Original Article

Analysis of *CSN2* variants in Friesian Holstein cows and their association with milk protein allergy and production traits

Mohammad Ilham Dhiaurridho[1], Firmansyah Tristadika Prakosa[1], Firna Fauziatul Karimah[1], Salsabilla Ramadhana[1], Ine Febriantama[1], Thoriq Aldri Bramastya[1], Ahmad Pramono[1], Muhammad Cahyadi[1,2*]

[1]Department of Animal Science, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Jawa Tengah, 57126
[2]Halal Research Center and Services, Institute for Research and Community Service, Universitas Sebelas Maret, Surakarta, Jawa Tengah, 57126

*Correspondence: mcahyadi@staff.uns.ac.id

Abstrak

Tujuan: Penelitian ini bertujuan untuk menganalisis variasi *CSN2* pada sapi Friesian Holstein (FH) Indonesia dan asosiasinya dengan alergi protein dan sifat produksi susu.

Metode: Total DNA genome diekstraksi dari 12 ekor sampel darah sapi FH Indonesia. Ekson 7 *CSN2* diamplifikasi menggunakan *novel primer* untuk menghasilkan produk PCR sebesar 683 bp. Pasangan primer yang digunakan adalah 5'-ACCCCAATTTCTTAACCAAACCA-3' sebagai primer *forward* dan 5'-CATCAGAAGTTAAACAGCACAGT-3' sebagai primer *reverse*. Produk PCR di-*sequencing* untuk mengetahui urutan nukleotida ekson 7 dari *CSN2* dan data urutan nukleotida dianalisis menggunakan Bioedit versi 7.2.5. Hardy-Weinberg equilibrium (HWE) dihitung dan *one-way analysis of variance* (ANOVA) dilakukan untuk analisis asosiasi *CSN2* dengan alergi protein dan sifat produksi susu.

Hasil: Dua polimorfisme pada ekson 7 dari *CSN2*, yaitu c.350A>C and c.516G>C teridentifikasi pada penelitian ini. Substitusi adenin (A) menjadi sitosin (C) pada c.350A>C mengubah kodon asam amino histidin (CAU) menjadi prolin (CCU), dan substitusi guanin (G) menjadi sitosin (C) pada c.516G>C mengubah kodon asam amino arginin (AGG) menjadi serin (AGC). Frekuensi genotipe CC untuk c.350A>C SNP adalah 33% dan sapi FH dengan genotipe CC mampu menghasilkan susu A2 yang mampu menurunkan resiko intoleransi laktosa akibat alergi protein susu. Analisis statistik menunjukkan bahwa polimorfisme c.350 A>C dan c.516 G>C ekson 7 *CSN2* tidak berasosiasi dengan sifat produksi susu sapi FH Indonesia.

Kesimpulan: Sapi dengan genotipe CC berdasarkan polimorfisme c.350A>C mampu menghasilkan susu varian A2 yang dapat meminimalisir resiko alergi protein susu bagi konsumen. Polimorfisme *CSN2* tidak berasosiasi dengan sifat produksi susu pada populasi sapi FH Indonesia yang digunakan pada penelitian ini.

Kata Kunci: *CSN2*; Friesian Holstein; Intoleransi laktosa; Susu A2; Produksi susu

Abstract

Objective: The objective of this study was to analyze *CSN2* variants in Indonesian Friesian Holstein (FH) cow and their association with milk protein allergy and production traits.

Methods: Genomic DNA was extracted from bloods of twelve Indonesian FH cow. Exon 7 of the *CSN2* was amplified using novel primer pair to produce 683 bp amplicon. The primers were 5'-ACC CCAATTTCTTAACCAAACCA-3' as a forward primer and 5'-CATCAGAAGTTAAACAGCACAGT-3' as a reverse primer. The PCR products were sequenced and nucleotide sequences of *CSN2* was analyzed using Bioedit version 7.2.5. Moreover, Hardy-Weinberg equilibrium (HWE) was calculated and one-way analysis of variance (ANOVA) was conducted to associate between *CSN2* variants with milk protein allergy and production traits.

