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Comparative Analysis of Rapid and Less Invasive Methods for A2A2 Dairy Cattle Genotyping and A2 Milk Purity Detection

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Abstract: In this study, two methods for assessing the purity of A2 fermented milk and β-casein genotypes in dairy cows were examined. The need for rapid and precise methods for herd screening and A2 milk quality control justified this study. Accordingly, an ELISA test was developed to identify β-casein A1 in fermented milk, and a commercial Lateral Flow Immunoassay (LFIA) was evaluated to determine A2A2 genotypes and the purity of A2 milk. The results demonstrated 100% sensitivity and specificity of the ELISA test in identifying β-casein A1. The LFIA test successfully identified A2A2 genotypes and confirmed the purity of A2 milk, with a minimum detectable contamination of 5% for raw milk and 10% for fermented milk. Both tests exhibited 100% sensitivity and specificity, resulting in positive and negative predictive values of 100%. The positive likelihood ratio was infinite, while the negative was zero, indicating a precise and reliable test with no false diagnoses. Compared to traditional genotyping, these methods proved to be more practical and showed potential for large-scale screening. It was concluded that ELISA and LFIA are valuable tools for ensuring the quality and authenticity of A2 milk, meeting the demands of producers and consumers for safe and healthy dairy products.

Keywords: A2A2 dairy cows; genotyping; ELISA; LFIA; comparison of methods; facilitated analysis

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1. Introduction

Millions of people around the world consume milk and its derivatives daily, taking advantage of its nutritional richness as an excellent source of proteins, fats, and micronutrients [1–3]. Milk is composed of two main groups of proteins: caseins and whey proteins [4]. The four types of casein (αs1-casein, αs2-casein, β-casein, and κ-casein) are encoded by the genes CSN1S1, CSN1S2, CSN2, and CSN3, respectively, and located in a cluster within a 250 kb region on chromosome 6 [5]. Among them, β-casein is the main protein found in milk [6]. Different mutations in the bovine CSN2 gene have resulted in 12 genetic variants of β-casein, including A1, A2, A3, B, C, D, E, F, G, H1, H2, and I, with A1 and A2 being the most common variants [5,7,8]. Cows with the A1/A1 homozygous genotype and A1/A2 heterozygous genotype produce A1 milk, while cattle with the A2/A2 genotype produce A2 milk [2,8,9].

The A1 and A2 variants do not differ nutritionally; the distinction between them lies in a single-nucleotide polymorphism (SNP) at the amino acid residue at position 67 of the peptide chain. In A1 milk, this residue is histidine (His67), while in A2 milk it is proline (Pro67) [5,10–13]. It is believed that the A2 variant was initially present in all herds; however, a few thousand years ago, a natural mutation from proline to histidine occurred in European dairy cattle, resulting in the presence of the A1 allele in various breeds as we know them today [4,14,15].

During the gastrointestinal digestion of dietary proteins, enzymatic action can result in the release of biologically active peptides [9,16,17]. During the digestion of A1 bovine milk, the presence of the amino acid histidine allows the proteolytic cleavage of β-casein by human gastrointestinal enzymes, releasing a bioactive peptide known as β-casomorphin-7 (BCM-7) [12,18]. The release of BCM-7 has been reported in A2 bovine milk, although less pronounced than in A1 milk [19]. Studies indicate that the BCM-7 peptide has opioid activity, with affinity for μ-opioid receptors (MORs), found in various regions of the body [14,20].

Several studies have also reported correlations between the consumption of A1 cow's milk and dairy products with various human health conditions, due to the release of the BCM-7 peptide during digestion, including type 1 diabetes, cardiovascular disorders, gastrointestinal problems, inflammatory responses, and autism spectrum disorders [3,14,15,21–30]. Awareness of the adverse effects of BCMs on human health has generated a growing demand for exclusively A2 dairy products, both among companies and dairy consumers [2,10,12]. As a result, producers have started selecting females with the A2/A2 genotype [4,9,31], leading to a steady increase in the global A2 milk market in recent years [3], with estimates that this market will grow exponentially until 2029 [4]. Thus, labeling indicating the presence of β-casein A2 has become common in various dairy products, often associated with nutritional benefits [5].

In addition, recent studies [32], show that animals with the A2A2 β-casein genotype (CSN2) exhibit superior genetic indices, including total performance index (TPI), net merit (NM), and predicted transmitting abilities for protein and productive life (PL). These findings reinforce that selection based on casein genes can optimize the efficiency and longevity of dairy herds, offering significant economic benefits and contributing to the genetic improvement of dairy animals.

In this context, a variety of analytical methods have been employed to study and identify the genetic variants of β-casein [18]. This includes techniques such as liquid chromatography–tandem mass spectrometry (LC-MS/MS) [33], reverse-phase high-performance liquid chromatography (RP-HPLC) [34], electrophoresis and high-resolution accurate mass spectrometry (HRAMS) [35], and mid-infrared spectroscopy and chemometrics [10]. In addition to these, various DNA-based genotyping methods have been described, including allele-specific polymerase chain reaction (AS-PCR) [18,23], allele-specific competitive replication system (ACRS-PCR) [18,36], restriction fragment length polymorphism (RFLP-PCR) [18,34,37], single-strand conformation polymorphism analysis (SSCP-PCR) [7], amplification refractory mutation system (ARMS-PCR) [34], and gene sequencing [38,39].

