



DNA extraction procedures and validation parameters of a real-time PCR method to control milk containing only A2 β -casein

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ABSTRACT

Bovine milk mainly contains two types of β -casein: A1 and A2 variants. In recent years, a new variety of cows' milk has emerged in the dairy sector called "A2 milk". This novel product is characterised by the absence of A1 β -casein, which has been associated with possible gastrointestinal discomfort due to β -casomorphin-7 (BCM-7) release during gastrointestinal digestion. In this context, methods to verify the A1 allele absence in A2 milk are required as a quality control in the A2 milk commercialisation. Therefore, the aim of the present study was to develop a locked nucleic acid (LNA) probe-based duplex real-time PCR (qPCR) assay for A1 allele detection in A2 milk samples. Firstly, four DNA isolation methods from milk somatic cells were optimised and evaluated. The results suggest that the commercial kit *NucleoSpin Tissue* was the most suitable method in terms of DNA quality and amplifiability for downstream applications. Then, optimisation and validation of the qPCR assay were carried out. For both A1 and A2 alleles, the absolute limits of detection of this qPCR assay were 7.3 DNA copies/reaction (2×10^{-5} ng DNA) and 30.4 DNA copies/reaction (0.1 ng DNA) at a 95% confidence level with synthetic reference DNA samples and heterozygous genotyped DNA sample, respectively. The relative limits of detection were 2% (15 copies) and 5% (152 copies) for the A1 allele in A2 samples at 95% confidence with synthetic reference and genotyped DNA samples, respectively. The qPCR assay was robust, with intra- and inter-assay variability below 4.3%, and specific, differentiating between A1 and A2 alleles with 100% genotyping accuracy. In conclusion, this cost-effective and fast method could be used to discriminate A1 allele in A2 samples and, consequently, to verify the A1 allele absence in "A2 milk" by screening commercial products on the market.

1. Introduction

Bovine milk and other dairy products are essential food resources for a large number of people. Milk contains a great source of energy, proteins, and micronutrients, but it is not easily digested by some people (Bell et al., 2006). Some diseases and allergies imply the exclusion of milk from the diet and its replacement by vegetable beverages. These milk substitutes are manufactured foods that need the addition of several additives to reach a nutritional composition similar to that of cow's milk (Silva et al., 2020).

A intolerance of milk consumption has been related to β -casein protein fraction that represents 30% of the total protein contained in bovine milk (Brooke-Taylor et al., 2017). β -casein presents two main variants, A1 and A2, differed by a single-nucleotide polymorphism (SNP) at position 67 in the gene coding for β -casein: from CCT on the A1 allele to CAT on the A2 allele. The mutation resulted in an amino acid change: proline on the A2 β -casein was replaced by histidine on the A1

β -casein (Bell et al., 2006). It has been reported that the A1 and A2 β -caseins are digested differently in the gut because of a structural dissimilarity (Asledottir et al., 2018). During digestion, the A1 β -casein releases the bioactive peptide β -casomorphin-7 (BCM-7) whilst the A2 β -casein does not. BCM-7 can influence the nervous, endocrine, and immune system by activating gastrointestinal tract μ -opioid receptors (de Gaudry et al., 2019). Thus, BCM-7 is thought to be responsible for potential adverse outcomes associated with A1 β -casein consumption, and it has been highly discussed in the literature (Brooke-Taylor et al., 2017). Despite the uncertainty still exist, A2 milk, produced by homozygous A2A2 cows and thus only containing A2 β -casein, has emerged as an alternative product in the dairy sector (Alfonso et al., 2019; Benti-voglio et al., 2020; Oliveira Mendes et al., 2019).

A2 milk could be an opportunity for some consumers to recover the consumption of a natural food, which provides a protein of high biological value, instead of consuming manufactured and additive fortified vegetable beverages. However, A2 milk authentication is essential to

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verify the absence of A1 β -casein and, consequently, decrease consumer confidence decline and possible frauds in the commercialisation. For this purpose, the development and standardisation of analytical methods are required.

A2 milk authentication using molecular methods such as chromatographic (Oglobline et al., 2022) and isoelectric focusing techniques have been recently developed (Mayer et al., 2021). Alternatively, DNA-based methods for β -casein A1 and A2 allele detection can be employed based on the analysis of DNA of milk somatic cells in cows (Giglioti et al., 2020, 2021; Mayer et al., 2021). Moreover, to increase the specificity of the qPCR assays, locked nucleic acid (LNA) probes can be used (Giglioti et al., 2021, 2022; Puente-Lelievre & Eischeid, 2021). LNA probes improve stability and mismatch discrimination thankful for the formation of a methylene bridge between the 2' oxygen and the 4' carbon of the pentose ring that results in higher affinity for the complementary DNA region (You et al., 2006). In this context, the objective of the present study was to develop a duplex real-time PCR assay with LNA probes for A1 allele detection in A2 milk samples to corroborate its usefulness in A2 milk authentication.

2. Materials and methods

2.1. DNA isolation

The isolation of highly purified DNA from milk is one of the essential steps in an authentication process by PCR-based method. For this purpose, DNA samples from somatic cells of fresh commercial milk were isolated according to four different procedures. The number of bulk milk samples needed for a representative statistical analysis was calculated. The variable used for the sample size calculation was DNA purity assessed by the ratio of absorbance at 260 nm and 280 nm ($A_{260/280}$) using a spectrophotometer (Thermo Scientific, Madrid, Spain) (Nejati, Junne, Kurreck, & Neubauer, 2020). For the analysis, *pwr.t.test* tool of R statistical package was used (difference to be detected: 0.2, level of significance: 5%, power: 70%, two tailed t-student test), using a 0.2 value for standard deviation (SD) of $A_{260/280}$ ratio according to the reviewed literature (Pokorska et al., 2016; Usman et al., 2014; Wassermann, 2020). Under these conditions, 14 milk samples were required for the analysis.

Before DNA isolation, a milk sample pre-treatment was required to remove fat and proteins that could affect DNA isolation (Usman et al., 2014). The pre-treatment step was common to all the four methods. Fresh commercial bulk milk samples were pre-treated based on the method described by Yap et al. (2020). Briefly, 2×10 ml milk samples were centrifuged at 4500 g for 20 min at 4°C, the fat was removed by a sterile spatula and the supernatant was discarded. The two somatic cell pellets were pooled and resuspended in 800 μ l phosphate-buffered saline (PBS, pH 7.4), before centrifugation at 4500 g for 1 min at room temperature. Subsequently, the supernatant was discarded and the pellet was washed twice in 1 ml of PBS. The somatic cells pellet was then stored at -20°C for 20 min and unfreeze pellets were processed according to each method.

