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Authentication of fresh goat cheese by *Taq*Man based real-time PCR

Nachweis von frischem Ziegenkäse durch TaqMan-basierte Real-Time PCR

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Summary

The most prevalent form of adulteration found in milk and dairy products involves the addition of cow milk to goat milk and dairy products. Detecting such adulteration is crucial in order to prevent health issues, particularly allergies, and to safeguard consumers against financial losses. This research aimed to examine the extent of cow and goat milk mixing at various percentages: 100%, 90%, 75%, 25%, 10%, 5%, 1%, and 0%. To accomplish this, fresh cheese samples were experimentally produced using these milk mixtures, and the levels of cow milk percentage and cow DNA content were determined using *Taq*Man real-time PCR. The results indicated that the presence of cow milk mixed with goat milk at concentrations as low as 1% and with cow DNA levels of 0.01 ng could be detected in cheese samples. In conclusion, *Taq*Man-based real-time PCR demonstrates high sensitivity and can be regarded as a reliable method for identifying the presence and proportions of cow in cheese samples obtained through the blending of cow and goat milk, thereby ensuring protection against economically driven adulteration and promoting consumer safety.

Keywords: Authentication, goat cheese, cow milk, *Taq*Man Real-Time PCR

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Introduction

The global increase in population and subsequent rise in food demand have contributed to a surge in food adulteration. Consequently, consumers are increasingly seeking accurate labeling of food products to ascertain their origin, safety, and quality. It has been observed that adulteration is more prevalent in foods with higher value and nutrient content, as unscrupulous producers seek unfair economic gain (Woolfe and Pimrose, 2004; Borkova and Snaselova, 2005; de la Fuente and Juarez, 2005; Kamal and Karoui, 2015; Kalogianni, 2018). Milk and dairy products, owing to their huge demand, are particularly susceptible to adulteration. Sheep and goat milk, in particular, are perceived as healthier and more nutritious alternatives to cow milk, leading to increased consumer demand (Mayer, 2005; Kamal and Karoui, 2015; Caira et al., 2017). The most common form of adulteration in dairy products involves the blending of cow milk with sheep or goat milk products, as it is cheaper and more readily available in larger quantities. Given that cow milk proteins are among the most common food allergens, especially in children (Bartuzi et al., 2017), accurate labeling assumes even greater significance.

Goat milk holds a significant position in terms of nutrition due to its protein, vitamins, minerals, higher levels of short and medium-chain fatty acids, and small fat globules that offer enhanced digestibility (Haenlein and Anke, 2011; Golinelli et al., 2014; Di Pinto et al., 2017). In comparison to cow milk, goat milk contains higher levels of six out of the ten essential amino acids. Moreover, it surpasses cow milk in terms of mono- and polyunsaturated fatty acids and medium-chain triglycerides, known for their potential health benefits, particularly in preventing cardiovascular diseases (Tomatake et al., 2006; Nunez-Sanchez et al., 2016). Due to the cost-effectiveness and availability of cow milk, there may be a temptation for producers to adulterate goat milk with cow milk (Guo et al., 2019). Additionally, various types of cheese are produced by mixing cow, sheep, and goat milk in specific proportions. Consequently, to instill consumer confidence, the indicated proportions on the label must be verified.

Species identification plays a crucial role in the authentication of animal-derived food products. In the case of products derived from sheep and goat milk, it is essential to accurately detect and quantify the presence of cow milk to prevent economically motivated adulteration, mitigate the risk of food allergies, and ensure compliance with regulatory standards. To achieve these objectives, the development of rapid, sensitive, and reliable analytical methods is necessary to enable regulatory authorities to detect such adulteration (Rentsch et al., 2013). In the past, conventional PCR (Golinelli et al., 2014; Kumar et al., 2014; Keyvan et al., 2017) and real-time PCR (qPCR) methods employing EvaGreen or SYBR Green have been used for authentication of various meat and dairy products (Agrimonti et al., 2015; Seçkin et al., 2017; Li et al., 2019). However, in recent years, the *Taq*Man-based qPCR method, which offers enhanced specificity and effectiveness compared to dye-based qPCR, has emerged as the preferred choice for meat and dairy product authentication (Rentsch et al., 2013; Guo et al., 2019; Guo et al., 2020; Biçer and Sönmez, 2022).