Each of these methods has strengths in terms of accuracy for identifying β-casein genotypes; however, many require extensive processing times, specialized training, and advanced equipment, presenting cost and complexity challenges for large-scale, rapid applications [6,34]. Our comparative analysis reveals that, while traditional methods like AS-PCR and RFLP-PCR are highly precise, they tend to be slower and more labor-intensive. In contrast, techniques such as SSCP-PCR and mid-infrared spectroscopy with chemometrics provide faster results and require less manual intervention, making them more suitable for high-throughput settings. Nonetheless, choosing the appropriate method depends on balancing the need for precision, speed, and cost-effectiveness based on specific genotyping requirements (e.g., A2A2 genotyping) and operational context (e.g., farm or laboratory settings) to ensure optimal support for genetic selection initiatives.

Genetic sequencing focused on nucleotide mutations is considered the gold standard for identifying A2A2 animals due to its high accuracy [40,41], but it has drawbacks such as cost, complexity, and the need for specific biological samples like blood, buccal swabs, hair bulbs, or ear tissue. Non-invasive methods, such as hair bulbs and buccal swabs, are less invasive but often yield lower DNA quality, increasing error chances. Invasive methods, such as blood and ear tissue, provide higher DNA quality, making them more reliable despite the added collection difficulty and cost. Laboratory genotyping requires

specialized personnel and centralized testing, complicating mass screening. Point-of-care (POC) methodologies enable decentralized mass screening and should be developed for global herd testing and quick A2 milk genetic selection. Ensuring A2 milk purity is crucial for quality control in the dairy industry, necessitating accurate methods to verify milk and dairy products. This growing demand for precise evaluation methods is driven by the increasing focus on A2 milk production and the need for stringent quality control. Therefore, the aim of this study was to evaluate different rapid methods for identifying A2A2 animals, as well as assess various methods for verifying the purity of A2 milk (absence of β-casein A1) in raw milk and fermented milk.

In the present study, an ELISA test was developed for the detection of β-casein A1 in fermented milk, creating an important methodology for the quality control of this type of food. Additionally, this work evaluated the sensitivity and specificity of a commercial Lateral Flow Immunoassay (LFIA) specific for A2 milk detection for the identification of A2A2 animals and the verification of A2 milk purity. The data were compared with other techniques, such as traditional genotyping and other ELISA-based methodologies, and the results were analyzed and discussed. This study aims to benefit the management of A2 milk-producing herds, provide an industrial solution for batch analysis of milk, and offer a reliable alternative for consumers to identify milk and dairy products according to their nutritional preferences.

2. Materials and Methods

2.1. Material

Chemicals and the anti-IgY-HRP secondary antibody were purchased from Sigma-Aldrich® (Cotia, SP, Brazil). The IgY anti-A1 primary antibody, the ONE STEP TMB Linear substrate, and the rapid test (LFIA) A2-MiLK TEST® were provided by Scienco Biotech (Lages, SC, Brazil). The following reagents were used in this study: 0.05 M carbonate–bicarbonate solution (pH 9.6), 0.1 M NaOH, PBS, 1% bovine serum albumin (BSA), IgY anti-A1 primary antibody, 0.05% Tween-20, anti-IgY-HRP secondary antibody, TMB substrate, and sulfuric acid (H_2SO_4).

2.2. Selection of Dairy Cow Samples for Genotyping

Milk samples were collected from dairy cows on three farms, two located in the state of Santa Catarina and one in the state of Minas Gerais, Brazil. Collection was carried out manually and individually by each farmer, without the use of milking machines, to prevent contamination. Clean and dry containers were used, and the milk was obtained by mixing a squirt from each teat of the animal. After collection, the milk was immediately labeled and frozen until subsequent analysis. At one farm, located at geographic coordinates −28.46926833441343 and −49.306805225131185, milk samples were collected from 34 previously genotyped animals of the Jersey breed. The genotyping of these animals was performed by Zoetis (Morumbi, SP, Brazil) using hair samples. At another farm, located at coordinates −28.581369733535674 and −49.18977477310808, milk samples were collected from 28 previously genotyped animals of the Holstein breed. These animals were genotyped by STgenetics (Indaiatuba, SP, Brazil) using ear cartilage samples. At a third farm, located at coordinates −19.708631 and −45.472717, milk samples were collected from 69 animals of the Girolando (1/2 blood) breed. These animals were genotyped by Laboratório Raça (Goiânia, GO, Brazil) using samples of hair, blood, and semen. In total, 131 milk samples were collected from 131 animals. In addition to the milk samples, the owners of the three farms provided the genotyping reports.

2.3. ELISA Test for Identification of β-Casein A1 Dairy Products

Enzyme-Linked Immunosorbent Assay (ELISA) tests were conducted on the collected milk samples to identify A2A2 animals, following the protocol described by Jesus et al. [6]. Additionally, ELISA tests were employed to detect β-casein A1 contamination in A2 milk. For this analysis, milk from A2A2-genotyped animals was mixed with varying proportions

of milk from A1A1-genotyped animals, and the assay adhered to the previously described protocol by Jesus et al. [6]. The absorbance results were then used to generate a calibration curve, enabling the determination of the percentage of β-casein A1 in A2 milk, with the aim of developing a protocol for analyzing A2 milk mixtures.