With respect to DNA extraction methods, methods 1 and 2 were a direct applications of a commercially available kits, *NucleoSpin Tissue* (Usman et al., 2014; Vougiouklaki et al., 2020) and *NucleoSpin Blood* (Macherey-Nagel, Düren, Germany), both kits are based on the binding of DNA to a silica gel membrane to get high-quality DNA. The main difference between them was the incubation time after adding proteinase K, which is not necessary for *NucleoSpin Blood* and it was 2 h for the *NucleoSpin Tissue* kit. Method 3 was a modification of the *NucleoSpin Blood* kit to increase DNA yield and purity according to Psifidi et al. (2010), in which more time of incubation with the elution buffer was assayed to allow DNA to elute properly from the silica membrane. Finally, method 4 was an in-house protocol developed by Pokorska et al. (2016), which requires a relatively small amounts of milk with reduced cost and time of analysis. The procedures of each the extraction methods

Table 1

Primers and LNA probes and used in the duplex real-time PCR assay.

| Target | Oligonucleotide | Sequence (5'-3') ^a | Amplicon size (bp) |
|--------|-----------------|--|--------------------|
| CSN2 | Forward primer | CAGTCTCTAGTCTATCCCTTCC | 74 |
| | Reverse primer | GTTTGAGTAAGAGGAGGGATGT | |
| | A1 LNA probe | /5HEX/ CC+A+TC+C+A+T+AA+C+AG/ 3IABkFQ/ | |
| | A2 LNA probe | /56-FAM/ CC+A+TC+C+C+T+AA+CA/ 3IABkFQ/ | |

^a Locked nucleic acid (LNA) bases are represented by "+" and the detected polymorphism in bold.

are detailed in Supplementary material.

Additionally, the DNA used in duplex qPCR assay development and validation was isolated from raw milk of individual cows with known genotype for β -casein (A1A1, A1A2, and A2A2) (EuroG MD Microarray), supplied by a commercial farm.

2.2. Selection of primers, locked nucleic acid (LNA) probes and controls for the duplex real-time PCR assay

Primers were designed based on a region of the CSN2 gene coding for bovine β -casein (Bos Taurus β -casein GenBank: X14711.1) containing the A1 polymorphism (GenBank: MK426695.1) and the A2 polymorphism (GenBank: MK426696.1) (Table 1). A 74 bp region of the CSN2 gene was amplified. Oligonucleotides for qPCR were designed using the online PrimerQuest Tool on the IDT website (<https://sg.idtdna.com/PrimerQuest/Home/>).

Additionally, to detect A1 and A2 polymorphisms, specific locked nucleic acid (LNA) probes were used (Puente-Lelievre & Eischeid, 2021). These probes were complementary to the region of the CSN2 gene, which contains A1 and A2 polymorphisms. A1 LNA probe was complementary to A1 allele and was labelled with HEX reporter dye, whilst A2 LNA probe was complementary to the A2 allele and was labelled with FAM reporter dye (Table 1). These probes were designed and synthesised by Integrated DNA Technologies, Inc. (<http://www.idtdna.com/>). To check the correct functioning and design of the primers and LNA probes, an *in-silico* analysis was previously performed using the IDT OligoAnalyzer tool (<https://eu.idtdna.com/pages/tools/oligoanalyzer>).

Two synthetic reference DNA samples (gBlocks Gene Fragments, IDT, Coralville, USA) were produced (CD.GT.QWDC2715.6.1 WT and CD.GT.QWDC2715.6.2 MUT) each containing the DNA region of the CSN2 gene with the specific sequence of either allele (A1 and A2). These synthetic reference DNA samples were used as controls for qPCR assay validation, thus preventing the occurrence of false positives in the results. The sequences of the gBlocks Gene Fragments are shown in S1 Table of Supplementary material.

2.3. Analysis of DNA samples isolated from milk somatic cells

2.3.1. DNA concentration, quality and amplificability

The concentration and quality of DNA isolated from milk somatic cells was determined using a spectrophotometer (Thermo Scientific, Madrid, Spain) (Nejati, Junne, Kurreck, & Neubauer, 2020). Concentration of DNA samples was assessed by measuring the absorbance at 260 nm and the quality of DNA samples was estimated through $A_{260/280}$ ratio (Gallagher & Desjardins, 2006). Ratio values between 1.8 and 2 were considered optimal, whilst lower values indicated the presence of other contaminating compounds such as proteins (Desjardins & Conklin, 2010; Sukumaran, 2010).

The correct amplification of DNA isolated from milk somatic cells was analysed via SYBR green-based qPCR assay in a CFX96 Touch Real-Time PCR Detection System (BioRad, Munich, Germany). A total of 14

Table 2

Mixtures of decreasing concentrations of A1 allele in A2 allele (%) of synthetic reference DNA samples and genotyped DNA samples for standard curves preparation.

| Proportion of A1 in A2 (%) | Reference DNA samples | | Genotyped DNA samples | |
|----------------------------|-----------------------|-----------------------|-----------------------|------------------|
| | A1 allele copy number | A2 allele copy number | A1A1 copy number | A2A2 copy number |
| 100 | 734 | 0 | 3041,41 | 0 |
| 50 | 367 | 367 | 1520,71 | 1520,71 |
| 40 | 293 | 440 | 1216,57 | 1824,85 |
| 30 | 220 | 514 | 912,42 | 2128,99 |
| 20 | 147 | 587 | 608,28 | 2433,13 |
| 10 | 73 | 660 | 304,14 | 2737,27 |
| 5 | 37 | 697 | 152,07 | 2889,34 |
| 2 | 15 | 719 | 60,83 | 2980,59 |
| 1 | 7 | 726 | 30,41 | 3011,00 |
| 0 | 0 | 734 | 0 | 3041,41 |

DNA samples extracted with each DNA extraction method were evaluated. Amplification reaction in triplicate was performed via SYBR green-based qPCR assay in a total volume of 10 µL, comprising 5 µL of TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara Bio, Otsu, Japan), 0.3 µM of each primer (IDT, Coralville, USA), 2 µL of template DNA and 2.4 µL Nuclease-free water (Cytiva, Amersham Place, United Kingdom). Two synthetic DNA gBlocks Gene Fragments each containing the DNA region of the CSN2 gene with the specific sequence of either allele (A1 or A2) were used as positive control for the reaction (Liao & Liu, 2018). Thermal cycling conditions were as follows: initial denaturation step at 95 °C for 30 s, followed by 40 cycles including denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 1 min and finally amplicon melting curve from 65 to 95 °C (with increments of 0.5 °C/5 s). The dissociation curves were examined in the presence of a single product.