Results: Two polymorphisms, c.350A>C and c.516G>C, were identified in the *CSN2* exon 7. Base substitution from adenine (A) to cytosine (C) of c.350A>C changed amino acid codon from histidine (CAU) to proline (CCU), and base substitution guanine (G) to cytosine (C) of c.516G>C changed amino acid codon from arginine (AGG) to serine (AGC). The CC genotype frequency for c.350A>C SNP was 33% and they were able to produce A2 *CSN2* variant which is favorable for preventing lactose intolerance. In addition, there were no association between c.350 A>C and c.516 G>C SNP of the *CSN2* with milk production traits.

Conclusions: In conclusion, A1 and A2 variants of *CSN2* were identified in Indonesian FH population and they did not associate with milk production in Indonesian FH.

Keywords: *CSN2*; Friesian Holstein; Lactose intolerance; A2 milk; Milk production

INTRODUCTION

Cow's milk is one of the exclusive products because of its exquisite taste and rich nutrients. Milk has a complete nutritional element containing 87.2% water, 3.5% protein, 3.7% fat, 4.9% lactose, and 0.07% minerals. These nutrients are a necessity for humans which are easily absorbed by the body [1]. However, some people are not able to digest lactose and milk protein properly. The condition of a person who cannot digest lactose is called lactose intolerance which is occurred when the body experiences lactase deficiency and lactose malabsorption with symptoms of gas in the stomach, bloating, abdominal pain, or diarrhea [2]. Cases of lactose intolerance in Indonesia are still often encountered. In Jakarta, a total of 21% children aged 2-11 years and 58% children 6-11 years were lactose intolerant. Moreover, 63.2% of Manado peoples suffer from lactose intolerance [3]. Beta-casein protein in milk may cause lactose intolerance [4].

Beta-casein protein makes up 30% of the total protein in cow's milk and has two dominant carrier gene variants, namely A1 and A2 [5]. A1 beta-casein is the most abundant variant in the milk of dairy cattle from northern Europe: Friesian, Ayrshire, British Shorthorn and Holstein. A large proportion of A2 beta-casein is found in the milk of Channel Island cattle: Guernsey and Jersey, and in Southern France: Charolais and Limousin [6], and in Zebu cattle, native to Africa [7]. Beta-casein A1 gene with the Histidine amino acid is a wild-type milk protein in Friesian Holstein (FH) cattle before the mutation of the Proline amino acid caused the emergence of the A2 beta-casein gene in FH cattle [7]. Beta-casein A1 gene contains beta-casomorphin-7 (BCM-7), inhibiting intestinal absorption compared to the beta-casein A2 gene [8]. The characteristics of BCM-7 can affect lactase production and activity, thus worsening hypolactasia and resulting in lactose malabsorption and inflammation [9]. The presence of BCM-7 in A1 milk also causes a delay in gastrointestinal transit, resulting in an increased opportunity for lactose fermentation in the intestine [10]. In addition, digestion is disturbed due to inflammation of the large intestine due to the fermentation of lactose into a mixture of hydrogen gas, carbon dioxide, and methane by microbes. These gases cause the stomach to become bloated. Both unabsorbed lactose and gaseous fermentation products will increase osmotic

pressure, drawing water into the intestines, therefore, water flows in and causes diarrhea [11,12]. Considering these effects, it is necessary to provide information on mapping the beta-casein gene as an attempt to prevent cases of diarrhea by cause of lactose intolerance in dairy cattle in Indonesia.

