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Detection of alleles and genotypes of beta-casein and their effects on milk traitsin Friesian X Bunaji cows

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Abstract
Over the last decades, the Beta-Casein (β-CN) variants A^1, A^2 and B have received attention from the research community, producers, and consumers because of the influence of the variants on milk production traits, milk gelation performance and the health benefits of β-CN variant A^2 over A^1. The study examined and analysed the incidence of β-casein variants A^1, A^2, and B and their genotypes in Friesian X Bunaji cows; it also investigated how the identified genotypes affected milk yield, milk pH and composition traits.Thirty (30) Friesian X Bunaji cows were used to collect blood and milk samples. Twenty (20) ml of milk from each cow was used for analysis of percentages of protein, fat, lactose, solid-not-fat (SNF), salts and pH, usingLactoscan milk Analyzer. After which total solid (TS) was calculated from contents of fat and solid-not fat. Three SNPS (CNS2_67, CSN2_106 and CSN2_122) at the beta casein locus were genotyped using the Allele-specific single-base primer extension techniques via SNPs Genotyping SequenomMassARRAY® system (iPLEX GOLD method). The results indicated that the beta casein gene locus was polymorphic and had three genetic variants A^1, A^2, andBwith the frequency of 15.0, 83.0 and 1.7 percent respectively; there were four genotypes identified A^1A^1(2 cows), A^2A^2(22 cows), A^1A^2(5 cows) and A^2B (1 cow) with frequency of 6.7, 73.3, 16.7 and 3.3 percent respectively. The Variant A^2 was the most common at the beta casein gene locus followed by A^1 and the least was B; the most common genotype identified was A^2A^2 followed by A^1A^2,A^1A^1 an lastly A^2B. A relatively low frequency of 15.0 percent recorded for CSN2 A^1 allele indicated that the milk from Friesian X Bunaji cows might consist of a relatively low percent of the deleterious peptide of beta-casomorphin-7 opioid and has low risk of infection if consumed. The high occurrence of the desired A^2 allele (83.0 percent) in Friesian X Bunaji cows was outstanding. The beta casein genotypes significantly affected daily milk yield (P<0.01) and fat content of milk (P<0.01). Cows carrying beta casein genotype A^2A^2 produced the highest amount of daily milk yield and the least was produced by those carrying A^1A^1; while those carrying the genotype A^1A^2 produced milk with the highest amount of fat and those carrying A^2A^2 produced milk with the minimum fat content. It is concluded that in Friesian X Bunaji cows, there is high occurrence of the desired beta casein A^2 allele; the genetic variants of the beta casein gene associate with the daily milk yield and content of milk fat and may be used as a marker during selection of breeding animals.

Key words: *Friesian X Bunaji cows, genetic polymorphism, beta-casein gene, genotyping, milktraits*

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I. Introduction

Over the last decades, milk protein genetic polymorphism has received research interest because of the associations of their genotypes with milk production traits, its manufacturing properties and consumers' health (Hristov *et al*., 2013). Thus, these genes are useful markers for supplementing conventional selection method in dairy cattle breeding (Contreras *et al.*, 2011). The study of the genetic polymorphism of milk proteins started in 1955when Aschaffenburg and Drewry (1955) discovered β-LG variants. So far in Bos's genus, the nomenclature of the milk proteins shows the presence of 10 α_{S1}-casein, 15 β-casein, 5 α_{S2}-casein, 13 κ-casein, and 12 β-LG variants and 4 α-LA variants *(*Caroli *et al*., 2009; Gallinat*et al., 2013)*. However, only about 12 genetic variants are common in most milk (Caroli *et al*., 2009).

The β-CN variants A^1, A^2 and B have received attention from the research community, producers, and consumers because of the influence of the variants on milk production traits, milk gelation performance and the health benefits of β-CN variant A^2 over A^1 (Adzhubei*et al*., 2013). In bovine milk, beta-Casein (β-CN) contains approximately 45 percent of the total casein fraction; it is the most abundant protein; the secondmost polymorphic milk protein gene (Miluchová*et al*., 2014). The reference protein of β-CN is β-CN A^2-5P;its molecular weight is 23,983 Daltons; it has 209 amino acid residues. So far, previous studies have identified15

genetic variants of β-CN that include: A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2, I, J, K and L in the Bos genus (Farrell Jr *et al*. 2004; Caroli *et al*., 2009; Singh *et al.* 2015). However, the most common genetic variants of the beta-casein gene are A^1, and A^2 while variants A^3 and B are of lesser occurrences in cattle (Caroli *et al*., 2009;Gallinat*et al*., 2013). Previous studies have established that β-CN variants A^2 is the variant of ancestral origin of the Bos genus from which variant A^1 mutated latter in the European cattle breed (Bos taurus) (Dinc*et al*., 2013; Malarmathi *et al*., 2014).The differences between allele A^2 and A^1 is in one amino acid substitution at positions 67 (His/Pro) while the differences between A^2 and A^3 is amino acid replacement at position 106(Gln/His) of the beta casein polypeptide chain (Miluchová *et al*., 2014; Massella *et al*.,2017). The difference between A^1 and B variants is amino acid change at position 122 (Ser/Arg) of the β-casein. While allele B differs from A^2 in having a prolinein position 67 and an arginine replaces serine at position122 (Vallas *et al*., 2012).

The mutations that cause these differences are due to a single nucleotide polymorphism (SNP) of the β-casein gene; accordingly, the β-casein A^1 allele varies from the A^2 allele via an A→C replacement at nucleotide position 304 of the cow β-casein referencesequence (GenBank, NM_181008). While the B allele varies by 2 non-synonymous mutations from the A^2 allele via an A→C transition at nucleotideposition 304, resulting in the amino acid exchange Pro→His, and a C→G transition at nucleotide position 470, resulting in a Ser→Argreplacement. These variations in the amino acidsequence leads to changes in the secondaryshape of the expressed protein (Kaminski *et al*., 2007).

In the small intestine of humans, the presence of histidinein variant $A^1$of beta casein causes the release of a bioactive peptidecalled beta-casomorphin-7 (BCM-7) opioid; while the presence of proline invariant A^2 forms another peptide called beta-casomorphin-9 (BCM-9)(Thorsdottir*et al*., 2000). The peptide BCM-7 opioid has low molecular weight which causes it to cross the bloodbrainbarrier and binds to μ/k/δreceptors that are situated in part of the immune system, the gastrointestinal system, the central nervous system,and all these have an impact on consumer's health(Barnett *et al.,* 2014; Pal *et al*., 2015). These diseases include type 1 diabetes (DM-1), coronary heart disease (CHD), gastrointestinal discomforts, neurological disorders, sudden infant death syndrome (SIDS), and autism (Laugesen and Elliott, 2003;Pal*et al*., 2015).