Furthermore, ELISA assays were conducted to detect β-casein A1 contamination in fermented milk samples, with the objective of developing a methodology for the quality control of A2 fermented milk, ensuring its purity. In this case, commercial A2 fermented milk (Letticoa2 (L20240711:36)) was used, mixed in known proportions with regular fermented milk (Yakult (L25.06.240837C)). The fermented milk products were purchased from a local store in the city of Lages, Santa Catarina, Brazil. The samples were subjected to ELISA analysis according to the following protocol. Polystyrene plates were coated with 96 µL of coating buffer, to which 4 µL of the fermented milk sample, which had been previously diluted in NaOH at a 1:10 ratio, was added. The plates were then incubated overnight at 4 °C to allow for the immobilization of A1 casein in the wells. After immobilization, the plates were blocked for 1 h at 37 °C with 200 µL of blocking buffer containing 1% BSA in PBS to prevent nonspecific binding. Subsequently, the anti-β-casein A1 IgY antibody was diluted in PBS containing 1% BSA and 0.05% Tween-20, at a 1:1500 dilution. This solution was added to the wells and incubated for 45 min at 37 °C, allowing the specific antibody to bind to the captured A1 β-casein. After incubation with the primary antibody, the plates were washed three times with washing buffer (PBS containing 0.05% Tween-20) to remove unbound antibodies. Then, the secondary anti-IgY-HRP antibody was diluted in PBS + 1% BSA + 0.05% Tween-20, at a 1:7500 dilution, and added to the wells. The plates were again incubated for 45 min at 37 °C, protected from light. After incubation with the secondary antibody, the plates were washed five times with washing buffer to remove any unbound secondary antibody. To develop the reaction, 50 µL TMB substrate was added to each well and incubated for 15 min at room temperature. The reaction was then stopped by the addition of 50 µL of H_2SO_4. Finally, the optical density (OD) of each well was measured in an ELISA plate reader at 450 nm. The color intensity developed is directly related to the amount of A1 β-casein present in the fermented milk samples, thus allowing quantification through this sensitive and specific immunological method. A calibration curve with the obtained absorbances was generated.

2.4. LFIA Test for Identification of A2A2 Genotype

To evaluate the A2A2 genotype using a point-of-care method, the A2-MiLK TEST® from Scienco Biotech was employed. The procedure followed the manufacturer's protocol. First, milk was manually collected in a separate, clean flask. Then, ten drops of the collected milk were added to a diluent tube using a plastic pipette provided in the kit. The tube was shaken to ensure homogeneity of the solution. Next, the test cassette was placed on a flat surface, and two drops of the diluted milk solution were pipetted onto the cassette. The solution was allowed to migrate along the device for 20 min at room temperature. The results were interpreted by observing the lines on the cassette: According to the manufacturer, the appearance of one pink line in the test area (T) and another in the control area (C) indicated A2 milk, produced by an A2A2 animal. Conversely, the appearance of only one pink line in the control area (C) indicated that the milk was not A2, produced by animals of genotype A1A1 or A1A2.

2.5. LFIA Test for Purity Evaluation

To verify the purity in milk and fermented milk samples, the A2-MiLK TEST® from Scienco Biotech was also used. Samples from cows with confirmed genotyping for A1A1 and A2A2 milk were selected, as well as commercial fermented milks, including A1 fermented milk from the Yakult brand (L25.06.240837C) and A2 fermented milk from the Letticoa2 brand (L20240711:36). Formulations were then prepared with varying percentages of A1A1 milk added to A2A2 milk (0%, 5%, 10%, 15%, 20%, 50%, and 100% (v/v)), and A1 fermented milk added to A2 fermented milk (0%, 5%, 10%, 15%, 20%, and 100% (v/v)). The procedure

followed the manufacturer's protocol. Ten drops of the milk mixture were added to a diluent tube using a sterile plastic pipette provided in the kit. The tube was shaken to ensure homogeneity of the solution. Next, the test cassette was placed on a flat surface, and two drops of the diluted milk solution were pipetted onto the cassette. The solution was allowed to migrate along the device for 20 min at room temperature. The results were interpreted, according to the manufacturer´s protocol, by observing the lines on the cassette: The appearance of one pink line in the test area (T) and another in the control area (C) indicated 100% A2 milk. Conversely, the appearance of only one pink line in the control area (C) indicated the presence of A1 β-casein, meaning the A2 milk was not pure, containing a percentual of contamination with A1 β-casein.