Advances in molecular biology technology have made the process of identifying a gene easier with a high degree of accuracy using the polymerase chain reaction (PCR) technique. Previous research has succeeded in identifying the *CSN2* polymorphism using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) technology in a population of Slovak Pinzgau cattle [13] and dairy cattle breed in the Czech Republic [14]. In addition to the RFLP technique, the DNA sequencing technique for determining the nucleotide sequence (adenine, guanine, cytosine and thymine) of a DNA sample can identify numerous gene variations in one reaction [15]. Rangel *et al.* [16] and Sebastiani *et al.* [17] succeeded in finding *CSN2* polymorphisms in populations of Gir and Guzera dairy cattle and Italian FH dairy cattle using DNA sequencing techniques. Up to these days, the mapping of the *CSN2* in the FH dairy cattle population in Indonesia has not been discovered as a basis for determining milk variants that are friendly to cases of diarrhea due to lactose intolerance. Therefore, the goal of this study was to analyze the *CSN2* variants in Indonesian FH dairy cattle as an attempt to prevent cases of diarrhea due to milk protein allergy and their association with milk production traits.

MATERIALS AND METHODS

Cows rearing and collection of blood sample

The materials used in this study were twelve Indonesian FH having milk production traits data of the first lactation at PT. Ultra Peternakan Bandung Selatan. They were fed with total mixed ration (TMR) containing both forages and concentrates, and received 58.81 kg per day. In addition, water was available *ad libitum.*

The process of taking blood samples was carried out in the jugular vein of cattle using an 18G venoject needle. The blood was collected in a vacutainer tube containing EDTA then stored in a refrigerator at 4°C until used for further analysis.

DNA extraction

The genomic DNA of Indonesian FH dairy cattle was extracted from blood samples using the High Salt Method according to Montgomery and Sise [18] at the UPT Integrated Laboratory of Sebelas Maret University. The DNA isolation process was carried out through several stages, starting with the lysis of red blood cells and protein lysis, followed by DNA precipitation and washing. The DNA quantification was carried out using a NanoPhotometer (P-Class®, Implen, Munich, Germany). DNA concentration (ng/μL) and DNA purity were obtained by comparing the optical density at 260 and 280 nm. The DNA concentration (ng/μL) and DNA purity were observed.

Primer design

The primer used in this study is a novel primer pairs compiled with a series of primer design stages. The primer design was prepared using the reference sequence of the *CSN2* in *Bos taurus* cattle obtained from the NCBI site with accession number NC_037333.1 and Ensemble with accession number ENSBTAG00000002632. The primers were designed to isolate exon 7 of the *CSN2*. The primers were designed using the Primer3 site (https://primer3.ut.ee) to obtain oligonucleotide forward and reverse primers. Forward primer sequence was 5'- ACC CCA ATT TCT TAA CCA AAC CA - 3' and reverse primer was 5'-CAT CAG AAG TTA AAC AGC ACA GT - 3'. The PCR product size was expected to be 683 bp covering complete exon 7 fragment.

DNA amplification

The PCR reactions were carried out using a MiniAmp® Thermal cycler machine (Thermo Fisher Scientific, Singapore) at the same laboratory as extraction DNA done. The total reaction volume in the microtube was 25 μL consisting of 12.5 μL MyTaq HS Red Mix (Bioline, London, UK), 9.5 μL nuclease free water (1st Base, Singapore), 1 μL forward and reverse primers, 1 μL DNA template. The PCR reaction begins with the initiation of denaturation at 95°C for 5 min, followed by 35 denaturation

cycles at 95°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 30 s. The PCR reaction was terminated with a final extension at 72°C for 10 min. The PCR results were electrophoresed using a submarine electrophoresis system (Mufid ex, Advance, Japan) on 2% agarose gel stained by FloroSafe DNA Stain for 30 min with a voltage of 110 Volts. A 100 bp marker ladder (Geneaid, Taiwan) was used as a standard for DNA band size. The agarose gel was then visualized using a gel documentation system (Glite UV, Pacific Image, Taiwan).

DNA sequencing

The PCR products of *CSN2* were sequenced using the services of PT. Genetika Science Indonesia. The DNA sequencing was conducted two-way reactions using forward and reverse primers. The DNA sequence was analyzed to determine the polymorphism (genetic variation) found in exon 7 of the *CSN2*. The *CSN2* sequence was analyzed using the supporting software BioEdit Sequence Alignment Ver. 7.2.5 and the Clustal Omega (www.ebi.ac.uk/Tools/msa/-clustalo). Individual genotype data was based on the alignment of the *CSN2* sequences and nucleotide sequence chromatograms. The *CSN2* sequences were deposited at NCBI website with accession number OK635333 to OK635337. Moreover, the nucleotide sequences were translated into amino acids using the Biomedicine DNA to Protein Translation software (http://bio.biomedicine.gu.se/edu/translat.html). This data became the basis to individually determine *CSN2* variant of Indonesian FH cattle used in this study.