In dairy cattle, β-casein has been investigated at the protein and DNA levels (Freyer *et al.,* 1999; Gallinat *et al.,* 2013). So far, several methods have been used for genotyping of polymorphism of major milk proteins in cattle (Hristov and Radoslavov, 2015). TheiPlexmassARRAY genotyping technique is one of the modern molecular genetic methods that takes advantage of polymorphism detected at the DNA level regardless of age, physiological status, and sex of the animal (Teneva and Petrović, 2010); this technique associates PCR and mass-spectrum.

Researchers have investigated the association of beta-casein genetic variants with milk traits and technological properties and the results are contradictory (Albarella*et al*., 2020, Asmarasari*et al*., 2020); some authors reported significant effects (Miluchova*et al*., 2014; Bugeac*et al*.2015) while other recorded non-significant effects (Ardicli*et al*., 2018;Čítek*et al*., 2019). For example, previous studies (Miluchová *et al*., 2014; Bugeac*et al*., 2015;Albarella *et al*., 2020) reported that at the CSN2 gene locus, the genotypes (A^1A^1, A^2A^2, A^1A^2) had significant effects on milk yield, contents of milk protein, fat, solid-nonfat, and total solids; cows carrying genotype A^2A^2produced highest milk, solid-nonfat and total solids while those with A^1A^1 produced the lowest milk; cow carry genotype A^1A^1 produced milk with highest fat and protein contentswhile those with genotype A^1A^2 produced milk with the lowest protein. On the contrast, other researchers (Ardicli*et al*., 2018; Nguyen *et al*., 2015; Čítek*et al*., 2019) found that cows with *CSN2* genotypes (A^1A^1, A^1A^2, A^1A^2) had non-significant effects on milk yield, crude protein percent, protein yield, fat percent, total solid and fat.

The annual domestic milk production in Nigeria stands at 0.6 million tons with a consumption of 1.7 million tons; to fill the gap in 2016, the government used approximately US$ 295 million to import dairy products (Federal Ministry of Agriculture and Rural Development (FMARD), 2016; PricewaterhouseCoopers (PwC), 2017). Consequently, the Nigeria dairy sector goal is to develop a domestic dairy value chain to satisfy domestic demand and reduce the nation's dependence on imported milk and/or dairy products (Food and Agricultural Organization (FAO report, 2016). One of the strategies to increase domestic milk production is through crossbreeding schemes between the indigenous and the exotic breed; Friesian sires and /or semen are the main dairy breed of cattle used in cross breeding of the Bunaji cows (Alphonsus, 2010; FMARD, 2016). This has resulted in increased milk protein content from 5.8±0.36 percent in Bunaji and 5.70±0.45 percent in Friesian to 6.4±0.27 percent in milk from Friesian X Bunaji cows; increased milk yield from 1.5-3.0kg/ cow/day in Banaji to 7-14kg/cow/day in Friesian X Bunaji cows (Alphonsus *et al*., 2012; Alphonsus *et al.,* 2016). Besides that, crossbreeding combines early sexual maturity and high milk yield of European dairy breeds with the adaptability, disease resistance and hardiness of the indigenous cattle (Geshaw*et al.,* 2011; Roschinsky*et al.,* 2015).Several studies have been accomplished in the Friesian X Bunaji cattle but there is no published information on the effects of β-casein genetic variants on milk traits, although improvement through artificial insemination has been broadly used (Alphonsus *et al.,* 2016).

Considering that Friesian X Bunaji cattle is one of common dairy cattle breeds in Nigeria; the aims of this study were to analyse the incidence of β-casein variants A^1, A^2, and B and their genotypes in Friesian X

Bunaji cows; investigate how the identified genotypes affect milk yield and composition traits. This is expected tounderstand: the biological significance of the beta casein genetic variants identified; their contribution to improvecattle selection and breeding programs; ways of having more healthier milk via reducing A^1 allele frequency in the population of Friesian X Bunaji cows.

II. Material and Methods

2.1.Location of the Experiment: The study was carried out at the Dairy Research Programme farm of the National Animal Production Research Institute (NAPRI), Ahmadu Bello University (ABU), Shika, Zaria, Kaduna State, Nigeria. Shika is geographically located in the Northern Guinea Savanna between latitude 11^o 12'59''N and longitude 07^o 33'40''E at an elevation of 702 m above sea level (Google Earth, 2012). The mean annual rainfall is 1,100 m and it begins from May and end in October. Dry, cool weather harmattan extend from mid-October – January while the dry season from February-May. Mean relative humidity is 72 percent during the rainy season and 21 percent during the harmattan with a daily mean temperature of 24.8 ^{o}C and 14 ^{o}C respectively.

2.2. Ethical Statement: The study was implemented following an approved guideline by the Ahmadu Bello University (ABU) Committee on Animal Use and Care (ABUCAUC), Zaria, Nigeria.

2.3. Experimental Animals and Management: Animals used in this experiment were 30 Friesian X Bunaji cows that differed in their lactation stage (early=6, mid=9, late=15) and parity (1=10, 2-3=14, ≥4=6) kept at the Dairy farm of NAPRI. These cows were kept under semi-intensive system and fed on the same diet.The study covered the months of January to November in the dry and wet season; that involved follow-up and collection of milk and blood sample. The cows were allowed to graze on paddocks of established pasture consisting of variety of grasses such as elephant grass (*Pennisetum purpuneom*), Giant star grass (*Cynodonplectostacynum*), Gamba grass (*Andropogon gayanus*), Guinea grass (*Pancium maximum*) (Tanko *et al.*, 2014). In the dry season, hay, silage, and cotton seed cake were offered to the cows. Furthermore, the cows had access to fresh water and mineral salt blocks *ad-libitum*. Milking was done twice daily at 7:00 am and 4:00 pm using a milking machine and each cow offered 2.0kgs of concentrate feed at each milking. Calves were allowed to suckle their dams for seven days after calving and were then hand-fed with milk collected from the dam. Program veterinarians dipped the cows against ectoparasites twice a week during the rainy season and once during the dry season; each cow was treated against diseases on case-by-case basis.

2.4 Collection of Blood Sample: Blood samples were collected from 30 Friesian X Bunaji cowsvia venipuncture of *v. coccygea*with 23-gauge sterile needle and syringe and placed in tubes containing an anticoagulant (EDTA). From each cow 5 ml of blood was collected. The 30 blood samples were transported in an ice bag to a commercial laboratory (Bioinformatics Services) at Ibadan, Oyo State, Nigeria. Each blood sample on arrival at the laboratory was stored at 4 ^{o}C pending the extraction of the genomic DNA.

2.5 Laboratory Analysis of blood samples: Genomic DNA (gDNA) was extracted from each whole blood sample at a commercial DNA laboratory: Bioinformatics Services at Ibadan, Oyo State, Nigeria. Besides that, each gDNA was used for SNPs genotyping at a commercial Africa's Genomics Company: InqabaBiotec West Africa Ltd, Ibadan, Oyo State, Nigeria.