2.3.2. Statistical analysis

Results of the different DNA extraction methods (NucleoSpin Tissue, NucleoSpin Blood, Modified NucleoSpin Blood and Pokorska et al. (2016)) were analysed using simple one-way analysis of variance (ANOVA) (SPSS Inc., Chicago, USA), and multiple comparisons between groups were performed using Tukey's HSD test ($\alpha = 0.05$).

2.4. Optimisation of real-time PCR assay

The optimal primer concentration was determined using a SYBR green-based qPCR assay. For this purpose, a total reaction volume of 10 µL was used containing 5 µL of TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara Bio, Otsu, Japan), three different primer concentrations (0.2, 0.3, and 0.4 µM) (IDT, Coralville, USA), and different DNA quantities (30 ng in 2 µL, 45 ng in 3 µL and 60 ng in 4 µL). Thermal cycling conditions were: an initial denaturation step at 95°C for 30 s, followed by 40 cycles including denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min and finally amplicon melting curve.

Subsequently, the optimal LNA probes concentration was assessed with a duplex LNA probe-based qPCR assay for A1 and A2 alleles detection. A total reaction volume of 10 µL was used containing 5 µL of Premix Ex Taq (Probe real-time PCR) 2X (Takara Bio, Otsu, Japan), two different probes concentrations (0.2 µM and 0.15 µM), 0.4 µM of each primer and 2 µL of template DNA (both quantities defined in the previously assay). Amplification conditions were as follows: initial denaturation step at 95°C for 30s, followed by 40 cycles including denaturation at 95°C for 5s and annealing/extension at 60°C for 30s.

The optimised duplex LNA probe-based qPCR assay (10 µL) contained 5 µL of Premix Ex Taq (Probe real-time PCR) 2X (Takara Bio, Otsu, Japan), 0.4 µM forward and reverse primers, 0.2 µM A1 and A2 LNA probes, 2.4 µL Nuclease-free water (Cytiva, Amersham Place, United Kingdom) and 2 µL target DNA. All qPCR reactions were performed in a CFX96 Touch Real-Time PCR Detection System (BioRad, Munich,

Germany).

2.5. Validation of duplex real-time PCR assay for detection of A1 and A2 alleles of the β -casein

To validate LNA probe-based qPCR assay, the amplification efficiency, linear dynamic range, sensitivity, repeatability and specificity were evaluated following the U.S. Food and Drug Administration (2020) guidelines. For this purpose, two synthetic reference DNA containing A1 and A2 alleles of the CSN2 gene were separately 10-fold serially diluted in Nuclease-free water (Cytiva, Amersham Place, United Kingdom) from 7.34×10^6 to 7.34×10^{-2} DNA copies per reaction. Additionally, a A1A2 heterozygous DNA sample from a previously genotyped animal was 2-fold serially diluted in nuclease-free water (Cytiva, Amersham Place, United Kingdom) from 1946 copies to 30.41 copies per reaction. Furthermore, mixtures of decreasing concentrations of A1 in A2 (%) with both synthetic reference DNA and genotyped DNA samples were performed (Table 2). More details about the standard curve creation are placed in Supplementary materials.

2.5.1. Amplification efficiency and linear dynamic range

The amplification efficiency (E) refers to the average number of DNA copies per amplification cycle, which can assume values in the range of 90%–110% (Taylor et al., 2010; U.S. Food and Drug Administration, 2020). The efficiency of the duplex qPCR assay was determined using the four different standard curves previously detailed from the slope according to the formula (Friedman et al., 2014; Maier et al., 2021): $E = [10^{(-1/\text{slope})}] - 1$. Afterward, mean E and coefficients of determination (R^2) values of the 3 runs were obtained. By adjusting the E and R^2 values, the linear dynamic range of each standard curve was determined (Friedman et al., 2014; Maier et al., 2021). The linear dynamic range refers to the minimum and maximum target DNA concentrations which can be reliably detected (Bustin et al., 2009; U.S. Food and Drug Administration, 2020).

2.5.2. Sensitivity: limit of detection (LOD) and limit of quantification (LOQ)

To determine the sensitivity of the duplex real-time PCR assay, the limit of quantification (LOQ) and limit of detection (LOD) of each standard curve were calculated. LOQ is understood as the lowest amount of target DNA that can be reliably quantified at an acceptable level of precision, accuracy and repeatability (Chen et al., 2020; U.S. Food and Drug Administration, 2020). Additionally, the LOQ should be the minimum target DNA concentration included in the linear dynamic range (Chen et al., 2020). According to U.S. Food and Drug Administration (2020) guidelines, the LOQ should give a positive result and has a Cq coefficient of variation (CV) of no more than 0.5 Cq in all 12 DNA sample replicates. Furthermore, LOD is understood as the lowest amount of target DNA at which an amplification product is detected with a probability of at least 0.95 (at a 95% confidence level) (U.S. Food and Drug Administration, 2020). Absolute and relative sensitivities were determined (Chen et al., 2020). To evaluate the absolute LOD and the LOQ of the duplex qPCR assay, standard curves with serial dilutions of synthetic reference DNA samples and heterozygous (A1A2) genotyped DNA sample were used. Alternatively, to evaluate the relative LOD and LOQ of the duplex qPCR assay, standard curves with decreasing mixtures of A1 in A2 (%) with synthetic reference DNA samples and with genotyped DNA samples were used.

2.5.3. Precision: intra-assay and inter-assay repeatability

To determine the precision of the duplex qPCR assay, intra-assay and inter-assay repeatability were calculated. Repeatability refers to the degree of agreement between successive and independent results obtained using the same method with identical test material under the same conditions (apparatus, worker, laboratory and short time intervals) (NordVal International, 2018). In this sense, intra-assay

Table 3

Comparison of four DNA extraction methods using two spectrophotometer measurements (concentration in ng/μl and quality in A_{260/280}) and real-time PCR results (Cq value).

| Ítem | NucleoSpin Tissue | NucleoSpin Blood | Modified NucleoSpin Blood | Pokorska et al. (2016) | P-value |
|-------------------------------|---------------------------|---------------------------|---------------------------|----------------------------|---------|
| Concentration, ng/μl | 15.60 ± 3.75 ^b | 8.96 ± 2.75 ^b | 5.20 ± 2.66 ^b | 220.8 ± 174.2 ^a | <0.001 |
| Quality, A _{260/280} | 1.93 ± 0.13 ^{ab} | 2.05 ± 0.16 ^a | 1.79 ± 0.19 ^b | 1.10 ± 0.11 ^c | <0.001 |
| Cq value | 22.86 ± 0.4 ^b | 22.79 ± 0.68 ^b | 26.22 ± 0.58 ^a | n/a | <0.001 |

Cq = Cycle number of crossing quantity; n/a = no amplification.