2.6. Sample Analysis and Statistical Analysis

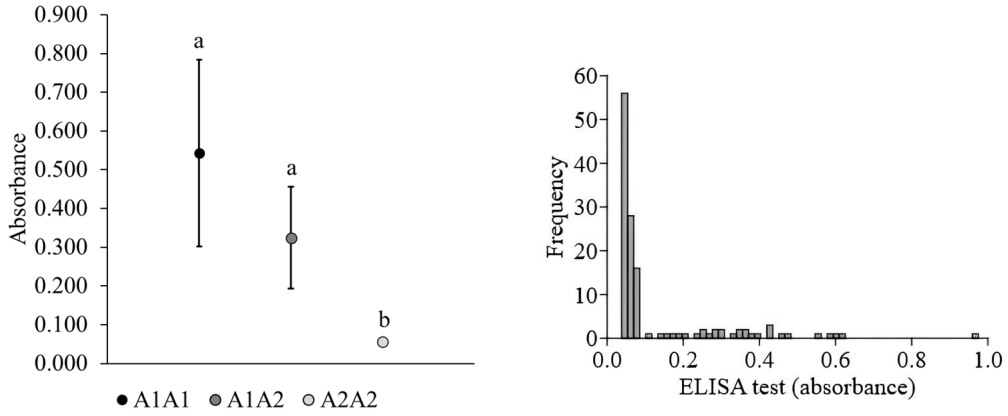
All experiments were performed in duplicate. The sensitivity and specificity of the samples were calculated as $S = [TP/(TP + FN)] \times 100$ and $E = [TN/(TN + FP)] \times 100$, where TP is the number of true-positive samples, TN is the number of true-negative samples, FP is the number of false-positive samples, and FN is the number of false-negative samples [42]. Data normality was assessed using the Shapiro–Wilk test. For data with a normal distribution, statistical differences between samples were evaluated using an analysis of variance (ANOVA), followed by Tukey's test. For data which did not follow a normal distribution, the Kruskal–Wallis test was applied, followed by Dunn's test. The confidence interval for statistical tests was set at 95%. Differences were considered statistically significant when $p < 0.05$. The presence of outliers was also checked. All statistical analyses were performed using the PAST 4.3 software.

3. Results

3.1. Identification of β-Casein Phenotypes by ELISA and LFIA Tests

A total of 131 raw milk samples from cows previously genotyped for β-casein A1A1, A1A2, and A2A2 were used. The milk samples had their β-casein phenotypes analyzed by ELISA test, and the results are shown in Figure 1. The ELISA test (Figure 1a) showed a clear differentiation in absorbance values between the groups with A1 phenotype (A1A1 and A1A2) and A2 phenotype (A2A2). The absorbances of the A1A1 and A1A2 raw milk samples ranged from 0.33 to 0.975 and 0.104 to 0.609, respectively. For the A2A2 raw milk samples, the absorbances ranged from 0.038 to 0.082, with one sample showing an absorbance of 0.195, characterized as an outlier (absorbance above the calculated upper limit of 0.105). The ELISA absorbance histogram (Figure 1b) shows that most samples have values concentrated between 0.04 and 0.08, indicating a predominantly low absorbance in the A2 raw milk samples. In contrast, a smaller number of samples show higher absorbance values, between 0.30 and 0.60, representing the A1 raw milk samples with higher absorbance response. The highest peak of the histogram is around 0.05, reflecting the high frequency of A2 raw milk samples with this absorbance value. Some raw milk samples, with absorbance values above 0.5, are considered outliers and may indicate significant differences in the composition or response of A1 raw milks compared to A2 raw milks.

Table 1 presents a comparison between different methods for identifying A2 raw milk, including animal genotyping by gene sequencing, the ELISA test, and the LFIA test. The results show that, out of a total of 131 raw milk samples from cows genotyped by gene sequencing, 4.58% had the A1A1 genotype, 18.32% had the A1A2 genotype, and 77.10% had the A2A2 genotype. The tests used showed consistency, with a sensitivity and specificity of 100% for A2A2 genotype detection. A representative image of the LFIA results from A1A1, A1A2, and A2A2 cows is shown in Figure 2. The LFIA results from the 131 genotyped animals demonstrated that all A2A2 animals were correctly identified, with no false positives. This yielded a specificity and sensitivity of 100% (Table 1). Both the ELISA test and the LFIA test corroborate the results of gene sequencing, correctly identifying the A2A2 genotypes. This demonstrates that these methods have the potential to identify cows that produce A2 milk.



(**a**) (**b**)

Figure 1. Raw milk samples had their β-casein phenotypes analyzed by ELISA test: (**a**) absorbance values (mean ± SD) of raw milk samples obtained by the ELISA test designed to detect A1 and A2 β-casein, wherein different lowercase letters indicate significant difference ($p \leq 0.05$) by Dunn's test; and (**b**) ELISA absorbance histogram.

Table 1. Comparison between tests for the identification of A2A2 cows/raw milk.

Number of Cows [1]	Genotype—Gene Sequencing [2]	ELISA Test [3]	A2-MiLK TEST® [3]	Sensitivity	Specificity
6	A1A1	No-A2	No-A2	100%	100%
24	A1A2	No-A2	No-A2	100%	100%
101	A2A2	A2	A2	100%	100%

[1] Total of 131 cows/raw milk samples. [2] Sample used in the test—hair bulb or ear cartilage. [3] Sample used in the ELISA and LFIA—raw milk.

Figure 2. Results of the LFIA test from the comparison stage between tests to identify A2A2/raw milk cows.