2.6. Genomic DNA extraction and quantification: The gDNA was extracted from 5ml of whole blood each from 30 cows using a QUICK-DNA MINIPREP KIT Cat No. D3024 (Manufactured by Zymo Research) and the manufacturer's protocol were followed. The quality or purity of each extracted gDNA was assessed using a Nanodrop Spectrophotometer; protein contamination was evaluated using the ratio of absorbance at 260 nm and 280 nm. Besides that, gel electrophoresis was used to assess the integrity of the extracted gDNA. (Black and Foarde, 2007). The samples that indicated an optical density (OD) ratio (260 nm/280 nm) of between 1.8 and 2.2 were reserved for further analyses, and diluted to a concentration of 50 ng/µL. The 30 samples of gDNA were forwarded to InqabaBiotec West Africa Ltd for SNPs genotyping.

2.7. SNPs Genotyping SequenomMassARRAY® system (iPLEX GOLD Technique):

Three non-synonymous missense SNPs (CSN2_67; CSN2_106 and CSN2_122) identified by Ketto *et al*. (2017) and 30 gDNA belonging to Friesian X Bunaji cows were genotyped using the MassArray genotyping platform the SequenomMassARRAY® system (iPLEX GOLD; Sequenom, San Diego, CA, USA) following manufacturer's protocols. The method is based on the analysis of DNA products using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Gabriel *et al*., 2009). PCR was used to amplify the area of DNA containing the SNPs, for each SNP, a primer extension reaction was performed to generate allele-specific DNA products, and the size and quantity of each allele specific product was determined via chip-based mass spectrometry.

The PCR amplification of the targeted region of gDNA containing the SNPs was accomplished in a 5µl total volume of reaction system comprising of 20 ng of gDNA, 0.5U HotstarTaq (Qiagen), 0.5µl 10×PCR buffer, 0.1µl dNTPs and 0.5 pmol of each primer. PCR reactions were carried out in a PTC-100 PCR instrument

(Eppendorf) with the following conditions: 4 minutes of denaturation at 94°C, 35 cycles of 20 seconds at 94°C, 30 seconds at 56°C and 1 minute at 72°C and a final extension at 72°C for 3 minutes. After which 2µl shrimp Alkaline Phosphatase (SAP) (SEQUENOM) was used to clean the PCR products. The single base extension used 2µl EXTEND Mix (SEQUENOM) consisting of 0.2µl iPLEX termination mix, 0.94µl Extend primer Mix and 0.041µl iPLEX enzyme and was accomplished through the following steps: initial denaturation at 94°C for 30 seconds, followed by 40 cycles of three steps amplification profile of 5 seconds at 94°C, additional 5 cycles of 5 seconds at 52 °C and 5 seconds at 80°C and a final extension at 72 °C for 3 minutes. Resin purification was used to clean the PCR products and then it was analysed by means of MassARRAY Analyzer Compac (SEQUENOM) and software TYPER (SEQUENOM). Table 1, represents the marker IDs and primer IDs and their sequences adapted from previous publication (Ketto *et al*., 2017).

Table 1. Single nucleotide (SNP ID) polymorphism and primer sequences for the genotyped markers.

Gene	SNP ID	Position (bp)	Forward primer sequence	Reverse primer sequence	Extended primer sequence
CSN2	CSN2_67	87181453	ACGTTGGATGCCAAAGTGA AGGAGGCTATG	ACGTTGGATGTCAACATCAG TGAGAGTCAG	ATCAGTGAGAGTC AGGCTCTG
	CSN2_106	87181501	ACGTTGGATGTCAACATCA GTGAGAGTCAG	ACGTTGGATGCCAAAGTGAA GGAGGCTATG	GCTATGGCTCCTA AGCA
	CSN2_122	87181619	ACGTTGGATGTAAAATCCA CCCCTTTGCCC	ACGTTGGATGAGAGGAGGG ATGTTTTGTGG	TTTGTGGGAGGCT GTTA

Source: Ketto et al., 2017

2.8 Collection of Milk Samples: Twenty millilitres (20) of milk were collected once from one of the morning milks of each of the 30 Friesian X Bunaji cows for analysis of milk traits (two duplicates). Besides that, information on each cow's parity (1, 2-3 and ≥4) and lactation stage (early (7- 90 days), mid (91-180 days) and late (181-305 days) were recorded. Furthermore, the average daily milk yield of each cow was calculated from the farm records.

2.9 Laboratory Analysis of Milk Samples: Twenty millilitres (20 ml) of milk were collected from each cow, frozen at 4^{o}C and transported in an ice bag to the laboratory at Centre of Excellence in Agriculture Development and Sustainable Environment (CEADESE) Central Laboratory, Federal University of Agriculture, Abeokuta, Nigeria. The 20ml of milk from each cow was used for duplicates analysis of percentages of protein, fat, lactose, solid-notfat (SNF), Salts and pH usingLactoscan milk Analyzer. After which total solid (TS) was calculated using the following formula:

$$\%\,Total\ solid = \%SolidNotFat + \%Fat$$

2.10. Statistical analyses: Genotyped of three SNPs belonging to *CSN2 (CSN2_67, CSN2_106, CSN2_122)* gene via the IPLEX massARRAY assays:The Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) mass spectra plotted allelic peak intensity (*y*-axis) against mass (Daltons) (*x*-axis); while the MassARRAY Typer software analysed the allele peak intensities; cluster plots were produced which showed the genotype calls at the SNPs CSN2_67, CSN2_106, CSN2_122 loci of the 30 Friesian X Bunaji cows. Furthermore, massARRAY Typer software enabled the recording of the resulting parameters on Microsoft excel format for each SNP: number of alleles; call rate, total number of cows; observed and expected homozygotes genotypes; observed and expected heterozygotes genotypes, the alleles frequencies (p and q), and p-value for Hardy Weinberg Equilibrium.

Calculated Chi-square ($\square^{2)}$ test to verify departure from Hardy-Weinberg proportion

Then, the above-mentioned data from Excel spreadsheet was used to test the differences between observed and expected genotype frequencies for each SNP using Chi-square ($\square^{2)}$ test to verify departure from Hardy-Weinberg proportion at the significance levels of P<0.05 and P<0.01 using the following formular:

$$\mathcal{X}^2 = \sum \frac{(Observed - Expected)^2}{Expected}$$

Furthermore, some population genetic indices: gene heterozygosity (He), gene homozygosity (Ho), and Fixation Index (FIS) were triangulated via POPGENE32 software version 1.32(Yeh *et al*., 2000).

Calculated allele and genotypes frequencies for the most common genetic variants:

In addition, the Microsoft Excel spreadsheet gave summaries of the genotypes present at each SNP locus; this information was used to calculate the genotypes frequencies for the following genetic variants: Variants A^1 and A^2 were calculated from SNP CSN2_67 (one point mutation); Variants A^2 and A^3 were calculated from SNP CSN2_106 (one point mutation) and Variants A^2 and B were calculated from SNP CSN2_122 (one point

mutation).The allele frequencies and genotypes frequencies of the genetic variants present in the 30 Friesian X Bunaji cows were recorded.