Calculation of the allele and genotype frequencies

Allele and genotypes frequencies of *CSN2* polymorphisms within exon 7 were analyzed using the following formula:

$$H = p^2(n) + 2pq(n) + q^2(n)$$

where H is the expected value, n is the total sample, p is the frequency of the 1st allele and q is the frequency of the 2nd allele [19]. The Chi-square test (X^2) was used to test whether the alleles and genotypes of the *CSN2* were in accordance with Hardy-Weinberg equilibrium law (HWE) based on the following formula [20]:

$$X^2 = \sum \frac{(O - E)^2}{E}$$

The X^2 value represents calculated Chi-square, O is the genotype value obtained from observations, and E is expected value. The calculated value of X^2 is compared with the X^2 table at 5% alpha. Allele and genotype frequencies were within Hardy-Weinberg equilibrium if the probability value is less than 3.84.

Association between *CSN2* polymorphism and milk production traits

One-way analysis of variance was carried out to evaluate association between *CSN2* genotypes with milk production traits of Indonesian FH cattle. If there were significant differences among genotypes, Tukey test was conducted using MINITAB version 14.0 software (Minitab Inc., USA). Association analysis was performed using the following model:

$$Y_{ij} = \mu + G_i + \varepsilon_{ij}$$

where Y_{ij} is the phenotype of the kth animal, μ is the population mean, G_i is the fixed effect of genotype, and ε_{ij} is the residual error associated with the k_{th} animal.

RESULTS

DNA concentration

Twelve DNA samples with a total volume of 20 μL were obtained through an extraction process. DNA quantification revealed the concentration and purity of all DNA samples. The average concentration of the resulting sample was 61.33 ng/μL with an
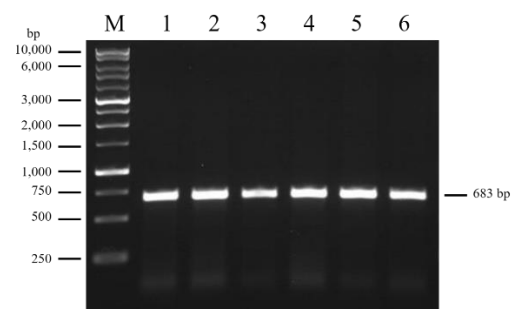

Figure 1. The PCR product of CSN2 using novel primer. M is 1 kb marker ladder, 1-6 are PCR products.

Table 1. The DNA concentration and purity of Indonesian FH cattle

Sampel code	Sample ID	Concentration (ng/μl)	Purity (A260/A280)
a	3055	60	1.818
b	3103	51.5	1.407
c	3426	53	1.898
d	802170	55.5	1.370
e	802482	58	1.902
f	802655	88.5	1.566
g	802964	62.5	1.894
h	803026	55	1.864
i	803064	56.5	1.915
j	803322	60	1.846
k	806008	75.5	1.798
l	806114	60	1.818

average purity of 1.758. The results of DNA extraction and quantification are presented in Table 1.

Amplification of *CSN2*

The primer used in this study was a novel primer that successfully amplified the exon 7 of CSN2 at 683 bp (Figure 1). The DNA band lies between 600-700 bp based on the 1 kb marker ladder used. The PCR product was specific, bright, and uniform for the twelve samples used in this study.