3.2. ELISA and LFIA Tests on Raw Milk Samples to Assess Purity

The applicability of the ELISA method in identifying the purity of raw A2 milk was evaluated and compared to the LFIA test. For this, A2A2 milk samples were contaminated with 0% (negative control), 5%, 10%, 15%, 20%, 50%, and 100% (*v*/*v*) (positive control) of raw A1A1 milk. The ELISA test absorbance results of the samples can be seen in Figure 3, and the LFIA test results can be seen in Table 2. For the ELISA test, the initial average absorbance of A2 milk was 0.046 ± 0.001, progressively increasing with the contamination of A1 milk ($p \leq 0.05$), reaching 0.124 ± 0.007 with 15% contamination. The average

absorbance for A1 milk was 0.338 ± 0.002. The ELISA method demonstrated efficacy in detecting A2 milk contamination with A1 milk when the latter was present in a proportion of 15% or higher. The LFIA test detected the presence of A1 milk when added to A2 milk in concentrations of 5% or higher.

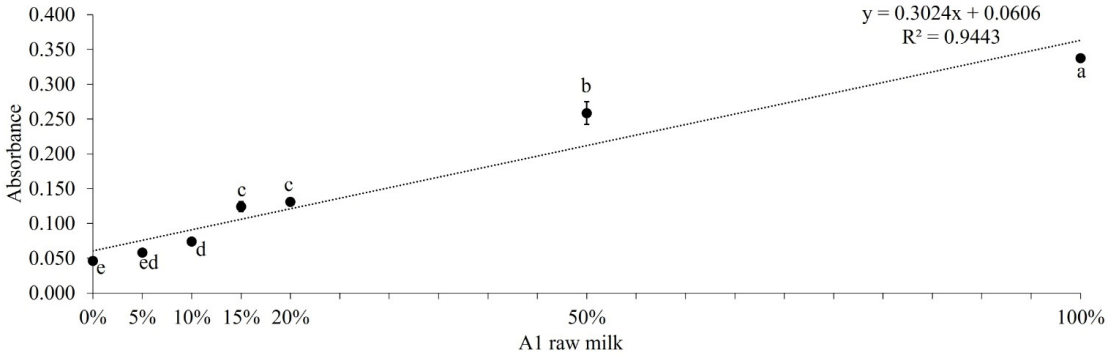


Figure 3. Absorbance values (mean ± SD) of A2A2 raw milk samples contaminated with A1A1 raw milk, obtained by the ELISA test designed to detect β-casein A1 and β-casein A2. Note: different lowercase letters indicate a significant difference ($p \leq 0.05$) by Tukey's test.

Table 2. Comparison between traditional genotyping methods, ELISA test, and LFIA test.

Aspect	Traditional Genotyping	ELISA (Enzyme-Linked Immunosorbent Assay)	LFIA (Lateral Flow Immunoassay) A2 Milk Test
Method	Direct DNA analysis	Detection of A1 β-casein using immunological reactions	Detection of milk proteins
Process	Use of laboratory techniques to extract and analyze DNA	Use of specific antibodies to detect and quantify A1 β-casein	Point-of-care testing, with no need for lab equipment
Result	Identification of genotypes A1A1, A1A2, or A2A2	Quantification of A1 β-casein concentration in milk	Identification of A2A2 animals and milk mixture purity
Precision	High accuracy in determining genotypes	High accuracy in measuring A1 β-casein concentration	100% A2A2 detection 95% purity detection
Application	Genetic studies and heredity analyses	Milk quality control and selection for human consumption	Milk quality control and heredity analyses
Benefits	Specific and direct, useful for detailed genetic studies	Fast, practical, and suitable for quality monitoring with moderate costs	Point-of-care testing, fast, cost-effective, and suitable for quality monitoring
Disadvantages	Costly and time-consuming, requiring specialized equipment and knowledge	Less specific compared to direct genotyping	Does not differentiate among A1A1 and A1A2 genotypes

3.3. ELISA and LFIA Tests on Fermented Milk Samples to Assess Purity

The applicability of the ELISA method in identifying the purity of A2 fermented milk was evaluated and compared with the LFIA test. For this, A2 fermented milk samples were contaminated with 0% (negative control), 5%, 10%, 15%, 20%, and 100% (positive control) of A1 fermented milk. The LFIA test results are presented in Figure 4, while the absorbance results from the ELISA test are displayed in Figure 5. The A2 fermented milk samples showed a significant increase in average absorbance ($p \leq 0.05$), starting from 10% contamination with A1 fermented milk, rising from 0.044 ± 0.003 in the negative control to 0.105 ± 0.001 in the fermented milk contaminated with 10% A1 fermented milk. With contamination at 15% and 20% of A1 fermented milk, the average absorbance values increased to 0.148 ± 0.018 and 0.162 ± 0.013, respectively, while the sample with 100% A1 fermented milk showed absorbance values of 0.348 ± 0.008. The LFIA test detected

the presence of A1 fermented milk when added to A2 fermented milk at concentrations of 10% or higher (Figure 6). These results demonstrate that the ELISA method is capable of detecting the presence of A1 fermented milk in A2 fermented milk when added in a proportion of 10% or higher, a value equal to that obtained with the LFIA test.