There is an increasing consumer interest in goat milk and its derivatives. Since goat milk is produced in smaller quantities and is relatively more expensive than cow milk, there is a risk of adulteration by adding cow milk to goat milk products. Therefore, it is crucial to accurately determine the extent of cow milk addition using reliable and sensitive methods. This study aimed to determine the cow milk level in experimentally produced fresh cheese samples by mixing cow and goat milk with the *Taq*Man qPCR method.

Material and Method

Cow and Goat Milk Supply and Cheese Production

Raw cow and goat milk, sourced from Selcuk University Faculty of Veterinary Medicine Farm (38°02‘08“N; 32°30‘22“E) and a private Saanen goat farm (37°49‘59.5“N; 32°32‘23.2“E) in Konya, Turkey, respectively, were utilized to experimentally produce cheese mixtures. The milk samples were mixed at various percentages: 100%, 90%, 75%, 25%, 10%, 5%, 1%, and 0%, totaling 2 liters. Subsequently, cheeses were produced following the protocol reported by Garcia-Gomez et al. (2019), with the process conducted in triplicate. In brief, the milk was heated to 35 °C, and 220 IMCU/mL microbial protease (Intermak, Konya, Turkey) was added at a 1:10 dilution to facilitate coagulation within approximately 40 min. Additionally, 0.02% $CaCl_2$ was introduced to obtain the curd. Following enzymatic coagulation, the curd was cut into approximately 1 cm^3 dimensions and left for 15–20 min. It was then pressed for around 8 h and stored at 4 °C for 24 h before analysis.

Genomic DNA Extraction of Cheese Samples

DNA extraction from cheese samples was conducted using a modified version of the procedure outlined by Murphy et al. (2002). Briefly, 1 g of cheese was dissolved in 2 mL of lysis buffer containing 200 mM NaCl, 100 mM Tris-HCl, 0.1% SDS, 5 mM EDTA, and 1% Triton X-100. Subsequently, 10 µL of Proteinase K (Zymo, D3001–2; 20 mg/mL) was added, followed by overnight incubation at 55 °C. After incubation, a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, Sigma-Aldrich, A2279) was added to the samples, which were then centrifuged at 28,000 ×g for 10 min. The upper phase was combined with 150 µL of 3 M sodium acetate and 400 µL of 100% ethanol (prechilled at –20 °C). The DNA was precipitated, washed with 70% ethanol, dried, and finally dissolved in 40 µL of TE buffer. The DNA samples were stored at –20 °C until further analysis.

Real-Time PCR Protocol

The obtained DNA samples were diluted to a concentration of 20 µg/µL using TE buffer. The analysis of cheese samples was conducted utilizing the cow milk detection real-time kit (SNP Biotechnology, Ankara, Turkey, Cat. No: 403R-10–01). The authentication of cheese samples was achieved by employing cow-specific primers and probes, with the internal amplification control (IAC) employed to eliminate false negative results. The quantification of cow DNA was determined using carboxyfluorescein (FAM) stain, while the IAC amplifications were assessed using hexachlorofluorescein (HEX) stain. For the analysis, 5 µL of DNA was added to a 20 µL reaction mixture. The reaction protocol commenced with enzyme activation at 95 °C for 3 min, followed by 32 cycles of 15 s at 95 °C and 60 s at 60 °C (Lightcyler Nano 1.0 Roche). Each reaction included positive and negative controls, and the analysis was performed in triplicate.