Values are means ± standard error.

^a, ^b, ^cMeans with different lowercase superscripts within a row are different ($P < 0.05$).

variability describes the variability of replicates performed in the same experiment and inter-assay variability describes the variability between experiments performed on different days (Bustin et al., 2009; Kralik & Ricchi, 2017). Intra-assay variation (CV values) was calculated considering four replicates in a single run and inter-assay variation (CV values) using three independent runs with four replicates each (Bustin et al., 2009; Kralik & Ricchi, 2017).

2.5.4. Specificity

Analytical specificity evaluates the detection of the target DNA relatively to non-targeted DNA (Bustin et al., 2009; Martins et al., 2019). It can be defined as the absence of interferences and cross-reactions between primers, probes and target DNA (Bustin et al., 2009). The desire number of positive samples in exclusivity tests is zero, i.e., no interference is expected (Johnson et al., 2013). To determine the specificity of the duplex qPCR assay, allelic discrimination graphs with A1A1, A1A2, and A2A2 genotyped DNA samples were constructed. The objective of this assay was to determine whether the A1 and A2 LNA probes were specific enough to detect the A1 allele, A2 allele, or both, depending on the DNA sample type used.

3. Results and discussion

3.1. Selection of the optimal method for DNA isolation from somatic milk cells

The isolation of DNA in molecular analytical methodologies requires steps able to remove several inhibitor compounds of the qPCR reaction, such as fats, proteinases, EDTA, phenol, and high concentrations of Ca²⁺ (Hedman & Rådström, 2013; Liao & Liu, 2018). Milk, in particular, is a challenging food matrix due to its physical and chemical characteristics, especially its fat, protein and calcium constituents that act as PCR inhibitors. Calcium ions in milk compete with Mg²⁺ for the binding site on the polymerase and proteinases (plasmin) in milk can cause the degradation of polymerase, thereby inhibiting PCR (Hedman & Rådström, 2013). Additionally, milk somatic cells, which consist of polymorphonuclear neutrophilic leukocytes, macrophages, lymphocytes, and small amount of mammary epithelial cells, are the source of genomic DNA (Liao & Liu, 2018). Milk usually contains low somatic cell counts, ranging from 2×10^4 to 2×10^5 cells per milliliter milk in healthy quarters of dairy cows, making the DNA extraction from milk relatively difficult (Liao & Liu, 2018; Usman et al., 2014). The results of the four DNA isolation procedures are shown in Table 3.

In reference to the DNA concentration, a significantly higher quantity of DNA was obtained from milk somatic cells with the method of

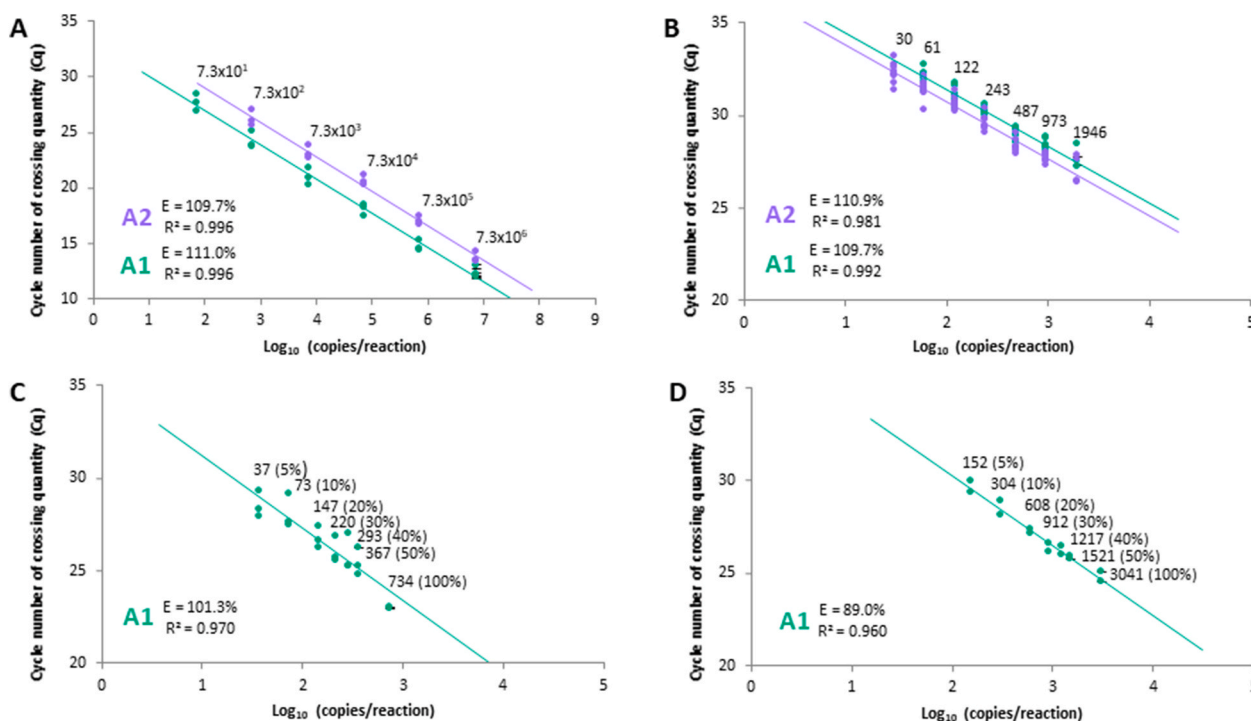


Fig. 1. Duplex real-time PCR standard curves using (A) pure A1 or A2 allele reference DNA samples, (B) pure A1 or A2 heterozygous (A1A2) genotyped DNA samples, (C) decreasing mixtures of A1 in A2 (%) of reference samples (D) decreasing mixtures of A1 in A2 (%) in heterozygous (A1A2) genotyped samples. All replicate of three runs are represented in each case. E = efficiency.

Pokorska et al. (2016) with respect to the other methods ($P < 0.05$). In addition, it is noteworthy that using modified *NucleoSpin Blood* method a very small amount of DNA was obtained (5.2 ng/ μ L).