Sequence analysis of *CSN2*

The PCR product of *CSN2* exon 7 was confirmed having the size of 683 bp and in accordance with the expected size of PCR product. Nucleotide sequence analysis of
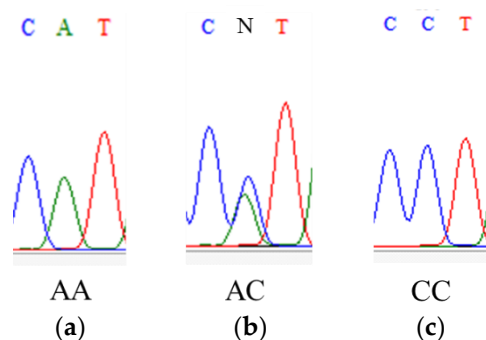
twelve samples showed there were two points of mutation in *CSN2* exon 7 specifically located at the 218th and 384th from forward primer, namely c.350A>C and c.516G>C, respectively. The c.350A>C SNP was a missense mutation that substitutes adenine (A) to cytosine (C) for determining A2 variant, and c.516G>C SNP was also a missense mutation that substitutes guanine (G) to cytosine (C) determining B variant. Polymorphisms of *CSN2* are shown in Figure 2 and Figure 3.

Allele and genotype frequencies of *CSN2*

Allele frequencies were analyzed based on two variations of *CSN2*. The alleles found at the *CSN2* exon 7 were A and C alleles for c.350A>C SNP. The frequency of C allele was
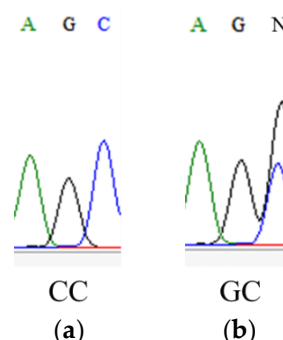

Figure 2. Genotype of c.350A>C SNP found in Indonesian FH cows based on *CSN2* sequencing data: (**a**) AA Genotype; (**b**) AC Genotype; (**c**) CC Genotype

Figure 3. Genotype of c.516G>C SNP found in Indonesian FH cows based on *CSN2* sequencing data: (**a**) CC Genotype; (**b**) GC Genotype

Table 2. Allele and genotype frequencies of SNP identified in exon 7 of *CSN2*

SNP	Genotype frequency			Allele frequency		X^2
c.350A>C	AA (n)	AC (n)	CC (n)	A	C	
	0.167 (2)	0.5 (6)	0.33 (4)	0.417	0.583	0.921
c.516G>C	GG (n)	GC (n)	CC (n)	G	C	
	0	0.167 (2)	0.833 (10)	0.083	0.918	0.753

Table 3. Base substitutions, amino acid changes, and *CSN2* variants found in Indonesian FH

SNP	Genotype	Amino acid	*CSN2* variant
c.350A>C	AA	Histidine	A1
	CC	Proline	A2
c.516G>C	GG	Arginine	B
	CC	Serine	A1

0.583 which is higher than A allele. Consequently, three genotypes were found based on this SNP, namely, AA, AC, and CC. In addition, G and C alleles were found for c.516G>C SNP where C allele was higher than G allele. However, only two genotypes (GC and CC) found for this SNP. Based on calculation, the polymorphisms of *CSN2* exon 7 were both in accordance with HWE law. A heterozygous AC was the most common genotype in this study, followed by CC and AA genotypes. In term of c.516G>C SNP, CC genotype was dominant (Table 2).

Translation of *CSN2* exon 7

The amino acid sequence of *CSN2* exon 7 was determined based on the nucleotide sequence obtained. The nucleotide sequence translation showed that A to C substitution of c.350A>C SNP caused the amino acid codon change at amino acid number 117 of beta-casein protein form histidine (CAU) to proline (CCU). In addition, G to C substitution of c.516G>C SNP changed amino acid codon at amino acid number 172 of beta-casein protein from arginine (AGG) to serine (AGC) (Table 3). The change in histidine amino acid to proline is the basis for determining A1 or A2 milk variants. FH cattle with AA genotype based on c.350A>C SNP produced A1 milk variant, on the other hand FH with CC genotype produced A2 milk variant milk. Moreover, FH cattle with GG genotype based

on c.516G>C SNP produced B milk variant which is not found in this study.