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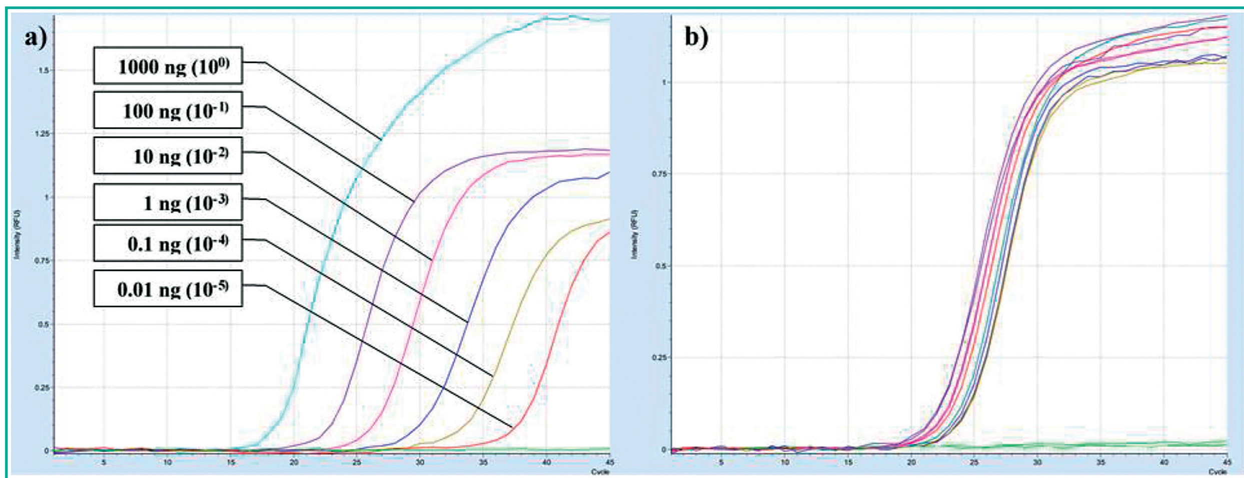

FIGURE 1: *(a): Amplification plots for DNA dilutions obtained from the positive control (0.001 ng DNA not detected); (b): Internal amplification control for cow DNA.*

Results

To authenticate the cheese samples, the limit of detection (LOD) of the employed primers and probes was determined using serially diluted DNA samples (10^0 (1000 ng), 10^{-1} (100 ng), 10^{-2} (10 ng), 10^{-3} (1 ng), 10^{-4} (0.1 ng), and 10^{-5} (0.01 ng)) from the positive control. As depicted in Figure 1 (a), the LOD was determined to be 0.01 ng of cow DNA. The amplification plots of the IAC, utilized to eliminate false negative results, exhibited consistent Ct values across all samples, as shown in Figure 1 (b). The Ct values (mean ±standard deviation [SD]) of the serially diluted DNAs are presented in Table 1. Ct values increased from 17.70 to 36.86 as the DNA concentration decreased from 1000 ng to 0.01 ng. No Ct value was obtained for the 0.001 ng DNA sample and the negative control.

In Figure 2 (a and b), amplification plots of the qPCR were generated using the Ct values associated with the proportions of cow milk in the cheese samples, along with the IAC. The calibration curve exhibited a slope of –7.1901, and the corresponding correlation coefficient was 0.8926 (Fig. 3). As indicated in Table 2, the Ct values increased from 21.29 to 35.77 as the cow milk levels decreased from 100% to 1% in the mixtures. No Ct value was obtained in cheese samples produced solely from 100% goat milk. As depicted in Figure 2, even a 1% concentration of cow milk could be detected in the cheese samples derived from the mixed milk.

TABLE 1: *The Ct values for the sensitivity of cow DNA detection.*

Input DNA amount (ng)	Cow – FAM Mean Ct[a]	IAC – HEX Mean Ct[a]	Cow – FAM SD[b]	IAC – HEX SD[b]
1000	17.70	21.24	0.02	0.03
100	22.10	21.43	0.00	0.01
10	25.68	22.04	0.07	0.03
1	29.55	23.49	0.02	0.15
0.1	32.82	23.69	0.27	0.03
0.01	36.86	22.38	0.16	0.08
0.001	n.d.	22.44	n.d.	0.03

[a] Cycle threshold; [b] Standard deviation of two replicates, FAM: carboxyfluorescein, HEX: hexachlorofluorescein, IAC: internal amplification control, n.d.: not detected

Discussion

PCR techniques based on the amplification of species-specific DNA sequences have proven effective in detecting different milk types within mixtures (Poonia et al., 2017). The quantification of various milk types in dairy products can be achieved using qPCR methods that rely