The ratio $A_{260/280}$ reflects DNA purity. Ratio values between 1.8 and 2 were considered optimal, whilst lower values indicated the presence of other contaminating compounds such as proteins (Desjardins & Conklin, 2010; Sukumaran, 2010). Therefore, *NucleoSpin Tissue* and modified *NucleoSpin Blood* methods extracted the purest DNA, although no significant differences were detected with respect to *NucleoSpin Blood* method ($P > 0.05$). The in-house protocol developed by Pokorska et al. (2016) showed least desirable results with a $A_{260/280}$ ratio of 1.1, and with significant differences with respect to the other methods ($P < 0.05$). Regarding C_q values, lower values are considered more suitable since they are associated with larger amounts of amplifiable DNA (Liao & Liu, 2018). In this sense, both *NucleoSpin Tissue* and *NucleoSpin Blood* methods presented the best results, with lower C_q values ($P < 0.05$). In contrast, DNA samples obtained using Pokorska et al. (2016) method did not amplify correctly by qPCR and, therefore, no C_q values were obtained. This seems to be related to the poor purity of these samples, which may have contained a large number of qPCR inhibitors compounds, which could not be removed by this method (Hedman & Rådström, 2013; Liao & Liu, 2018).

The results indicated that *NucleoSpin Tissue* and *NucleoSpin Blood* methods showed the best results in terms of DNA quality and amplifiability. Nevertheless, although not statistically significant, DNA yield in milk samples isolated with *NucleoSpin Tissue* method (15.6 ng/ μ L) was higher than with *NucleoSpin Blood* method (8.96 ng/ μ L), which can be valuable for downstream applications considering the small amount of DNA isolated from this matrix. Thus, it was determined that the more suitable DNA extraction method for DNA isolation from milk somatic cells was the commercial *NucleoSpin Tissue* kit. These results agree with those obtained by Psifidi et al. (2010) with ovine milk samples, although in this study the quality of the extracted DNA fitted better with the optimal values for $A_{260/280}$ ratio, probably due to the higher fat content of ovine milk compared to bovine milk.

3.2. Real-time PCR validation for detection of A1 and A2 alleles of the β -casein

3.2.1. Amplification efficiency and linear dynamic range

The four standard curves required for the validation of duplex qPCR assay for A1 and A2 allele discrimination of the CSN2 gene are presented in Fig. 1. To obtain correct qPCR assay validation, efficiency values were adjusted in the range of 90%–110% and R^2 values were established at around 0.98 (Taylor et al., 2010; U.S. Food and Drug Administration, 2020).

Efficiency values of the qPCR using the synthetic reference DNA samples revealed an averaged value of 111.0% for the A1 allele through HEX reporter screening ($R^2 = 0.996$) and 109.7% for the A2 allele through FAM reporter detection ($R^2 = 0.996$) (Fig. 1A). With respect to the heterozygous (A1A2) genotyped DNA sample, the efficiency was 109.7% for A1 allele ($R^2 = 0.992$) and 110.9% for A2 allele ($R^2 = 0.981$) (Fig. 1B). In another similar study developed by Manga and Dvořák (2010), a hydrolysis probes system (Taqman) assay was developed using a heterozygous genotyped DNA sample. This assay showed a 96.5% efficiency through YEC reporter screening (for A1 allele) and 94.1% efficiency through FAM reporter detection (A2 allele).

Furthermore, efficiency values with standard curves of mixtures of A1 in A2 (%) were 101.3% using synthetic reference DNA samples ($R^2 = 0.970$) (Figs. 1C) and 89.0% using genotyped DNA samples ($R^2 = 0.960$) (Fig. 1D), both through HEX reporter screening. These results were similar to those obtained in previous studies (Manga & Dvořák, 2010) and all of them were adjusted to the desirable range for qPCR efficiency. Based on the qPCR reaction, the linear dynamic range of the assays was determined. The linear dynamic range of serial dilutions with synthetic reference DNA samples was from 7.3×10^6 to 73 DNA copies/reaction

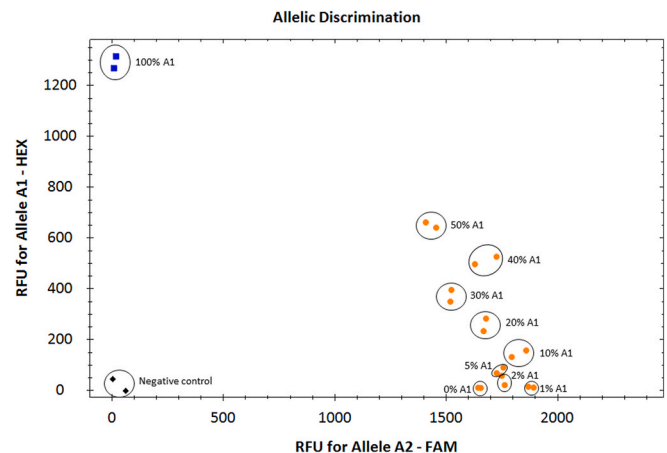


Fig. 2. The analytical sensitivity test for A1 allele identification in A2 DNA samples for duplex LNA probe-based real-time PCR assay using decreasing mixtures of A1 in A2 (%) synthetic reference DNA samples. Synthetic reference DNA samples initially contained a 100% A1 allele (734 copies), and the percentages of A1 allele were progressively decreased: 50% (367 copies), 49% (293 copies), 30% (220 copies), 20% (147 copies), 10% (73 copies), 5% (37 copies), 2% (15 copies), 1% (7 copies). Two replicates of each sample are represented. HEX reporter RFUs were plotted on the ordinate axis, whilst FAM reporter RFUs were plotted on the abscissa axis. RFU = Relative Fluorescence. The blue squares represent synthetic reference DNA containing A1 allele of the CSN2 gene with 7.34×10^2 DNA copies/reaction (positive control), the black rhombus represent samples with no DNA (negative control) and the yellow dots represent DNA mixtures of decreasing concentrations of A1 in A2 (%).

for A1 allele through HEX reporter screening, and from 7.3×10^6 to 730 DNA copies/reaction for the A2 allele through FAM reporter detection. In the case of heterozygous genotyped DNA samples, the linear dynamic range was from 1946 copies to 30.4 DNA copies for both A1 and A2 allele. Mixtures of A1 in A2 (%) using both reference DNA and genotyped DNA samples showed a linear dynamic range from 100% to 5% for the A1 allele, through HEX detection dye.

3.2.2. Sensitivity: limit of detection (LOD) and limit of quantification (LOQ)

The accuracy and precision of LOD and LOQ values establishment increase with replication. Nevertheless, there is no a definite level of replicates and, recommendations vary between different studies (Klymus et al., 2020; Kralik & Ricchi, 2017). In this work, following the FDA guidelines, 12 replicates for each sample were used. Furthermore, absolute and relative sensitivities were determined as described in Chen et al. (2020).