Analysed the Effects of Beta-Casein genotypes on the milk yield and composition traits: These effects were analysedvia the MIXED procedure of Statistical Analysis System (SAS), Version 9.0 (SAS, 2002), where the effect of cowwas treated as a random effect; while the genotypes were taken as fixed effects. However, the effects of lactation stage and parity were found to be non-significant and consequently, were omitted from further statistical analysis. In addition, the less frequent genotype (<4 percent) of β-CN (A^2B) wasexcluded from the statistical analysis. Based on the above, the fixedeffects of the beta casein genotypes on the milk yield and composition traits were tested in Linear Mixed Model 1:

$$Y_{ijk} = \mu + \beta CNgen_i + Cow_j + \varepsilon_{ijk}$$

Where:
Y_{ijk} = dependent variables include milk yield and composition traits;
μ= the overall mean;
$\beta CNgen_i$= the fixed effect of ith*β-CN* genotype (i=A^2A^2 or A^2A^1 or A^1A^1)
Cow_j = the random effect of jth cow (j= 1 to 30) N ~ (0, σ^2_{cow}),
ε_{ijk} = the random residual effect N ~ (0, σ^2_{ε}).
Furthermore, the values were presented as least squares means and their differences were tested using Tukey-Kramer procedure of SAS (2002), which adjusted tests for multiple comparison and unequal subgroup sizeat $p \leq 0.05$ and $p \leq 0.01$ levels as described in Kramer (1956). In addition, the differences between the least squares means for each fixed effect were tested with the Probability of Difference (PDIFF) option of the mixed procedure of SAS.

III. Results and Discussions

3.1 iPlexMassARRAY results for three SNPs genotyped at beta casein gene locus

At the SNP CSN2_67 locus: The results of this study presented that the SNP CSN2_67 locus was polymorphic and had two alleles G and T that determined genetic variants A^2 and A^1 respectively. Three genotypes GG (22 cows), GT (6 cows) and TT (2 cows) were determined from the allele's peak intensities corresponding to the genetic variants A^2A^2, A^1A^2 and A^1A^1 respectively (Plate 1a-d)

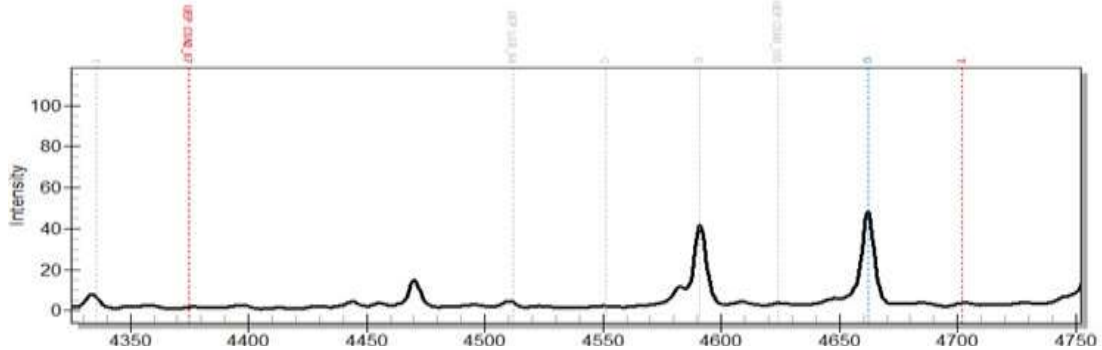

Plate 1a. MassARRAY spectrum plotting of allelic peak intensity versus mass (Daltons)indicated homozygosity of allele G at 4660 Da corresponding to genetic variant A^2at the SNP CSN2_67 locus in 30 Friesian X Bunaji cows.

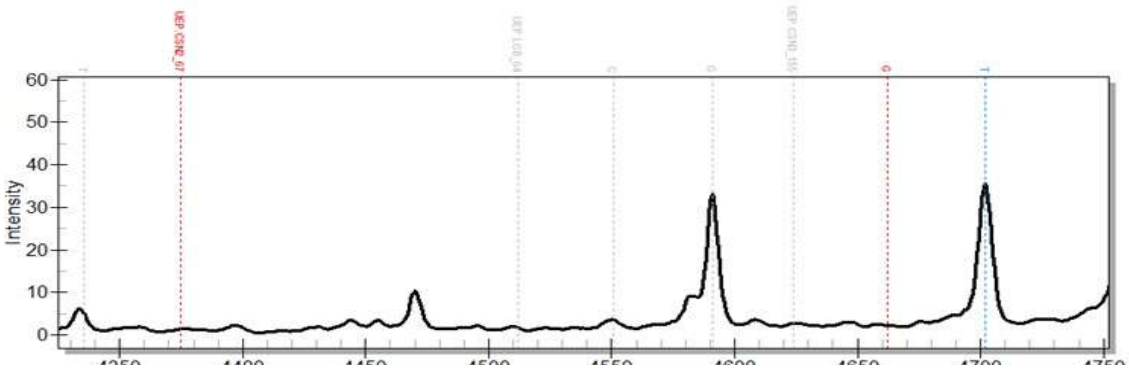

Plate 1b. MassARRAY spectrum plotting of allelic peak intensity versus mass (Daltons) indicated homozygosity of allele T at 4700 Da corresponding to genetic variant A^1 at the SNP CSN2_67 locus in 30 Friesian X Bunaji cows.

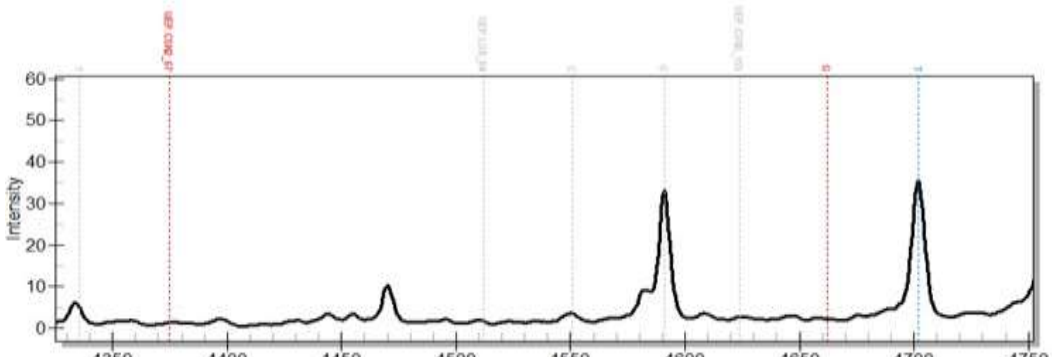

Plate 1c. MassARRAY spectrum plotting of allelic peak intensity versus mass (Daltons) indicated heterozygosity of allele G at 4660 Da and allele T at 4700Da corresponding to genetic variants A^2 and A^1 at the SNP CSN2_67 locusin 30 Friesian X Bunaji cows.