Association between *CSN2* variants with milk production traits

Effects of c.350A>C SNP and c.516G>C SNP of CSN2 on milk production traits including 305 days milk yield, total yield, average yield per day, and peak yield were conducted. Statistical analysis showed that there were no significant association between *CSN2* polymorphism and milk production traits in Indonesian FH cattle (Table 4).

DISCUSSION

The concentration and purity of DNA obtained in this study were considered acceptable and can be used for PCR reactions. Sambrook and Russell [21] stated that pure DNA has an absorbance ratio of 260/280 nm of 1.8 to 2.0. Absorbance values less than 1.8 can be due to contamination of carbohydrates, proteins, RNA and other organic materials. The amplification of gene fragment needs to use primers capable of amplify specific regions [13,22]. In general, the optimization of PCR process can be performed by varying the conditions used in the PCR process. Optimization conditions are closely related to factors such as the type of DNA polymerase; temperature; concentration, in this case, related to dNTPs, MgCl2 and DNA polymerase; PCR buffer and time. The bright

Table 4. Association of *CSN2* polymorphisms and milk production traits in Indonesian FH

Trait	c.350 A>C			*P*-Value
	AA	AC	CC	
AMY (kg)	5154.2 ±996.08	6530.97 ±1863.57	6959.68 ±624.81	0.39
TY (kg)	6356.5 ±137.88	8014.5 ±2333.83	8270 ±1402.13	0.51
AY (kg)	16.9 ±3.26	21.41 ± 6.1	22.8175 ±2.05	0.40
PY (kg)	30 .1 ±2.40	37.0833 ±4.93	31.975 ±3.11	0.10
Trait	c.516 G>C			*P*-Value
	GG	GC	CC	
AMY (kg)	-	6487.09 ±888.9	6435.88 ±1604.49	0.97
TY (kg)	-	7893 ±2035.05	7809.4 ±1952.72	0.96
AY (kg)	-	21.27 ±2.91	21.1 ±5.26	0.97
PY (kg)	-	30.55 ±3.04	34.95 ±4.92	0.26

AMY is 305 days accumulated milk yield; TY is total yield; AY is average yield per day; PY is peak yield

and specific DNA band can be yielded because each component of reaction worked well and in optimal conditions [23].

The frequency of C allele of the c.350A>C SNP was higher than A allele. This finding is supported by Huang *et al.* [24] who revealed that C allele frequency is 0.67 in FH cattle population and crosses between FH and Jersey. In addition, Rangel *et al.* [16] discovered that the frequency of the C allele in Gir cattle was 0.96 and 0.93 in Guzera cattle in Brazil; 0.68 in dairy cattle in the Czech Republic [14]; and 0.60 in the FH cattle population in Italy [17]. On the other hand, Miluchova *et al.* [13] reported that the frequency of C allele was lower than A allele in the Slovak Pinzgau cattle population, which was 0.44. Different results were obtained due to the different breeds of cattle, thus the difference in genetic character of *CSN2*. The high frequency of CC genotype did not follow the high frequency of C allele. This study reported that CC genotype frequency on Indonesian FH cattle was 0.33. Previous studies also found that CC genotype was not dominant [14,17,24].

Dairy cattle with CC genotype of the c.350A>C SNP produces A2 milk variant containing proline in the milk beta-casein protein which is well-known as mutant milk variant showing good effect to human health. On the other hand, dairy cattle with AA genotype produces beta-casein A1 variant with amino acid histidine is considered a wild-type milk variant in FH cattle [6,7]. The analysis of *CSN2* variant based on the possibility amino acid composition produced by individual FH cattle revealed that 33% of Indonesian FH cows used in this study produced A2 milk variant and 50% of them carried C allele that produced A2 milk variant. A1A2 cattle has the highest percentage value in the sample population, indicating that the heterozygosity value was relatively high, around 50% of the total sample population. This result indicated that there was no selection for specific traits related to *CSN2* genotype in this population. A1 milk contains beta-casomorphin-7 (BCM-7) peptide which is capable of causing inflammation and digestive system disorders. BCM-7 is the causative agent of lactose intolerance symptoms [9]. On the other hand, A2 cattle are able to produce much safer milk for people with lactose intolerance since it does not contain the BCM-7 peptide [4,25]. The A2 Indonesian FH cattle should be taken into account to be developed to improve their populations and to produce much more A2 milk variant. Thus, every people especially in Indonesia could enjoy milk without worrying about lactose intolerance symptoms.