The absolute LOD value of serial dilutions of synthetic reference DNA samples was 7.3 DNA copies/reaction (2×10^{-5} ng DNA) for both A1 and A2 alleles at a 95% confidence level. Additionally, absolute LOD value of serial dilutions of heterozygous genotyped DNA sample was 30.4 DNA copies/reaction (0.1 ng DNA) for both the A1 and A2 alleles. Manga and Dvořák (2010) using a heterozygous genotyped DNA sample, reached up to 3 ng DNA with ACSF-PCR assay and 0.03 ng DNA with Taqman-assay. In this sense, Taqman assay showed a 10-fold lower LOD than the 0.1 ng DNA obtained in this study. However, it is worth mentioning that 30.41 DNA copies/reaction (0.1 ng DNA) was the last serial dilution made in this duplex LNA probe qPCR assay and probably a lower absolute LOD value could have been obtained if more serial dilutions of the heterozygous DNA sample, would made. Absolute LOQ values were equal to the minimum target DNA concentration included in the linear dynamic range (Chen et al., 2020). For synthetic reference DNA samples, the absolute LOQ was 73 DNA copies/reaction for A1 and 730 DNA copies/reaction for A2 allele. The LOQ for heterozygous genotyped DNA samples was 30.4 DNA copies/reaction for both the A1 and A2 allele.

Relative LOD using synthetic reference DNA samples was 2% (15

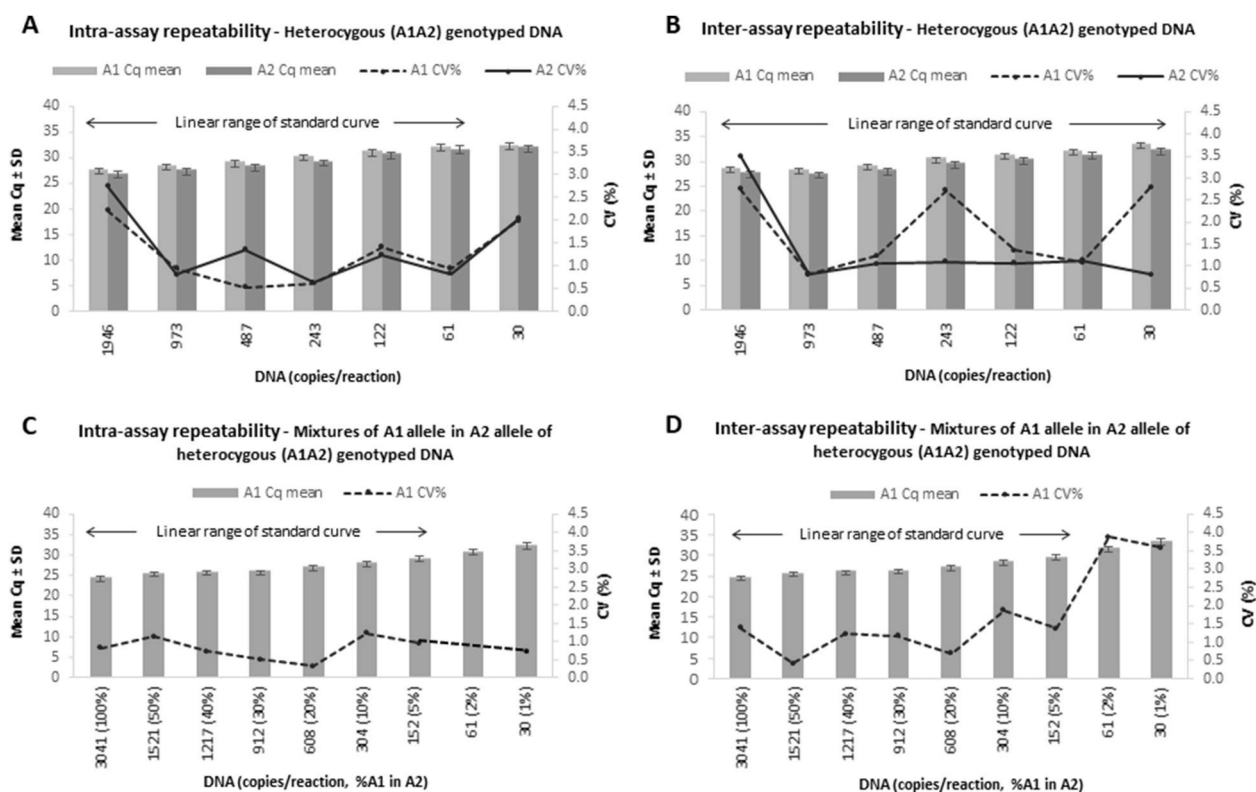


Fig. 3. Intra- and inter-assay repeatability of duplex real-time PCR assay for A1 and A2 allele discrimination of the CSN2 gene using pure heterozygous (A1A2) and decreasing mixtures of A1 in A2 allele (%) of heterozygous (A1A2) genotyped DNA samples. Cq = Cycle number of crossing quantity; CV = coefficient of variation; SD = standard deviation. The probes for A1 allele and A2 detection were labeled with HEX and FAM reporter dyes, respectively. The dilutions within the linear range of the standard curve are represented with the arrows. In C, for 61 (2%) copies/reaction, no CV (%) value was reported because only one sample amplified.

copies) for A1 allele in A2 samples. The analytical sensitivity test is presented in Fig. 2. The percentages of A1 in A2 allele were discriminated by their distribution along the axis of the A1 (HEX) and A2 (FAM) probes, with a correlation between the HEX probe signal reduction and the percentage of the A1 allele diminution.

In a previous study developed for detecting A1 and A2 allele, Gigliotti et al. (2020) reached up to 5% (50 copies) of the A1 allele with post-PCR high-resolution melting analysis (HRM) and 2% (10 copies) with the rhAmp method (uses RNase H2 to activate primers after they have bound to their target sites, reducing primer-dimer formation and improving reaction specificity). Thus, this qPCR assay shows increased sensitivity results respect to HRM analysis and similar with respect to rhAmp analysis. In a recently published paper, Gigliotti et al. (2021) also reported similar analytical sensitivity for LNA-probe and rhAmp methods but highlight the advantages of LNA-probe in terms of economic cost and primers or probe availability. With genotyped DNA samples, relative LOD was 5% (152 copies) for the A1 allele in the A2 allele at a 95% confidence level through HEX reporter screening. Here, a relative LOD of 10% (100 DNA copies) of A1 allele with HRM analysis and 2% (10 DNA copies) of A1 allele with rhAmp method was reported by Gigliotti et al. (2020). Respect to relative LOQ, a 5% A1 allele was detected in both mixtures of synthetic DNA and mixtures of genotyped DNA, through HEX reporter screening.