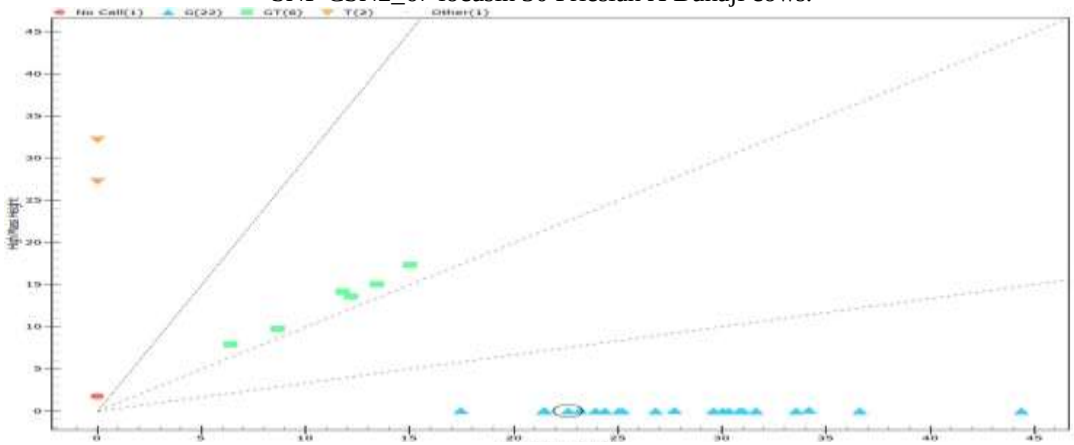

Plate 1d. The cluster plot indicated three genotypesof GG (22 cows), GT (6 cows) and TT (2 cows) corresponding to genetic variants A^2A^2, A^1A^2 and A^1A^1respectively at the SNP CSN2_67 locus in 30 Friesian X Bunaji cows.

At the SNP CSN2_106 locus: The results of this study presented that the SNP CSN2_106 locus was monomorphic and had one allele G that determine genetic variantA^2. One genotype GG (20 cows) was determined from the allele's peak intensity corresponding to the genetic variants A^2A^2(Plate 2a-b)

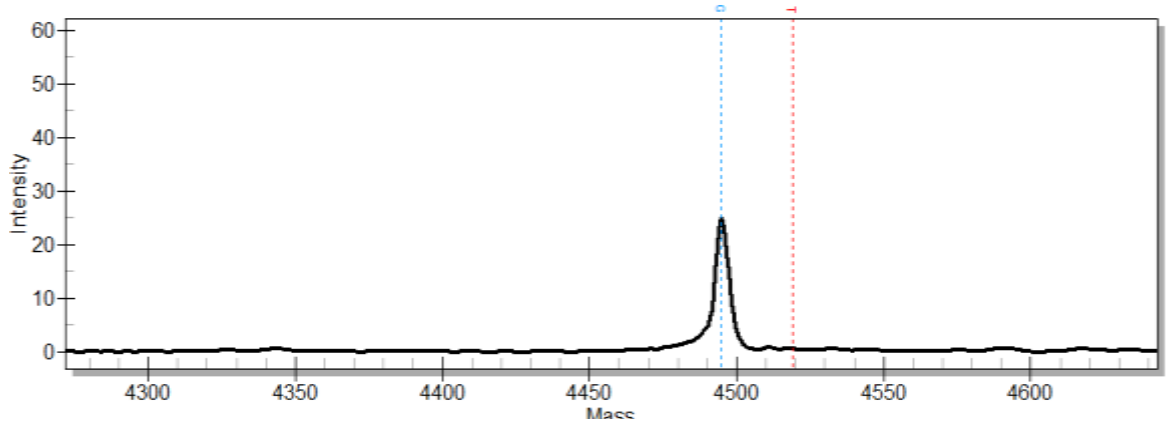

Plate 2a. MassARRAY spectrum plotting of allelic peak intensity versus mass (Daltons) indicated homozygosity of allele G at 4570 Da corresponding to A^2 at the SNP CSN2_106 locus in 30 Friesian X Bunaji cows.

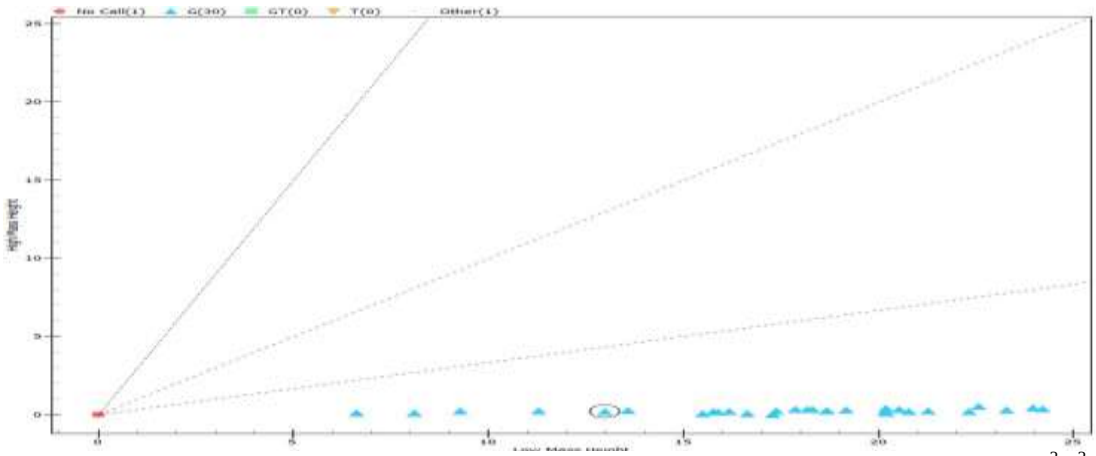

Plate 2b. The cluster plot indicated one genotype of GG (30 cows) correspondingto genetic variant A^2A^2at the SNP CSN2_106 locus in 30 Friesian X Bunaji cows.

At the SNP CSN2_122 locus: The results of this study presented that the SNP CSN2_122 locus was polymorphic and had two alleles G and C that determined the genetic variants A^2 and B respectively. Two genotypes GG (22 cows), and CG (6 cows) were determined from the allele's peak intensities corresponding to genetic variants A^2A^2 and A^2B respectively (Plate 3a-c)

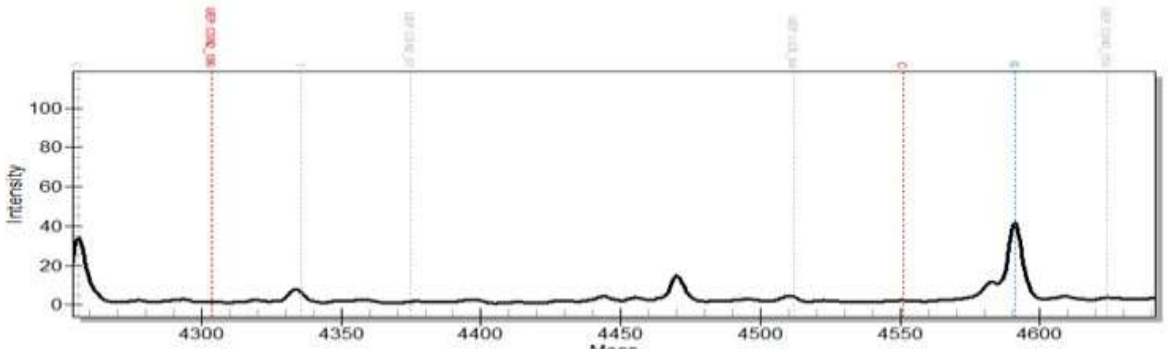

Plate 3a. MassARRAY spectrum plotting of allelic peak intensity versus mass (Daltons) indicated homozygosity of allele G at 4550 Da corresponding to genetic variant A^2 at the SNP CSN2_122 locus in 30 Friesian X Bunaji cows.