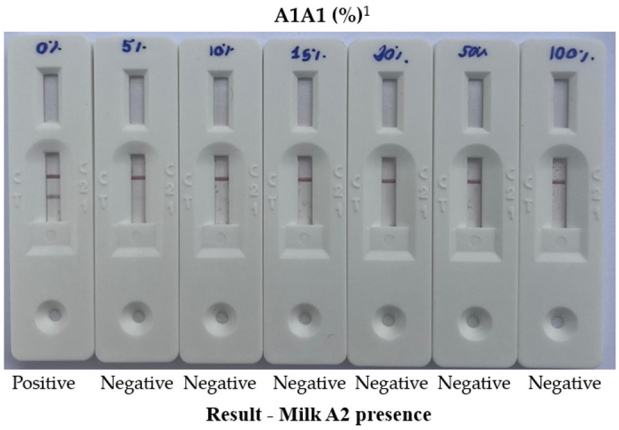


Figure 4. Results of the LFIA test for the detection of A2 β-casein in A2A2 milk contaminated with A1A1 milk. [1] % of A1A1 raw milk added to A2A2 raw milk.

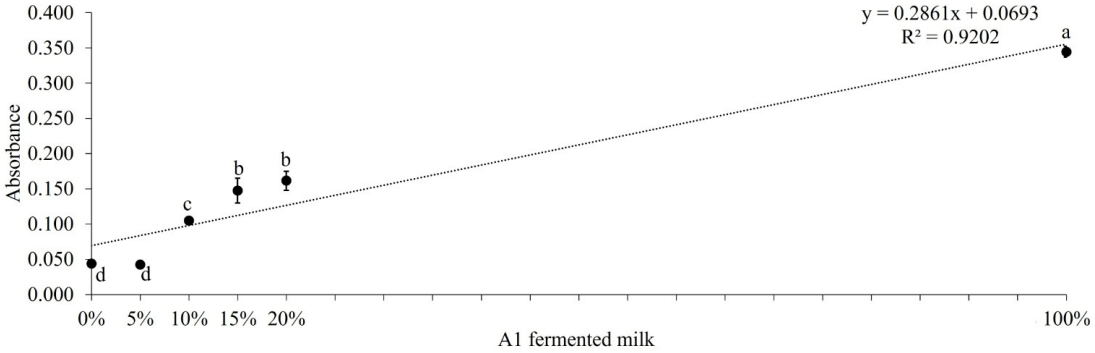


Figure 5. Absorbance values (mean ± SD) of A2 fermented milk samples contaminated with A1 fermented milk, obtained by the ELISA test designed to detect β-casein A1 and β-casein A2. Note: different lowercase letters indicate a significant difference ($p \leq 0.05$) by Tukey's test.

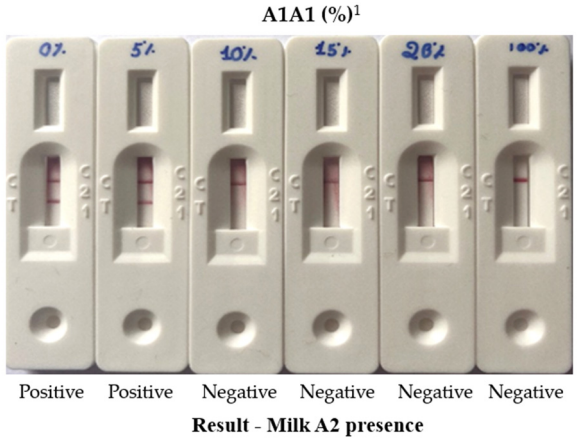


Figure 6. Results of the LFIA test for the detection of A2 β-casein in A2 fermented milk contaminated with A1 fermented milk. [1] % of A1 fermented milk added to A2 fermented milk.

4. Discussion

4.1. Importance of Non-Invasive ELISA Test and LFIA Test in the Detection of A1 β-Casein and Expansion to Other Dairy Products

In identifying phenotypes using the ELISA test, the skewed distribution with a bias toward low absorbance values suggests that most samples have a low absorbance response. This clear separation between the absorbance values of A1 and A2 raw milk allows for rapid classification, with A2 raw milk samples concentrated in a narrow range and A1 samples showing greater variability. The uniform distribution and defined peaks indicate that the ELISA test is sensitive and specific enough to distinguish the different phenotypes of raw milk [43]. This precision facilitates the quick identification of A2 raw milk, reduces classification errors, speeds up screening processes, ensures the consistency of the ELISA test, and provides a reliable basis for future studies. One advantage of the ELISA test is the ability to use the raw milk itself as a sample, which, combined with the quick turnaround of results, ensures the purity of the analyzed milk. This is advantageous because it eliminates the need to collect other samples from the animals, such as blood or cartilage, which are generally used for genetic sequencing tests. Additionally, the samples can be easily collected during conventional milking. Due to its efficient differentiation of β-casein A1 and A2 phenotypes, the ELISA method has the potential to be used for phenotyping dairy herds from milk samples collected from each animal. The accurate identification of animals with the A2A2 genotype is crucial for the production of A2 milk, preferred for its potential health benefits. The consistency of the methods tested in Table 1 reinforces the feasibility of implementing these techniques on a large scale to optimize A2 milk production. Since the phenotype is directly related to the genotype, this method shows potential for selecting animals with the A2A2 genotype and offers advantages by being less invasive and faster compared to DNA-based genotyping methods [44]. Identifying animals that produce milk with β-casein A2 allows producers to directly breed to increase the frequency of this genotype in the herd [13]. For example, cattle that produce A2 milk, and consequently have the A2A2 genotype, can be prioritized in mating programs during selection, leading to successful selective breeding [8,18]. In recent years, selecting the A2A2 allele has become a desirable trend in animal management to meet the growing market demand for milk and dairy products containing β-casein A2, referred to as "A2 protein", "A2 milk", or "β-casein A2 protein" [9,31]. The A2 milk market is increasing due to heightened consumer awareness of health and well-being, particularly in light of reports associating A1 casein with diseases such as type 1 diabetes, gastrointestinal symptoms, heart disease, and autism [3,15]. Additionally, the expanding market for A2 dairy products can provide new business opportunities and incentives for producers who invest in selecting a 100% tested herd [13].