Being at the point of commercialisation of A2 milk is increasing across the world (de Gaudry et al., 2019), accurate methods to verify the absence of minimal presence of A1 allele in A2 milk are required. In Oglobline et al. (2022), an overview of some quality control guidelines and specification limits to assist in protecting consumers and brands are established. However, there is no agreed definition for A1 free milk, nor regulatory and recommended methods. The duplex LNA probe-based qPCR assay developed in this study detected A1 allele at rates of 2% (15 copies) with synthetic reference DNA samples and 5% (152 copies)

with genotyped DNA samples. The qPCR assay with reference samples is ten times more sensitive than with genotyped DNA samples. This may be due to the fact that synthetic reference DNA samples were directly supplied by the manufacturer with a high degree of purity and quality. In contrast, genotyped DNA samples were obtained through DNA isolation from milk performed by an operator using NucleoSpin Tissue method. In that case, genotyped DNA samples may have lower purity values and, consequently, explaining the less qPCR sensitivity detected in the assays.

Literature studies using qPCR methods for A1 allele detection in A2 milk samples remain scarce. In a similar study developed by Manga and Dvorák (2010), a duplex Taqman probe-based qPCR assay was developed to detect A1 and A2 alleles. A 100% genotyping accuracy and a 100-fold greater degree of sensitivity with this method than with ACSR-PCR method were obtained. In Gigliotti et al. (2020), qPCR assay followed by HRM and rhAmp SNP genotyping assay was described to detect A1 allele in A2 samples and the use of rhAmp it was recommended to verify A1 allele absence due to increased sensitivity. Apart from qPCR methods, in Mayer et al. (2021) milk proteins were directly phenotyped through isoelectric focusing to discriminate A1 and A2 β -casein protein variants. However, most studies were focused on the genotyping of the CSN2 gene to determine the frequency of these genotypes in different cattle breeds (Masoumeh Firouzmandi et al., 2018; Mayer et al., 2021; Miluchová et al., 2014; Rangel et al., 2017; Vougiouklaki et al., 2020). Identification of animal genotypes may not be enough to A2 milk authentication (Gigliotti et al., 2020). In this manner, this study represents an attempt to standardise a duplex LNA probe-based qPCR assay for A1 allele detection in A2 milk samples. The results showed an adequate absolute and relative sensitivity of the qPCR assays, being able to detect a small amount of A1 allele in DNA samples. As was indicated by Oglobline et al. (2022), PCR-based methods, together with ELISA methods, are the most prominent candidates for sensitive, fast and

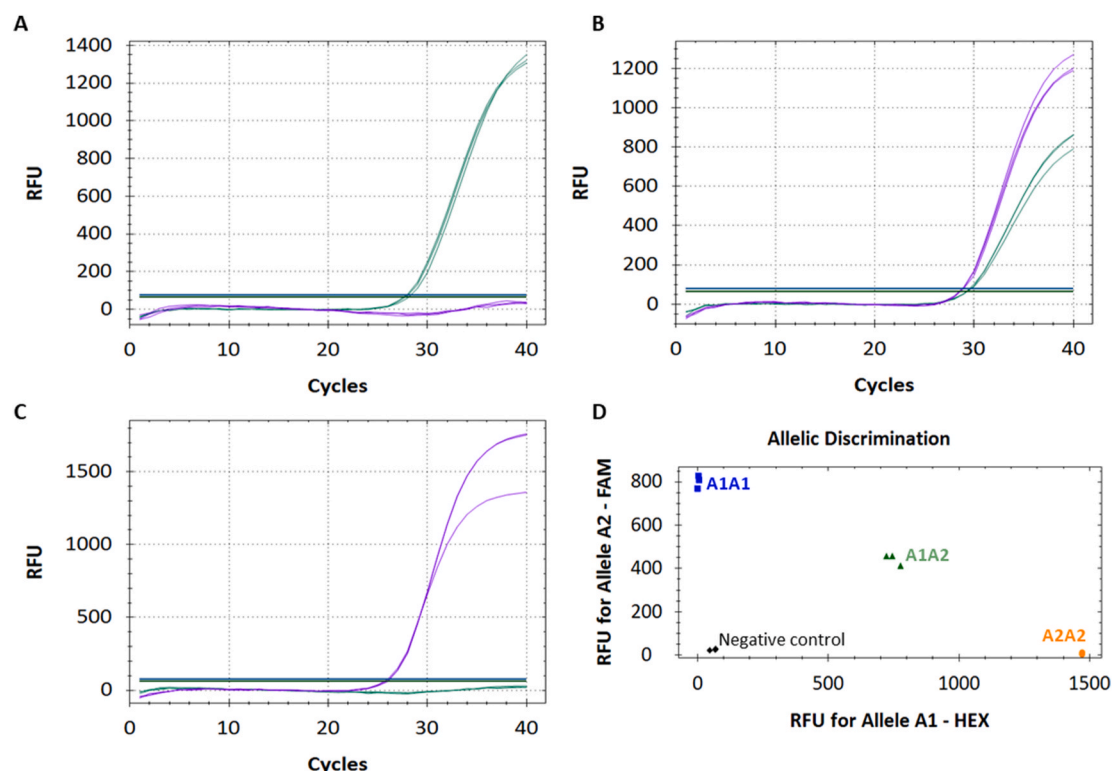


Fig. 4. Specificity test. (A) Amplification curve of the A1A1 genotyped DNA sample (B) Amplification curve of the A1A2 genotyped DNA sample (C) Amplification curve of the A2A2 genotyped DNA sample (D), Allelic discrimination graph of A1A1, A1A2, and A2A2 genotyped cow samples. No specific amplifications were not observed. In A, B, and C: green signal is obtained by HEX reporter screening (A1 probe) and purple signal is obtained by FAM reporter detection (A2 probe). In D: the blue squares represent the A1A1 genotype, the green triangles represent the A1A2 genotype, and the yellow dots represent the A2A2 genotype and black dots represent negative control (NTC). Data were plotted using RFU of the HEX and FAM reporter dye on the x, y axis of A1 and A2 allele. RFU = Relative Fluorescence Units.

cost-effective screening methods for testing A1-free dairy products. The sensitivity of DNA-based methods doesn't correspond exactly to the sensitivity of A1 β -casein detection, because of the variability in somatic cell count between animals and its relationship with milk yield. Nevertheless, PCR is postulated as an indirect milk contamination measurement, and is a highly sensitive approach for determining the purity of A1-free milk.