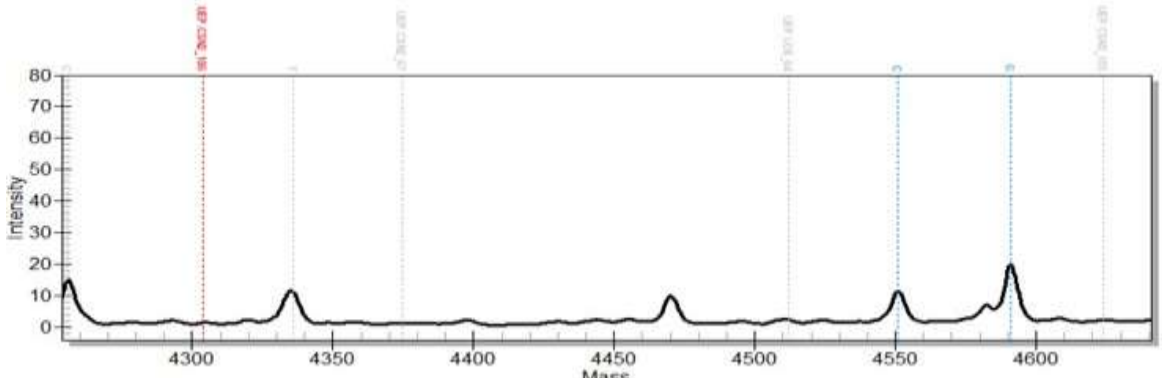

Plate 3b. MassARRAY spectrum plotting of allelic peak intensity versus mass (Daltons) indicated heterozygosity of allele C at 4550 Da and allele G at 4590 corresponding to genetic variants B and A^2respectively at the SNP CSN2_122 locus in 30 Friesian X Bunaji cows.

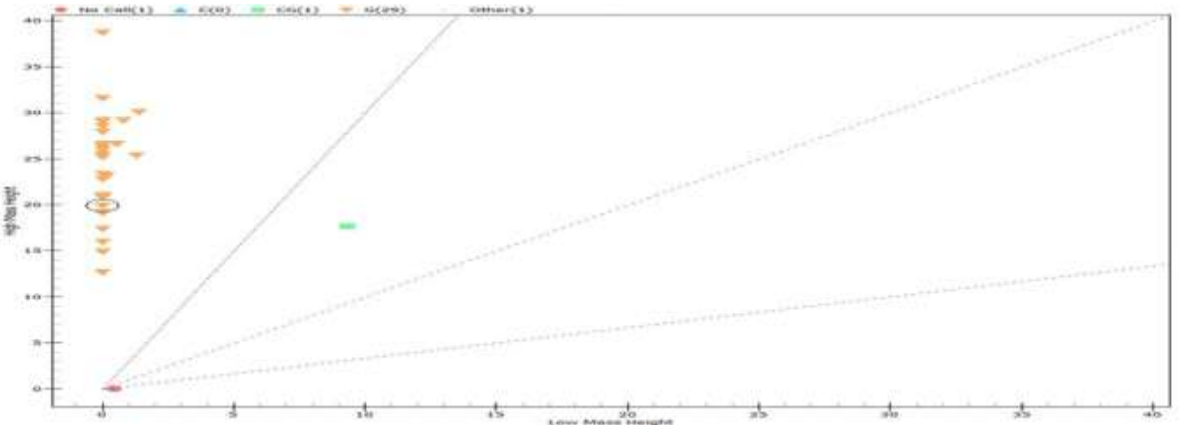

Plate 3c. The cluster plot indicated two genotypes of GG (29 cows) and CG (1 cow) corresponding to genetic variants $A^2A^2$and A^2B respectively at the SNP CSN2_122 locus in 30 Friesian X Bunaji cows.

3.2. Characteristics of Genotyping Results for three SNPs at beta casein locus

Table 2, shows the summary characteristics that includes SNP ID, polymorphism of the SNP, observed and expected homozygosity and heterozygosity, genetic variant involved, SNP call rate and Hardy- Weinberg Equilibrium (HWE) p-value for three SNPs locus of beta casein gene in 30 Friesian X Bunaji cows.

At the CSN2_67 locus: The results of the current study indicated that the SNP CSN2_67 was polymorphic and had two alleles G and T with frequency 0.83 and 0.17 respectively corresponding to genetic variants A^1 and A^2 respectively. Three genotypes were identified GG, GT, and TT with frequency 0.733, 0.200 and 0.067 respectively corresponding to genetic variants A^2A^2, A^1A^2 and A^1A^1 respectively. The call rate was 100 percent. The expected homozygosity showed a slight decrease resulting in homozygote deficiency while the expected heterozygosity showed a slight increase resulting in heterozygote excess. Heterozygote excess is usually associated with either crossbreeding, like two divergent populations coming together or heavy selection against inbreeding depression. A non-significant Hardy-Weinberg equilibrium P-value (0.13) was observed at the SNP CSN2_67 locus; this confirmed that the SNPCSN2_67 locus is at HWE.

At the CSN2_106 locus: The results of the current study indicated that the SNP CSN2_106 was monomorphic and had one allele G frequency 1.00 corresponding to genetic variants A^2. One genotype was identified GG with frequency 1.00 corresponding to genetic variants A^2A^2. The call rate was 100 percent. A non-significant Hardy-Weinberg equilibrium P-value (1.00) was observed at the SNP CSN2_106 locus; this confirmed that the SNPCSN2_106 locus is at HWE.

At the CSN2_122 locus: The results of the current study indicated that the SNP CSN2_122 was polymorphic and had two alleles C and G with frequency 0.02 and 0.98 respectively corresponding to genetic variants A^1 and A^2 respectively. Two genotypes were identified GG, and CG with frequency 0.967 and 0.033 respectively corresponding to genetic variants A^2A^2, A^2B respectively. The call rate was 100 percent. A non-significant Hardy-Weinberg equilibrium P-value (0.93) was observed at the SNP CSN2_122 locus; this confirmed that the SNPCSN2_122 locus is at HWE.

Table 2. Characteristics of three SNPs at beta casein gene loci assayed by iPlex mass ARRAY.