The use of the LFIA and ELISA tests to detect A2 milk may constitute an effective tool to prevent fraud in the A2 milk market, as demonstrated by the results of A2 raw milk contaminated with A1 raw milk. Due to the increasing demand and added value of A2 milk, there is a significant risk of adulteration, where A1 milk may be improperly mixed and sold as A2 milk. Ehling et al. [45] observed, in their study, that all fluid milk samples purchased from a local supermarket (n = 2; from two brands) and skimmed milk powder bought from commercial suppliers (n = 5; from various batches) labeled as A2 contained measurable levels of β-casein A1. Similarly, Mayer et al. [18] authenticated commercial A2 milk samples. Over a period of six months, four out of five milk samples analyzed were found to be adulterated, i.e., β-casein A1 was also detectable in these samples. After genotyping the cows, it was discovered that one farmer's herd included a cow with the heterozygous β-casein A1A2 genotype, whose milk was also used to produce the so-called "A2 milk". Milk adulteration generally aims to increase the volume of the product delivered to meet demand [46]. Additionally, fraud is one of the most serious problems faced by the dairy industry, as it not only results in significant financial losses for the industry, affecting producers and distributors, but can also pose health risks to consumers [47]. With their sensitivity and specificity, the evaluated tests demonstrate suitability for the identification

of β-casein phenotypes, ensuring that only genuine A2 milk is marketed. By implementing the analyzed tests, producers and regulators can ensure the authenticity of A2 milk, protect consumer confidence, and maintain market integrity. Additionally, using raw milk samples facilitates the continuous monitoring of milk purity throughout the production chain, from milking to final distribution.

The importance of extending adulteration detection to other dairy products, such as fermented milk, is undeniable. Popular derivatives like yogurts and cheeses are widely consumed and face similar risks of fraud as liquid milk. Rigorous tests for detecting β-casein A1 in these products are essential to ensure the authenticity and purity of A2 fermented milk. Expanding such measures across the entire dairy product chain would bolster consumer confidence and preserve market integrity by guaranteeing that all A2-labeled products meet the expected standards. The ELISA test has proven effective in identifying the presence of the A1 phenotype in fermented milks, demonstrating its potential for detecting adulteration. The increasing variety and volume of dairy products labeled as containing β-casein A2 reflect current market trends [4]. These products, often sold at a premium compared to conventional alternatives [18], underscore the need for standardized analytical methods to detect β-casein A1. This is particularly important given the absence of clear definitions, detailed specifications, and consistent regulatory frameworks for quality control in this category [32,48,49]. Implementing such methods is crucial to prevent both intentional and unintentional adulteration and safeguard product authenticity [18].

4.2. Advantages, Limitations, and Considerations for Practical Application and Cost-Effectiveness of the ELISA and LFIA Methodologies

Immunoassays are recognized for their sensitivity, precision, and broad applicability [49,50]. They provide faster results than DNA-based genotyping assays, which require a significant amount of time for the extraction and analysis of genetic material [34,44]. Additionally, milk sample collection for the ELISA and LFIA tests is less invasive compared to blood and tissue sample collection for DNA genotyping tests [36,51]. Despite the possibility of using milk samples for qPCR tests, the extraction of DNA from somatic cells in milk is challenging due to the presence of inhibitors such as fat, protein, and calcium, as well as the low number of these cells in milk from healthy herds, making it difficult to achieve a high yield of DNA [49]. In addition to simplified protocols for sample collection and preparation [48], ELISA and LFIA are recognized as two of the most economical and cost-effective methods for the direct measurement of β-casein A1 in milk samples [48,49]. Despite numerous advantages, the ELISA method has limitations, such as the fact that is not a POC assay and needs sample dilutions to fit the dynamic range, as well as strong alkaline solutions [44]. Regarding its advantages, the ELISA method described here is quantitative and, therefore, can determine the percentage of A1 casein contamination in A2 milk, while LFIA is not a quantitative method and cannot determine precisely the level of contamination. Similarly to the ELISA test, the LFIA test used for comparison in this study offers results over a short time, usually within minutes, allowing for a quick analysis compared to traditional genotyping methods, which are known to be time-consuming [10].

While the proposed rapid A2 milk testing method provides an accessible and practical solution for the on-site verification of milk purity, it is limited to testing lactating animals. Large enterprises frequently utilize genetic testing methods to not only differentiate between A1 and A2 milk but also perform strategic herd selection. This allows them to identify and select breeding cows from the calf stage, ensuring that only animals with desirable genetic profiles remain in the herd for future production.