3.2.3. Precision: intra-assay and inter-assay repeatability

The qPCR assay repeatability using serial dilutions of the heterozygous (A1A2) genotyped DNA sample showed a mean intra-assay variability of 1.11% (run 1), 2.49% (run 2) and 1.30% (run 3) for the A1 probe and 1.38% (run 1), 2.41% (run 2) and 1.41% (run 3) for the A2 probe (Fig. 3). The CV of the qPCR mean inter-assay variability was 1.66% for the A1 probe and 1.35% for the A2 probe. The qPCR assay repeatability using decreasing mixtures of A1 in A2 (%) with genotyped DNA samples showed a mean intra-assay variability of 0.81% (run 1), 0.88% (run 2) and 1.47 (run 3) for the A1 probe. The CV of the qPCR mean inter-assay variability was 1.16% for the A1 probe.

The LNA probe-based qPCR assay repeatability using serial dilutions of synthetic reference DNA samples showed a mean intra-assay variability of 0.80% (run 1), 2.21% (run 2) and 1.19% (run 3) for A1 probe (labelled with HEX dye) and 0.83% (run 1), 1.72% (run 2) and 2.13% (run 3) for the A2 probe (labelled with FAM dye) (see S1 Figure of Supplementary material). Mean inter-assay variability was 3.38% for A1 probe and 2.76% for the A2 probe. Furthermore, the qPCR assay repeatability using decreasing mixtures of A1 in A2 (%) with synthetic reference DNA samples showed a mean intra-assay variability of 1.26% (run 1), 1.57% (run 2) and 0.64% (run 3) for the A1 probe. Mean inter-assay variability was 2.88% for the A1 probe. It is remarkable that lower Cq variability values associated to the A2 probe, mainly inter-assay

variability, were observed with both synthetic and genotyped DNA samples. This may be because the A2 LNA probe showed an increased capacity of binding to the A2 allele, which results in a higher specificity than A1 probe.

These observed repeatability results agree with those obtained in other qPCR validation protocols. Martins et al. (2019) revealed maximum CV values of 1.39% and 3.67% for intra- and inter-assay repeatability, respectively. Additionally, Zheng et al. (2019) repeatability results showed lower variation with maximum CV values of 0.45% and 1.31% for intra- and inter-assay repeatability, respectively. In contrast, in Friedman et al. (2014) repeatability results were inadequate with CV values reaching 27%.

As previously mentioned, although the relative LOD was lower with synthetic reference DNA samples (2%) compared to genotyped DNA samples extracted in the laboratory (5%), probably due to the quality and quantity of the DNA, the intra- and inter-assay variability between these two types of samples were similar. Therefore, results of the qPCR assay developed in this work indicated adequate intra-assay and inter-assay repeatability with values under 25% of CV over the whole dynamic range (U.S. Food and Drug Administration, 2020). In conclusion, these data indicate consistent standard curves, which are particularly important to accurately determine the absence of A1 allele in A2 milk samples.

3.2.4. Specificity

In this study, DNA samples isolated from raw milk of individual cows with known genotype for β -casein (A1A1, A1A2, and A2A2) were used to determine qPCR assay specificity. Analytical specificity results were in accordance with those obtained in the previous genotyping assays (Gigliotti et al., 2020; Manga & Dvořák, 2010). The duplex qPCR assay could distinguish between A1A1 and A2A2 homozygous genotyped DNA

samples and A1A2 heterozygous genotyped sample (Fig. 4). It can be observed that with the A1A1 sample, only the green signal of the A1 probe (HEX reporter dye) was obtained (Fig. 4A), with the A2A2 sample only the blue signal of A2 probe (FAM reporter dye Fig. 4B) was detected, and the A1A2 heterozygous sample generated signal of both probes.

The adequate selectivity of the developed qPCR assay can also be shown in the allelic discrimination graph of A1A1, A1A2, and A2A2 genotyped cow samples (Fig. 4D). A1A1 homozygous sample (blue) was at the top, as it was detected only by the A1 probe (HEX reporter screening), generating 250 RFU on the ordinate axis. Additionally, A1A2 heterozygous sample (green) generated signal of both probes, whereas A2A2 homozygous sample (yellow), it was only detected by the A2 probe (FAM reporter detection), generating 700 RFU on the abscissa axis. Also, a negative control (black) with no DNA template, which did not generate signal of none probe, was used to validate the assay.

Therefore, A1 and A2 allele discrimination using the LNA probes (Puente-Lelievre & Eischeid, 2021; You et al., 2006) was possible because each probe was highly specific and complementary to each allele. This method can be used instead of DNA sequencing and other PCR-based techniques not only for A2 milk authentication but also for the control of animals producing for certified A2 cow milk.

4. Conclusions

Firstly, this study has provided evidence that the isolation of DNA samples with high purity, yield and integrity is an essential and previous step required during the optimisation and validation PCR-based protocols. The extraction of DNA from milk somatic cells is challenging not only due to the presence of PCR inhibitors such as fat, protein and calcium, but also because of the small number of somatic cells in milk from healthy herds. Thus, it is difficult to obtain a high efficiency in DNA yield. The commercial *NucleoSpin Tissue* kit was determined to be the most suitable DNA isolation method from milk somatic cells in terms of quality and amplifiability of the DNA. Then, a duplex real-time PCR using highly selective LNA probes for the A1 allele detection in A2 milk samples was developed and validated. The relative limits of detection for the A1 allele in the A2 allele were 2% (15 copies) with synthetic reference DNA samples and 5% (152 copies) with genotyped DNA samples at 95% confidence. The results also indicated that the qPCR assay was robust, with intra- and inter-assay variabilities below 4.3%. This cost-effective and fast method could be an important tool to discriminate A1 allele in A2 samples and, consequently, to verify the A1 allele absence in “A2 milk” by screening a commercial products on the market. Moreover, it could be used in the authentication of individual cows, to reliably discriminate among A1A1, A2A2, and A1A2 genotypes of the CSN2 gene. A high binding capacity has achieved thanks to LNA probe employment, thus, LNA probe-based duplex real-time PCR is corroborated as a useful alternative to DNA sequencing techniques and other PCR-based methods for A1/A2 allele discrimination.

CRedit authorship contribution statement

L. Jiménez-Montenegro: Investigation, Formal analysis, Visualization, Writing – original draft. **J.A. Mendizabal:** Conceptualization, Writing – review & editing, Project administration. **L. Alfonso:** Conceptualization, Methodology, Writing – review & editing. **O. Urrutia:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration on competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2022.109259>.

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