Variable	SNP ID		
	CSN2_67	*CSN2_106*	*CSN2_122*
No of Allele	2	1	2
Call rate	100	100	100
No of cows	30	30	30
Observed Common	GG =0.733 (22)	GG=1.0 (30)	GG=0.967 (29)
Observed Heterozygotes	GT= 0.200 (6)	GT=0	CG=0.033 (1)
Observed Rare	TT=0.067 (2)	TT=0	CC=0
Allele Frequency (p)	G=0.83 (G)	G=1	G=0.98
Allele Frequency (q)	G=0.17 (T)	T=0	C=0.02
Expected Common	GG=0.694 (21)	GG=1.0 (30)	GG=0.967 (29)
Expected Heterozygotes	GT=0.278 (8)	GT=0	CG=0.033 (1)
Expected Rare	TT=0.028 (1)	TT=0	CC=0.00
HWEp	0.13 NS	1.00 NS	0.93 NS

SNP = Single nucleotide polymorphism; HWEp=Hardy-Weinberg equilibrium probability; NS= non-significant.

3.3. Allele and genotype frequencies of beta casein gene in 30 Friesian X Bunaji cows

Table3, indicates the results of genotype and allele frequencies, and Chi-square ($\square^2$) test for deviation from HWE for the most common genetic variants at the *CSN2/β-CN* gene locus (A^1, A^2, B) in 30 Friesian X Bunaji cows.

The 30Friesian X Bunajicows under this study were polymorphic and had three alleles A^1,A^2 and B at the beta-casein gene locus. The frequencies of the alleles A^2, A^1 and B were 83.3, 15.0 and 1.7 percent respectively; allele A^2 occurred most frequent followed by A^1 and B was the least common. These results agree with previous studies in Punganur and Ongole cattle (Srinivas *et al*., 2019), Czech Fleckvieh (Číteke*t al*., 2019), Holstein (Miluchová *et al*., 2014; Soyudal*et al*., 2018), Norwegian Red cattle (Ketto *et al*., 2017), Mexican Jersey (Zepeda-Batista *et al*., 2015), Sahiwal (Mir *et al*., 2014) and Slovak Spotted (Miluchová *et al*., 2014). This is attributed to the fact that variant A^2 is the original variant at beta casein gene locus and A^1 is a mutation that came up later in the European breed of cattle; in addition, most breeding programs have been favouring the cattle that are carrying variant A^2because it is free from deleterious peptide of beta-casomorphin-7 opioid and thus has low risk of infection if consumed (Dinc*et al*., 2013; Malarmathi *et al*., 2014). The crossing between Friesian (*B. taurus*) and Bunaji (*B. indicus*) had transferred the A^1 variant from *B. taurus* to *B. indicus* thereby integrated the exotic germplasm in Bunaji cattle. On the other hand, the results of current study disagree with the findings of previous researchers who found that the most common CSN2 genetic variant was A^1 in Holstein cattle (Vougiouklaki*et al.*, 2020), Hardhenucrossbred (Ramkaran *et al*., 2017) and this confirms the origin of variant CSN2 A^1in the European cattle breed which spread to other cattle through crossbreeding. The results of the current study suggest the superiority of variant A^2 at the beta casein locus in Friesian X Bunaji cows.

Furthermore, four genotypes homozygote A^2A^2 (22 cows), heterozygote A^1A^2 (5 cows), homozygote A^1A^1(2 cows) and heterozygote A^2B (1 cow) were detected and their frequencies were 73.3, 16.7, 6.7 and 3.3 percent respectively. The most frequent genotype was A^2A^2 (73.3 percent) followed by A^1A^2, while the least common was A^2B (3.3 percent) in the 30 Friesian X Bunaji cows. These results are constituent with previous findings inOngole and Punganur cattle (Srinivas *et al*., 2019) Czech dairy cattle (Číteke*t al*., 2019), Mexican Jersey cattle (Zepeda-Batista *et al*., 2015), Sahiwal cattle(Mir *et al*., 2014), and Slovak Spotted cattle (Miluchová *et al*., 2014). High occurrence of beta casein genotype A^2A^2 is attributed to the fact that most dairy cattle selection programs have been in favoured of cattle carrying the beta casein variant A^2 against those carrying variant A^1which produces the peptide BCM-7 opioid; on consumption of variant A^1 may result in diseases like type 1 diabetes (DM-1), coronary heart disease (CHD), gastrointestinal discomforts, neurological disorders, sudden infant death syndrome (SIDS), and autism (Laugesen and Elliott, 2003; Pal *et al*., 2015). On the other hand, these results do not agree with the findings of previous researchers who found that cows carrying beta casein genotype A^1A^2indicated the highest occurrences inHolstein (Soyudal*et al*., 2018; Vougiouklaki*et al.*, 2020) Holstein Friesian crosses (Srinivas *et al*., 2019) and Hardhenu crossbred (Ramkaran *et al*., 2017).

In the present study, a relatively low frequency of 15.0 percent recorded for CSN2 A^1 allele indicated that the milk from Friesian X Bunaji cows might consist of a relatively low percent of the deleterious peptide of beta-casomorphin-7 and thus has low risk of infection if consumed. The result of chi-square test ($\chi 2$) was non-significant (2.17) and indicated that the Friesian X Bunaji cows were in HW equilibrium at the *CSN2* gene locus.

Table 3. Genotype and allele frequencies of most common genetic variants at the beta casein gene loci in Friesian X Bunaji cows.

Gene	Genotype	No of cows	Genotype frequency	Allele	Allele frequency	Test HWE $\square^2$
Beta Casein /CSN2	A^2A^2	22	73.3	A^2	83.3	2.17 ns
	A^2A^1	5	16.7	A^1	15.0	
	A^1A^1	2	6.7	B	1.7	
	A^2B	1	3.3			

CSN2 = beta-casein; $\square^2$= chi square test; $\square^2$ = Chi square test; HWE=Hardy-Weinberg equilibrium; ns= non-significant chi square

3.4. Summary statistics for the random and fixed effects used in Linear Mixed Model 1

Table 4, shows the means and variance component estimates for the milk yield, milk pH and milk composition traits and significant of fixed effect in Linear Mixed Model 1.

The results of this research indicated that the estimates of variance within the cow were higher than the variance between the cows (within the beta casein genotypes) for daily milk yield and contents of salts; whereas, contents of fat, protein, lactose, solid-not fat, and total solid indicated lower variance estimates within the cow than between the cows (within the beta casein genotypes). Besides that, the content of milk total solid had zero variance estimate for the cow.

