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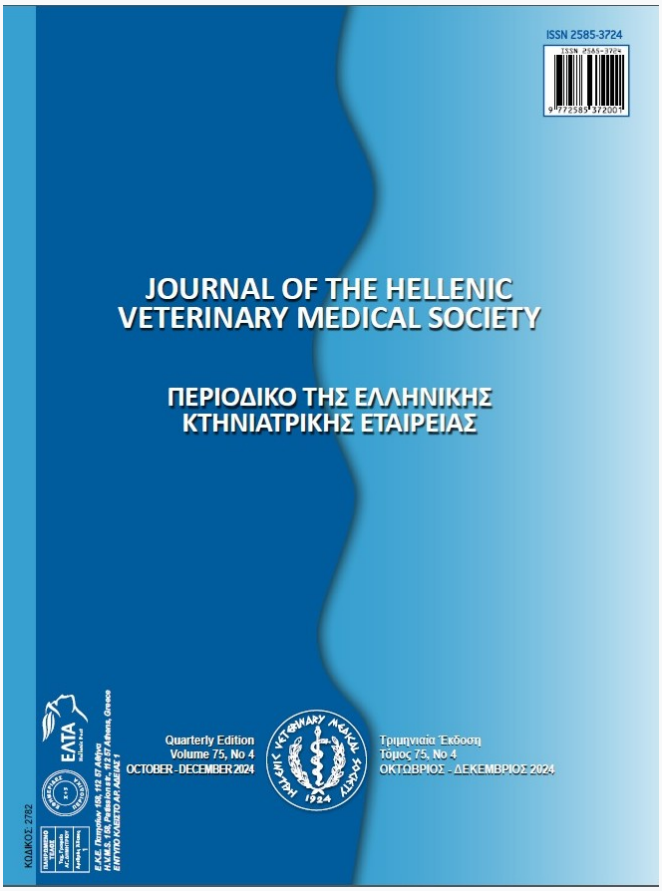

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Research article
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Determination of Different Types of Milk in Commercially Sold Goat Cheeses by Real-Time PCR Method

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ABSTRACT: Food mislabeling impacts consumer rights and informed choices, especially for premium products with designated origins. Ingredient substitutions can lower quality, dilute identity, and distort fair competition. Developed nations employ strict national and international regulations to combat this issue. This study aimed to accurately and reliably detect the presence of cow and sheep milk in cheeses labeled as "100% Goat Milk" using the RT-PCR method. 100 cheese samples with different production dates and batch numbers labeled "100% Goat Milk" were collected from markets. In the samples, RT-PCR TaqMan probe method was used to qualitatively detect the species-specific region in mitochondrial DNA and discrimination was made at the species level. In the study, it was determined that 76% of the cheese samples (76 out of 100 cheeses) labeled with the "100% Goat Milk" label did not comply with the expression on the label. Pure cow's milk was detected in 27% (27) of the cheese samples, pure sheep's milk in 4% (4), goat and cow's milk in 9% (9), cow and sheep's milk in 16% (16), and goat, sheep and cow's milk in 17% (17). The study reveals that cheeses containing goat milk are significantly adulterated and emphasizes the need for meticulous monitoring during production and sale. In conclusion, The RT-PCR method is recommended as an effective diagnostic method with the ability to detect low levels of sheep and cow milk in goat cheeses.

Keywords: Adulteration; goat milk; Real-Time PCR

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INTRODUCTION

The misidentification of food product ingredients has emerged as a growing concern, given that national and international regulations in many developed nations guarantee consumer protection and the right to make informed choices. "Premium products," like cheeses bearing a designation of origin, are particularly susceptible to adulteration through the replacement or exclusion of one or more valuable ingredients during the manufacturing process. This leads to substandard quality, loss of product identity, and unfair competition by producers who benefit economically from misleading labeling of food composition (Bottero et al., 2003; Kotowicz et al., 2007; López-Calleja et al., 2007; Darwish et al., 2009; Cottenet et al., 2011; Gonçalves et al., 2012).