However, the rapid A2 test offers substantial benefits for smaller producers, as it is simple, fast, affordable, and easy to implement, without the need for specialized laboratories or complex equipment. This decentralized approach empowers small-scale farms to independently assess their herds and make informed breeding decisions over time. By democratizing access to genetic insights, the rapid A2 test enables smaller producers to gradually work toward A2 milk production, an opportunity previously available mainly to

large enterprises. Thus, this method complements existing genetic selection programs, offering an accessible tool which enhances both daily management and long-term production goals for a broader range of producers

Additionally, these tests are designed to be easily used by non-specialized personnel, benefiting both producers and dairy farmers, as well as consumers, especially since they can be conducted directly on farms, industries, or at home. They are more cost-effective and less labor-intensive compared to more complex laboratory techniques such as PCR with restriction RFLP, high-resolution melting (HRM) genotyping, and SNP rhAmp genotyping, which involve extensive DNA extraction, sample pre-treatment, and data analysis procedures [44]. Among the limitations of the method, it is important to mention that it identifies A2A2 animals but does not distinguish between A1A2 and A1A1 animals; it also cannot analyze changes in the amino acid sequence of the target region, unlike DNA sequencing [39].

Genotyping provides a complete genetic profile by distinguishing A1A1 from A1A2 animals, which is valuable because A1A2 animals, when selectively bred with A2 semen, can gradually increase the A2A2 population, while A1A1 animals, lacking the A2 allele, do not contribute toward this goal. This approach supports a targeted breeding strategy that reduces the A1 allele over time. However, the rapid immunological tests presented here, while not differentiating between A1A1 and A1A2, offer the advantage of speed and cost-effectiveness, enabling broad and timely analysis across herds. Given that the frequency of A1A1 animals is very low in the population, the inability to distinguish between them has minimal impact on herd conversion outcomes and, in a broader context, is outweighed by the practical benefits of rapid testing.

This approach allows for a faster and more accessible selection process, facilitating the transition to an A2 herd without the higher costs and delays of full genotyping. Consequently, rapid tests and genotyping can complement one another according to the management strategy and needs of each producer, offering a balanced and economical approach which leverages each method's strengths to achieve A2 milk production.

Therefore, as mentioned earlier, due to the relatively low cost, effectiveness, and ease of use, the ELISA method and the commercial LFIA test are suitable for routine and large-scale screening of β-casein variants in milk and fermented milk.

4.3. Assessment of Sensitivity and Specificity of ELISA and LFIA Tests

In comparing the LFIA and ELISA methods for detecting milk purity, it is evident that each has distinct sensitivity levels. The LFIA method can detect contamination levels of 5% in raw milk and 10% in fermented milk, while the ELISA method can detect contamination levels above 10% in both raw and fermented milk. Although both the ELISA test and the LFIA test possess sensitivity and specificity in detecting β-casein A1 and A2 in milk, only ELISA can determine quantitatively the level of A1 contamination. Therefore, ELISA, with its precision and detailed quantification capability, is ideal for applications requiring reliable results [52]. Furthermore, both methods demonstrated 100% accuracy in identifying A2A2 animals, with no false positives recorded. This indicates that both LFIA and ELISA have the potential to detect A2A2 milk and, consequently, can effectively replace genotyping for this purpose. These findings underscore the practical utility of both methods in the dairy industry, providing alternatives for ensuring A2 milk purity and enhancing the efficiency of contamination detection and animal selection. To identify A2A2 animals, traditional genotyping methods, such as direct DNA analysis, are accurate but costly and time-consuming.

The analyzed methods are faster, more practical, and less costly, making them ideal for applications requiring frequent monitoring and quick decisions, such as in the dairy industry. In terms of cost, traditional genotyping involves higher expenses due to the complexity of the process and the need for specialized infrastructure, whereas ELISA and LFIA are more economical and simpler options. Table 2 presents a detailed comparison

between traditional genotyping methods, the ELISA test, and the LFIA test for detecting β-casein A1 in milk.

5. Conclusions

The methodologies analyzed for identifying β-casein genotypes in dairy cows and the purity of A2 fermented milk demonstrated significant effectiveness. This research was justified by the need for quick and accurate techniques for herd screening and A2 milk quality control. The methodology included the development of an ELISA test to detect β-casein A1 in fermented milk and the evaluation of the ELISA test and the LFIA test to identify A2A2 genotypes and the purity of A2 milk. The results showed that the ELISA test had sensitivity and specificity (100%) in detecting β-casein A1. The LFIA test (A2-MiLK TEST®) was effective in identifying A2A2 genotypes with 100% accuracy and also in verifying the purity of A2 milk with a minimum detectable contamination of 5% for raw milk and 10% for fermented milk. Compared to traditional genotyping, these rapid methods have proven to be more practical and show potential for large-scale screening. Therefore, ELISA and LFIA tests are valuable tools for ensuring the quality and authenticity of A2 milk, providing an efficient, cost-effective solution which meets the demands of producers and consumers for safe and healthy dairy products, while also supporting the growth of the A2 milk market. The proposed rapid A2 milk testing methods provide a practical and accessible solution for verifying milk purity directly on-site. However, they are limited to lactating animals. In contrast, blood-based methods allow for the early identification of desirable genetic traits, offering valuable insights for herd selection from the calf stage. Nevertheless, rapid A2 tests stand out in the production context due to their simplicity, speed, affordability, and decentralization, enabling anyone to perform the analysis without requiring specialized laboratories or complex infrastructure. This approach facilitates the continuous monitoring of A2 milk purity over time, complements genetic selection programs, and adds value to the daily management of herds.

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