The means recorded in this study were daily milk yield (7.52±2.49kg/cow/day), contents of fat (4.48±1.43percent), protein (3.23 ±0.23percent), lactose (4.86±0.34percent), solid-not fat (8.78 ±0.78percent), total solid (13.23±1.58percent) and salts (0.73±0.05percent) in milk and the milk pH (6.67±0.05). These values

are in line with the report of Alphonsus *et al*., (2014) who found that in Friesian X Bunaji cows the daily milk yield ranged from 4.04 to 12.00kg/cow/day, contents of milk fat (ranged =3.74 to 4.71 percent; mean =4.30 ± 0.10 percent), protein (ranged= 3.89 to 4.45 percent; mean=4.16 ± 0.07 percent) and lactose (ranged=3.88 to 4.66 percent; mean=4.29 ± 0.06 percent). Besides that, the values recorded for milk composition traits were within the recommended standard requirement for cow's milk components: for contents of fat (range 3.25 to 5.0 percent), solid-not fat (range 8.25 to 9.5 percent), total solid (range 12.5 to 14.5 percent), pH (range 6.5 – 6.7) (Anantakrishnan *et al*., 1993; FDA, 2010). The pH ranged of 6.5 – 6.7 reported in this study for cow milk indicated that the cowswere free from bacterial contamination or mastitis (Anantakrishnan *et al*., 1993).

Table 4. Means and variance component estimates for the milk yield, milk pH and milk composition traits and significant of fixed effect (Model 1).

Variable	N		σ^2 estimates		P-value
		Mean	Cow	Residual	β-CN
ADMY (kg)	29	7.52±2.49	1.416	0.677	(0.01) **
Fat (%)	29	4.48±1.43	0.185	0.348	0.01) **
Protein (%)	29	3.23 ±0.23	0.004	0.057	(0.35) ns
Lactose (%)	29	4.86±0.34	0.135	2.438	(0.37) ns
Solid-not fat (%)	29	8.78 ±0.78	0.267	0.400	(0.20) ns
Total solid (%)	29	13.23±1.58	0.000	1.926	(0.40) ns
Salts (%)	29	0.73±0.05	1.460	0.003	(0.40) ns
M-pH	29	6.67±0.05	0.007	0.009	(0.17) ns

M-pH= pH of milk; β-CN= beta-casein; **= Fixed effect is significant at $P \leq 0.01$; ns= Fixed effect is not significant at $P > 0.05$.

3.3. Effects of Beta-Casein (*β-CN /CSN2*) genotypes on milk yield, pH, and composition traits.

Table 5, represents the least square means and standard deviation of contents of daily milk yield, milk pH, contents of milk fat, protein, lactose, solid-not fat, total solid, and salts for beta-casein genotypes (A^1A^1, A^1A^2 and A^2A^2) in Friesian X Bunaji cows.

The findings of the current study, showed that the β-CN genotypes (A^1A^1, A^1A^2, and A^2A^2) had significant (P<.01) effects on daily milk yield, and content of milk fat but there were non-significant effects on milk pH, contents of protein, lactose, solid-not fat, total solid and salts in milk. The cows carrying homozygote A^2A^2 genotype produced the highest amount of milk per day (8.91±0.42kg/cow/day); the cows carrying heterozygote A^1A^2 (6.76±0.69kg/cow/day) had the least daily yield although not different from A^1A^1; the ranking for daily milk yield was A^2A^2>A^1A^1=A^1A^2. Furthermore, the cows carrying A^1A^2 genotype produced milk with the highest fat content (5.10±0.40 percent); the cows carrying genotype A^2A^2 produced milk with the least fat content; ranking recorded for content of fat was A^1A^2=A^1A^1>A^2A^2. These results support previous finding in Slovak Spotted, Pinzgau, and Holstein cattle (Miluchova*et al*., 2014) and Holstein-Friesian Cows (Bugeac*et al*., 2015); they also found that the β-CN genotypes (A^1A^1, A^1A^2, and A^2A^2) had significant (P<.05) effects on daily milk yield, and content of milk fat. On the contrary, these results disagree with previous studies in Holstein (Ardicli*et al*., 2018), Czech Simmental and Holstein cows(Čítek*et al*., 2020); they found that the β-CN genotypes (A^1A^1, A^1A^2, and A^2A^2) had non-significant influence milk yield, and content of milk fat. The current results agree with previous study who found that β-CN genotypes (A^1A^1, A^1A^2, and A^2A^2) had non-significant (P<.05) effects on contents of milk protein, lactose and total solid (Ardicli*et al*., 2018; Nguyen *et al.*, 2018; Čítek*et al*.., 2020). On the contrast, the present results disagree with previous reports that found significant effects of that β-CN genotypes (A^1A^1, A^1A^2, and A^2A^2) on contents of protein, solid-non-fat, and total solids (Day et al., 2015; Albarella *et al*., 2020) and lactose in milk (Bugeac*et al*., 2015)

Table 5. Effects of beta casein genotypes on milk yield, pH, and composition traits in Friesian X Bunaji cows

	β-CN (Beta Casein)			
Variable (%)	A^1A^1	A^2A^1	A^2A^2	P-value
No of cows	2	5	22	
ADMY	7.15±1.06^{ab}	6.76±0.69^{b}	8.91±0.42^{a}	(0.01) **
Milk pH	6.63±0.07	6.74±0.05	6.63±0.03	(0.17) ns
Fat	5.03± 0.61^{a}	5.10±0.40^{a}	3.80± 0.25^{b}	(0.01) **
Protein	3.23±0.19	3.11±0.13	3.32±0.08	(0.35) ns
Lactose	4.86±0.29	4.69±0.19	4.99±0.12	(0.37) ns
Solid-not fat	8.66±0.64	8.66±0.64	8.85±0.26	(0.20) ns
Total solid	14.06±1.09	13.53±0.70	12.84±0.45	(0.49) ns
Salts	0.72±0.04	0.70±0.03	0.75±0.02	(0.40) ns

N= Number of cows; β-CN= beta-casein; **= β-CNgenotypes are significant at $P \leq 0.01$; ns=β-CNgenotypes are not significant at $P > 0.05$; ab= Means with different superscript across roll differ significantly.

IV. Conclusions

Recently, the beta-casein polymorphism has attracted interest among researchers, milk producers and consumers because of the possible association between certainbeta casein genetic variants and milk quantitative and qualitative traits; human nutrition and health; technological properties of milk.

It might be concluded that the Friesian X Bunaji cowsproduce milk that meet the recommended standard for contents of milk fat, protein, solid-not fat, total solid and milk pH for cow's milk. The cows milk show superiority of variant A^2 (83.3) at the beta casein gene locus which prevents the release of bioactive peptide called beta-casomorphin-7 (BCM-7) opioid during human gastric and gut enzymatic digestion, therefore, the milk produced is safe for human consumption.The cows carrying the beta casein genotype A^2A^2 produce the highest amount of milk per day per cow (8.91±0.42 kg/cow/day $P<0.01$)) and milk with the least fat content (3.80± 0.25 percent $P<0.01$) that is within the recommended standard for production of dairy products like yoghurt and cheese. Including the animals carrying the beta casein genotype A^2A^2in the selection schemes in a long term may contribute toincrease milk production, meet the demand of domestic milk,andreduce the Country's dependent of imported milk and dairy products.

Considering the association of beta casein variants with gelation properties and most of the milk producedin Nigeria is used for production of fermented dairy products like yoghurt; it is recommended that the effects of beta casein genotypes on yoghurt parameters be investigated.

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