Although milk is generally considered a readily available food, milk from some animal species can be difficult to access (Bottero et al., 2003; Cheng et al., 2006; Pesic et al., 2011). Goat milk and its products have high prices due to seasonal production and small-scale farming, especially in developing countries. This high demand leads to the risk of adulteration with relatively cheaper cow's milk, damaging consumer confidence and creating health hazards. For example, consumption of goat's milk mixed with cow's milk poses a significant risk, especially for people with cow's milk allergy (Bottero et al., 2003; Cheng et al., 2006). Consequently, stringent quality control measures for raw goat milk and its products are imperative. The growing concern revolves around the increasing occurrence of adulteration, particularly with cow milk. This issue not only confounds consumers trying to make informed purchases but also violates the legislation in many countries (TFC, 2017; EC, 2019) and it is necessary to prevent illegal trade. By ensuring accuracy and transparency in labeling, consumer rights can be protected and the sale of fraudulent products can be prevented. Verifying the origin of milk types and ensuring traceability is one of the important steps to be taken to prevent this problem. Economic price pressure and incentives for alternative substitution negatively affect the quality of food of animal origin (Dias et al., 2009; Everstine et al., 2013; Golinelli et al., 2014).

To ensure a healthy and reliable food supply, there is a need to enhance collaboration and awareness efforts among authorities, producers, and consumers. Thus, by reducing problems about quality and labeling issues in animal-derived foods, a contribution can be made to a healthy and equitable food system (Ferreira and Caçote, 2003; Moatsou and Anifantakis, 2003; Haenlein, 2004; Hurley et al., 2004; Wen et al., 2020).

In particular, valuable cheeses, cheese brands, and those recognized for their production methods are being substituted with counterfeit products, undermining consumer confidence. Food fraudsters attempt to deceive consumers by using low-cost ingredients to produce products that resemble the appearance of the original cheese or by misleadingly applying labeling processes. As a matter of fact, this situation has been clearly demonstrated in some studies (Bottero et al., 2003; Gonçalves et al., 2012; I. M. López-Calleja et al., 2007; Agrimonti et al., 2015; Mašková and Paulíčková, 2006; Tuncay, 2023; Zengin and Kara, 2022).

In the Turkish Food Codex Communiqué on Cheese (TFC, 2015), if milk from different animal species is mixed in cheese production, the names of the species from which the milk is obtained are clearly stated next to the product name. For example, consumers should be informed with statements such as "produced from sheep, goat and cow milk". This practice ensures that customers have access to accurate and transparent information about the content and components of the products. The European Union's food safety policy aims to protect consumers not only from food pathogens but also from fraudulent species substitutions. Therefore, among the key priorities, ensuring proper labeling and traceability of food and food products is included. Accurate and reliable information on product labels helps consumers to make informed decisions about the content and origin of products. Similarly, compliance with the requirements of Regulation (EC) No 178/2002 of the European Commission, which establishes standards for food safety and traceability and mandates food businesses to adhere, holds significant importance. EU regulations also provide rules regarding the declaration of animal species in dairy-based foods. These rules provide consumers with access to accurate information and help protect them from fraudulent practices such as species substitution in food products. In this context, it is important for food and food labeling, traceability, and compliance with Regulation EU (B Regulation (Ec) No 178/2002 of the European Parliament and of the Council) to be supported by scientific studies (EC, 2002; TFC, 2015).

To combat the widespread issue of illegal mixtures in food production, the "Farm to Fork" concept is crucial for ensuring food authenticity. It involves

meticulously tracking and guaranteeing product authenticity from production to consumption. This necessitates rigorous verification of product definitions and labels, supported by innovative analytical techniques, especially for processed food components. These efforts enable transparent and reliable information about food content and composition, enhancing consumer trust and food safety (Rodríguez et al., 2004; Ghovvati et al., 2009; Haunshi et al., 2009; Di Domenico et al., 2017).

Today, in studies carried out in our country and the world, various analyses have been carried out to determine whether goat milk and milk of other animals are mixed or not. In these studies, it has been demonstrated that milk from different animal species can be fraudulently mixed with goat milk for the purpose of adulteration and deception (Bania et al., 2001; Chen et al., 2004; Mayer, 2005; Cheng et al., 2006; López-Calleja et al., 2007; Chávez et al., 2008; Ramírez et al., 2011; Rodrigues et al., 2012; Rodríguez-Zengin and Kara, 2022; Tuncay, 2023).

Protein-based methods can falter in matured cheeses or heated dairy products due to heat-induced protein changes (Mayer, 2005). Conversely, DNA-based methods, like PCR, remain reliable, detecting fraud or adulteration by amplifying and accurately identifying specific DNA fragments (López-Calleja et al., 2005; Mayer, 2005; Zengin and Kara, 2022; Tuncay, 2023).

It is known that this study is the first study in which adulteration of goat cheese was detected in Türkiye. Additionally, the use of the TaqMan probe Real Time-PCR (RT-PCR) analysis method in goat milk analysis adds further importance to this study.

