baba Ali A. Chemical composition and properties of milk from five goat breeds. *Milchwissenschaft*. 2005; 60: 140-3.
  11. Martin P, Ollivier-Bousquet M, Grosclaude F. Genetic polymorphism of caseins: a tool to investigate casein micelle organization. *Int Dairy J*. 2002; 9: 163-71.
  12. Haque E, Chand R. Antihypertensive and antimicrobial bioactive peptides from milk proteins. *Eur Food Res Technol*. 2007; 227(1): 7-15.
  13. Korhonen H, Pihlanto A. Bioactive Peptides: New Challenges and Opportunities for the Dairy Industry. *Aust J Dairy Technol*. 2003; 58(2): 129-34.
  14. Atanasova J, Ivanova I. Antibacterial peptides from goat and sheep milk proteins. *Biotechnol Equip*. 2010; 24: 1799-1803.
  15. Almaas H, Eriksen E, Sekse C. et al. Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. *Brit J Nutr*. 2011; 106: 896-905.
  16. Lahov E, Regelson W. Antibacterial and immunostimulating casein-derived substances from milk: Casacidin, isracidin, peptides. *Food Chem Toxicol*. 1996; 34: 131-45.
  17. Clare DA, Catignani GL, Swaisgood HE. et al. Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Curr Pharm Design*. 2003; 9: 1239-55.
  18. Shimizu M. Food-derived peptides and intestinal functions. *BioFactors*. 2004; 21: 43-7.
  19. Expósito IL, Recio I. Antibacterial activity of peptides and folding variants from milk proteins. *Int Dairy J*. 2006; 16: 1294-1305.
  20. Budiarti IK, Padaga MC, Fatchiyah F. Nutritional composition and protein profile of goat yogurt PE with double culture between *Streptococcus thermophilus* and *Lactobacillus* species. *Cukurova Med J*. 2013; 38(4): 681-6.
  21. Fatchiyah F, Raharjo SJ, Dewi FRP. Virtual Selectivity Peptides of CSN1S22 Protein of Local Goat Ethawah Breeds Milk Modulate Biological Mechanism of Calmodulin. *Int J Pharm Bio Sci*. 2015; 6(2): 707-18.
  22. Chotimah C, Ciptadi G, Setiawan B, et al. CSN1S2 protein of goat milk inhibits the decrease of viability and increases the proliferation of MC3T3E1 pre-osteoblast cell in methylglyoxal exposure. *Asian Pac J Trop Dis*. 2015; 5(3): 219-23.
  23. Fatchiyah F, Setiawan B, Suharjo S et al. The anti-osteoporosis effects of CSN1S2 protein of goat milk and yoghurt on a complete Freund's adjuvant-induced rheumatoid arthritis model in rats. *Biomarkers and Genomic Med*. 2015; 7: 139-46.
  24. Rohmah RN, Widjanto E, Fatchiyah F. Protective effect of CSN1S2 protein of goat milk on ileum microstructure and inflammation in rat-CFA-induced rheumatoid arthritis. *Asian Pac J Trop Dis*. 2015; 5(7): 564-8.
  25. Theolier J, Hammami R, Labelle P. et al. Isolation and Identification of Antimicrobial peptide derived by peptic cleavage of whey protein isolate. *J Func Food*. 2013; 5: 706-14.
  26. Morales G, Sierra P, Mancilla PA et al. Secondary metabolites from four medicinal plants from Northern Chile, antimicrobial activity and biotoxicity against *Artemia salina*. *J Chile Chem Soc*. 2002; 48(2): 13-8.
  27. Jorgensen, James H, John DT. Susceptibility Test Methods: Dilution and Disk Diffusion Methods. In: *Manual of Clinical Microbiology*. 11th Ed. Washington, DC: ASM Press; 2015: 1253-73.
  28. Fjell C, Hiss JA, Hancock REW. et al. Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov*. 2012; 11: 37-51.
  29. Roncada P, Piras C, Soggiu A. et al. Farm Animal Milk Proteomics. *J Proteomics*. 2012; 75: 4259-74.
  30. Ramunno L, Pauciullo A, Mancusi A. et al. Influence of genetic polymorphism of calcium-sensitive caseins on structural, nutritional, renneting and hypoallergenic properties of goat milk. *Sci Technol Lattiero-Casearia*. 2007; 58: 257-71.
  31. Batt CA, Tortorello M. *Encyclopedia of Food Microbiology*. 2nd Ed. USA: Elsevier Ltd. 2014 [Volume1]
  32. Ellison RT III, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest*. 1991; 88: 1080-91.
  33. Pellegrini A. Antimicrobial peptides from food proteins. *Curr Pharm Design*. 2003; 9: 1225-38.
  34. Murata M, Wakabayashi H, Yamauchi K. et al. Identification of milk proteins enhancing the antimicrobial activity of lactoferrin and lactoferricin. *J Dairy Sci*. 2013; 96: 4891-8.
  35. Floris R, Recio I, Berkhout B. et al. Antibacterial and antiviral effects of milk proteins and derivatives thereof. *Curr Pharma Design*. 2003; 9: 1257-75.