Interestingly, association analysis between *CSN2* polymorphism with milk production traits in Indonesian FH cattle was not significant in this study. Cattle with different genotypes statistically produced similar milk production traits. Therefore, there was no negative effect on milk production

when farmers or dairy industries developing A2 dairy cattle. This study is in agreement with several previous studies reported that *CSN2* variants did not associate with milk production traits in Mexican Holstein and dairy cattle in Czech Republic [14,26].

CONCLUSIONS

There were two *CSN2* variants found in Indonesian FH cows, namely A1, and A2 variants and they did not associate with milk production traits. A total of 33% of FH cows was able to produce A2 milk variant which is safer to be consumed and able to prevent diarrhea cases due to lactose intolerance. Efforts in developing dairy cattle producing A2 variant milk could be made by selecting and developing Indonesian FH dairy cattle with CC genotype to produce friendly milk (A2).

CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

REFERENCES

1. Haug, A., A. T. Høstmark, and O. M. Harstad. 2007. Bovine milk in human nutrition – a review. Lipids Health Dis. 6:1-6. Doi: 10.1186/1476-511X-6-25

2. Heyman, M. B., Committee on Nutrition. 2006. Lactose intolerance in infants, children and adolescents. Pediatrics. 118:1279-1286. Doi: 10.1542/peds.2006-1721

3. Sitepu, G. A. S., E. R. R. Putri, and Inayah. 2020. Isolasi enzim laktase untuk mengurangi kadar laktosa susu bagi penderita intoleransi laktosa. Prosiding Industrial Research Workshop and National Seminar (IRWNS). p. 720-724. Doi: 10.35313/irwns.v11i1.2106

4. Pal, S., K. Woodford, S. Kukuljan, and S. Ho. 2015. Milk intolerance, beta-casein and lactose. Nutrients. 7:7285-7297. Doi: 10.3390/nu7095339

5. Chia, J. S. J., J. L. McRae, S. Kukuljan, K. Woodford, R. B. Elliot, B. Swinburn, and K. M. Dwyer. 2017. A1 beta-casein milk protein and other environmental predisposing factors for type 1 diabetes. Nutr. Diabetes. 7:1-7. Doi: 10.1038/nutd.2017.16

6. Ng-Kwai-Hang, K. F. and F. Grosclaude. 2003. Genetic polymorphism of milk proteins. In: Fox, P. F., and P. L. H. McSweeney, editors, Advanced dairy chemistry-1 Proteins. Springer, Boston, MA. p. 739-816.

7. Truswell, A. S. 2005. The A2 milk case: A critical review. Eur. J. Clin. Nutr. 59(5):623-631. Doi: 10.1038/sj.ejcn.1602104

8. Lambers, T. T., S. Broeren, J. Heck, M. Bragt, and T. Huppertz. 2021. Processing affects beta-casomorphin peptide formation during simulated gastrointestinal digestion in both A1 and A2 milk. Int. Dairy J. 121:1-6. Doi: 10.1016/j.idairyj.2021.105099

9. Teagan, S. E., K. L. Dawson, J. L. Keenan, and A. S. Day. 2021. A simple method to generate β-Casomorphin-7 by in vitro digestion of casein from bovine milk. J. Funct. Foods. 85:1-8. Doi: 10.1016/j.jff.2021.104631

10. Thiruvengadam, M., B. Venkidasamy, P. Thirupathi, I. Chung, and U. Subramanian. 2021. β-Casomorphin: A complete health perspective. Food Chem. 337:127765. Doi: 10.1016/j.foodchem.2020.127765