This study aimed to determine the presence of sheep and cow milk in cheeses labeled as "100% Goat Milk" by RT-PCR method sensitively and reliably.

MATERIALS AND METHODS

Ethics committee

Approval was obtained from the Van Yuzuncu Yil University (Türkiye) Animal Researches Local Ethic Committee with the letter No: 2022/12-06 dated 01.12.2022.

Reference DNA

The pure reference goat, sheep, and cow DNA used in the study were obtained from DIAGEN (Türkiye).

Milk samples

100 cheese samples labeled as "100% Goat Milk," matured, with varying production dates and batch numbers, were collected from supermarkets.

Fifty two samples originated in Van province, and 48 samples were from other various provinces of Türkiye through the virtual market, i.e., Ankara (n=10), Antalya (n=8), Aydın (n=5), Hatay (n=5), Istanbul (n=10), Izmir (n=10).

DNA extraction

DNA Purification kit (GeneMATRIX FOOD-EXTRACT DNA Purification Kit, Poland) was used to extract DNA from the cheese according to the manufacturer's recommendation. For this purpose, 250 mg of cheese was weighed and 800 µl of lysis buffer was added, homogenized, and then transferred to a 1.5 ml Eppendorf tube, and then 25 µl of Proteinase K was added, vortex. The tubes to which proteinase K was added were incubated at 60°C for 45 min and then centrifuged at 11000 ×g for 1 min. 400 µL supernatant was transferred to another tube, and 200 µL binding buffer was added. After vortexing, it was transferred to a spin column and centrifuged at 11000 ×g for 1 min. The collecting tube was changed, 650 µL of wash buffer 1 was added, then centrifuged at 11000 ×g for 1 min, and the collection tube was changed, and wash buffer 2 was added. After centrifugation at 11000xg for 5 minutes, it was transferred to Eppendorf and 100 µl of elution buffer heated at 60°C was added and centrifuged. The obtained DNAs were stored at -20°C until the RT-PCR process.

RT-PCR reaction

RT-PCR TaqMan Probe commercial kits (DIAGEN, 2103,2104, 2110, Türkiye) that detects the NADH dehydrogenase (ND5) for cattle and sheep and the rRNA-ribosomal RNA for goat. The kit's sensitivity rate (0.1%) was determined in a previous study (Tuncay and Sancak, 2022). The RT-PCR TaqMan probe method in the kit qualitatively detects the species-specific (goat, sheep, cow) region in mitochondrial DNA and distinguishes at the species level. PCR mixtures consisting of 10 µL mix A, 5 µL mix B, and 5 µL DNA of each species were prepared separately according to the manufacturer's (DIAGEN, Türkiye) recommendations. The PCR mixture was subjected to pre-denaturation at 95°C for 5 min, and a total of 35 cycles of 95°C for 10 s denaturation, 59°C for 30 s annealing, 72°C for 5 s extension, and 25°C for 1 min final extension protocol was applied during the amplification phase.

Statistical analysis

Statistical frequency tests of the findings obtained in the study were performed with SPSS 13.0 package programme (SPSS, 2006).

RESULTS

It was determined that 24 (24%) of the 100 cheese samples collected were in compliance with the label, while 76 (76%) were not in compliance with the label. The analysis results of 100 cheese samples are given in Table 1.

DISCUSSION

In the food production processes, the adulteration of raw materials used for commercial purposes through illegal methods is a widely encountered problem. The "From Farm to Table" concept emphasizes the meticulous traceability and authenticity of all stages of a food product, starting from the production stages until it reaches the final consumer. Recently, food adulteration has achieved importance as one of the most current issues in this field (Ghovvati et al., 2009; Everstine et al., 2013; EC, 2015; Rahmati et al., 2016; Moyer et al., 2017)

Accurate food labeling is crucial for informed consumer choices (Herman, 2001). Mislabeled foods are a global concern, emphasizing the importance of ingredient quality and safety (Di Pinto et al., 2017). While RT- PCR is widely used for species identification in meat, its application in dairy products remains limited (Agrimonti et al., 2015; Di Pinto et al., 2017; Tuncay and Sancak, 2022).

In our study, 100 cheese samples labeled "100% Goat Milk" were examined. It was determined that 24% (24) of the samples did not comply with the statement on the label. Pure cow milk was detected in 27% (27) of cheese samples, pure sheep milk in 4% (4), goat and sheep milk in 3% (3), goat and cow milk in 9% (9), cow and sheep milk in 16% (16), and goat, sheep and cow milk in 17% (17).