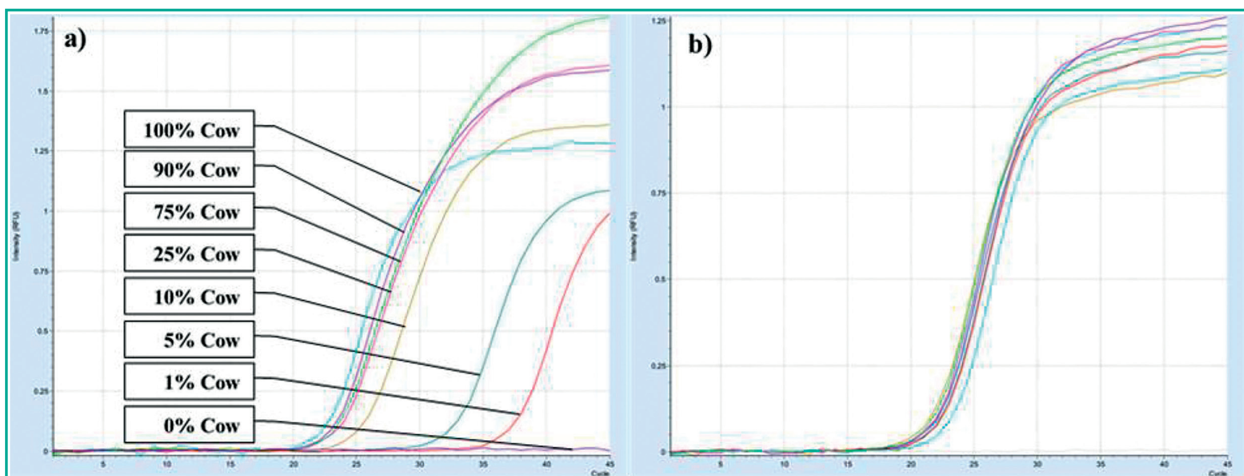

FIGURE 2: *(a): Amplification plots for cheese samples produced from mixed milk; (b): Internal amplification control for cheese samples.*

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TABLE 2: *Ct values for the detection of cow milk proportions in cheese samples.*

% Cow Milk	% Goat Milk	Cow – FAM Mean Ct[a]	IAC – HEX Mean Ct[a]	Cow – FAM SD[b]	IAC – HEX SD[b]
100	0	21.29	21.43	0.10	0.04
90	10	21.81	21.56	0.06	0.06
75	25	22.32	21.51	0.04	0.02
25	75	22.66	21.34	0.02	0.04
10	90	24.71	20.96	0.05	0.04
5	95	31.95	21.71	0.14	0.04
1	99	35.77	21.87	0.57	0.12
0	100	n.d.	21.17	n.d.	0.03

[a] Cycle threshold; [b] Standard deviation of three replicates, n.d.: not detected. Cow and goat milk were mixed with 100%, 90%, 75%, 25%, 10%, 5%, 1%, and 0% ratios, respectively, and cheeses were produced from these mixtures. The table shows the Ct values determined for different cow milk ratios from the individual reactions

was detectable. Therefore, the primer and probe utilized in this study are believed to be adequate for ensuring the authenticity of dairy products. The inclusion of an endogenous control is crucial to prevent false negative results that may arise from potential inhibitors (Hedman and Radström, 2013). Similar studies have emphasized the importance of employing an IAC to ensure the reliability of the method (Guo et al., 2018; Guo et al., 2019; Guo et al., 2020; Hai et al., 2020). In our study, the IAC was incorporated to ensure the reliability of our findings and eliminate false negatives. The amplification of our endogenous control was observed in all dilutions and mixtures. DNA isolation from cheese is considered a critical step as it is a sensitive process that directly affects the removal of PCR inhibitors, such as calcium ions (Rentsch et al., 2013). Furthermore, it is known that the DNA concentration obtained from cheeses (data not shown) does not correlate with the concentration of PCR-amplifiable DNA when measured using spectrophotometric methods. This discrepancy is attributed to the presence of non-specific high levels of bacterial DNA commonly found in raw milk. To address this issue, all extracted DNAs were reconstituted to a concentration of 20 µg/µL for analysis. It has also been reported that heat treatment and prolonged fermentation can impair DNA integrity and reduce the method‘s sensitivity (Guo et al., 2018). Dairy products generally exhibit relatively lower quality DNA, making its detection more challenging compared to meat products (Kesmen et al., 2009; Fang and Zhang, 2016). Therefore, fresh cheeses produced from raw milk were employed in this experimentally designed study.