11. Deng, Y., B. Misselwitz, N. Dai, and M. Fox. 2015. Lactose intolerance in adults: biological mechanism and dietary management. Nutrients. 7:8020-8035. Doi: 10.3390/nu7095380

12. Taylor, S. K. Dwyer, K. Woodford, and N. Kost. 2017. Systematic review of the gastrointestinal effects of A1 compared with A2 β-casein. Adv. Nutr. 8:739-748. Doi: 10.3945/an.116.013953

13. Miluchova, M., A. Trakovická, and M. Gabor. 2009. Analysis of polymorphism of beta casein of Slovak Pinzgau cattle by PCR-RFLP for alleles A1 and A2. Sci. Papers Anim. Sci. and Biotechnologies. 42:288-292.

14. Citek, J., L. Hanusova, L. Liskovcova, E. Samkova, O. Hanus, L. Hasonova, Z. Krizova, and L. Vecerek. 2019. Polymorphisms in CSN3, CSN2 and LGB genes and their relation to milk production dairy cattle in in the Czech Republic. Acta Univ. Agric. et Silvic. 67:19-24. Doi: 10.11118/actaun201967010019

15. Grada, A. and K. Weinbrecht. 2013. Next-generation sequencing: Methodology and application. J. Invest. Dermatol. 133:1-4. Doi: 10.1038/jid.2013.248

16. Rangel, A. H. N., L. G. Zaros, T. C. Lima, L. H. F. Borba, L. P. Novaes, L. F. M. Mota, and M. S. Silva. 2017. Polymorphism in the beta casein gene and analysis of milk characteristicsin Gir and Guzerá dairy cattle. Genet. Mol. Res. 16:1-9. Doi: 10.4238/gmr16029592

17. Sebastiani, C., A. Chiara, C. Marcella, T. Martina, C. Giulia, F. Stefano, and B. Massimo. 2020. Frequencies evaluation of β-casein gene polymorphisms in dairy cows reared in Central Italy. Anim. 10:252. Doi: 10.3390/ani10020252

18. Montgomery, G. W., and J. Sise. 1990. Extraction of DNA from sheep white blood cells. New Zealand J. Agric. Res. 33:437-441. Doi: 10.1080/00288233.1990.10428440

19. Nei, M., and S. Kumar. 2000. Moleculer evaluation and phylogenetic. Oxford University Press Inc., New York, USA.

20. Bruce, W. 1981. Basic population genetics. Columbia University Press, New York, USA.

21. Sambrook, J. and D. W. Russel. 1989. Molecular cloning: a laboratory manual. Cold-Spring Harbor Laboratory Press, New York, USA.

22. Vougiouklaki, D., D. Antonopoulos, S. Allexeli, and D. Houhoula. 2020. Identification of polymorphisms of gene CSN2 of b-casein in Greek Cow breeds (holstein) by restriction fragment length polymorphism. J. Agric. Sci. 12:32-39. Doi: 10.5539/jas.v12n11p32

23. Handoyo, D. and A. Rudiretna. 2000. Prinsip umum dan pelaksanaan polymerase chain reaction (PCR) [General principles and implementation of polymerase chain reaction]. Unitas. 9:17-29.

24. Huang, W., F. Peñagaricano, K. R. Ahmad, J. A. Lucey, K. A. Weigel, and H. Khatib. 2012. Association between milk protein gene variants and protein composition traits in dairy cattle. Int. J. Dairy Sci. 95:440-449. Doi: 10.3168/jds.2011-4757

25. Jianqin, S., X. Leiming, X. Lu, G. W. Yelland, J. Ni, and A. J. Clarke. 2016. Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. J. Nutr. 15:1-16. Doi: 10.1186/s1293 7-016-0147-z

26. Duifhuis-Rivera, T., C. Lemus-Flores, M. A. Ayala-Valdovinos, D. R. Sánchez-Chiprés, J. Galindo-García, K. Mejía-Martínez, and E. González-Covarrubias. 2014. Polymorphisms in beta and kappa casein are not associated with milk production in two highly technified populations of Holstein cattle in México. J. Anim. Plant. Sci. 24:1316-1321.