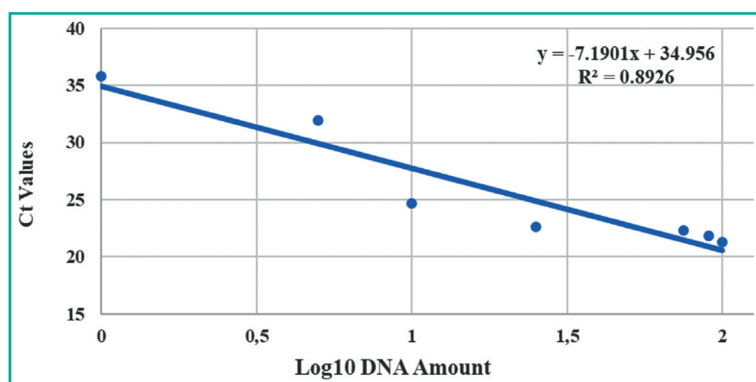

FIGURE 3: *Calibration curves for the quantification of cow DNA in cheese samples.*

on different bases. The initial publication by Lopez-Calleja et al. (2007) demonstrated the determination of animal species and their levels in dairy products through qPCR. While qPCR methods employing a fluorescent dye like SYBR Green have been utilized (Agrimonti et al., 2015), *Taq*Man-based qPCR appears to offer greater specificity. Furthermore, studies have revealed that simplex qPCR, utilizing primers and probes designed for individual targets, tends to exhibit greater sensitivity than multiplex qPCR for the identification of goat and cow milk (Guo et al., 2019). Hence, the current study employed the *Taq*Man-based simplex qPCR method. In a previous study, Guo et al. (2019) used a triplex *Taq*Man qPCR approach to confirm the authenticity of cheeses derived from mixtures of cow and goat milk, reporting the inability to detect cow milk at or below 5% concentration. The researchers noted that the adulteration of different species‘ milk at or above 10% could lead to unfair financial gain. Additionally, they reported the successful detection of 0.05 ng of cow DNA in cheeses, which was deemed sufficient for authentication in dairy products (Guo et al., 2019). The disparity in detection limits between studies is likely attributed to variations in DNA extraction methods and the utilization of simplex, multiplex, or triplex PCR methods. Hai et al. (2020) mixed cow and camel yogurts in various proportions and detected cow DNA in these mixtures using triplex real-time PCR. Conversely, Di Domenico et al. (2017) detected 0.025 ng of cow DNA in dairy products using *Taq*Man real-time PCR. In the present study, cow milk mixed with goat milk at concentrations of 1% and 5% could be successfully identified in cheeses. Guo et al. (2020) detected 0.005 ng and 0.01 ng of goat DNA in milk and cheese, respectively. In our study, as little as 0.01 ng of cow DNA

Conclusion

This study aimed to determine the presence of cow milk and evaluate the sensitivity of *Taq*Man qPCR in cheeses produced from a mixture of cow and goat milk. The findings revealed that cow milk mixed at a concentration of 1% with goat milk, along with 0.01 ng of cow DNA, could be successfully detected in the cheeses. Moreover, the inclusion of an internal amplification control ensured the reliability of the PCR amplification process, as evidenced by consistent amplification of the endogenous control across all dilutions and mixtures. This consistency further validates the accuracy of the methodology employed. While it is expected that economically motivated adulteration would involve the mixing of cow milk at levels of 10% or higher with other milk types for financial gain, it remains important to detect even trace amounts of cow milk to demonstrate the sensitivity of the method. In conclusion, the *Taq*Man qPCR approach employed in this study proves to be a sensitive, reliable, and reproducible method for safeguarding consumers against economically motivated adulteration and ensuring compliance with legal regulations.

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Conflict of Interest

Authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any studies with human or animal subjects.

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