Legislation in many European countries stipulates that the type of milk used in the production of cheese and other dairy products must be clearly labeled (Calvo et al., 2002). In Türkiye, not clearly stating the products in the food in the labeling regulation is considered adulteration, and legal action is taken for the companies detected (TFC, 2017). According to this information, adulteration was detected in 76 (76%) cheese samples in our study.

Bottero et al. (2003) stated in their study that 26.32% of the cheese samples they examined were incompatible with the label. This rate is lower than the label non-compliance rate of 76% in our study.

Mašková and Paulíčkov (2006) reported in their study that 17.65% of the goat and sheep cheeses examined contained undeclared cow's milk, although they were labeled as goat cheese and 14.29% as sheep cheese. These results are similar to the adulteration

Table 1 RT-PCR results of cheese samples labeled as "100% goat milk"

Province	n *	Milk origins detected by RT-PCR in cheese samples						
Ankara	10	Goat 3 (30%)	Sheep 1 (10%)	Cow 2 (20%)	Goat/Sheep 1 (10%)	Goat/Cow 1 (10%)	-	Goat/Sheep/Cow 2 (20%)
Antalya	8	Goat 1 (12.5%)	-	Cow 4 (50%)	-	Goat/Cow 1 (12.5%)	-	Goat/Sheep/Cow 2 (25%)
Aydin	5	Goat 1 (20%)	-	Cow 1 (20%)	-	-	Sheep/Cow 2 (40%)	Goat/Sheep/Cow 1 (20%)
Hatay	5	Goat 2 (40%)	Sheep 1(20%)	-	-	-	Sheep/Cow 2 (40%)	-
Istanbul	10	Goat 2 (20%)	-	Cow 2 (20%)	-	Goat/Cow 2 (20%)	Sheep/Cow 2 (20%)	Goat/Sheep/Cow 2 (20%)
Izmir	10	Goat 3 (30%)	Sheep 1 (10%)	Cow 3 (30%)	-	Goat/Cow 1 (10%)	Sheep/Cow 2 (20%)	-
Van	52	Goat 12 (23.08%)	Sheep 1 (1.92%)	Cow 15 (28.85%)	Goat/Sheep 2 (3.85%)	Goat/Cow 4 (7.69%)	Sheep/Cow 8 (15.38%)	Goat/Sheep/Cow 10 (19.23%)
Total	100	Goat 24 (24%)	Sheep 4 (4%)	Cow 27 (27%)	Goat/Sheep 3 (3%)	Goat/Cow 9 (9%)	Sheep/Cow 16 (16%)	Goat/Sheep/Cow 17 (17%)

*n: Number of cheese samples declared to be produced from pure goat milk.

rate in our study.

López-Calleja et al. (2007) stated in their study that 5 of 16 cheese samples (31.25%) were different from the animal species written on the label. This rate is higher than the cow milk adulteration rate of 27% in the given study.

In the study by Stănciuc and Râpeanu (2010), it was reported that 67.3% of sheep cheese samples and 20.3% of cow's milk and goat cheese samples were compliant with the label. These findings differ from the adulteration rates observed in the provided study.

Gonçalves et al. (2012) stated in their study that 12.5% of the 96 milk and dairy products examined were incompatible with the label. This rate is significantly lower than the label non-compliance rate of 76% in our study.

Khanzadi et al. (2013) study, it was stated that only 20% of sheep milk and products were compatible with the label, while 84% were incompatible. This study was found to have a higher non-compliance rate than the given study.

Agrimonti et al. (2015) reported in their study that 30.77% of the 26 dairy products examined were different from those stated on the label. Although this rate they reported is close to the adulteration rates in our study, our study only includes cheese samples.

Di Pinto et al. (2017) reported in their study that 72.5% of goat milk and products were not compatible with the label according to end-point PCR results, and this rate increased to 80% according to RT-PCR results. These results are very close to the label non-compliance rate of 76% in the given study.

Tsakali et al. (2019) state in their study that 90% of 40 milk and dairy products consumed in Greece are mixed with cow's milk. This result is similar to the adulteration rates in our study.

In their study, Zengin and Kara (2022) detected goat milk in 20% of goat cheese samples, a mixture of goat and cow milk in 38.33%, and pure cow milk in 41.67%. In sheep cheese samples, they detected sheep milk in 18.33%, a mixture of sheep and cow milk in 50%, and pure cow milk in 31.67%. Although these results are similar to the milk mixtures in the given study, there are differences in adulteration rates.

The differences observed between the studies are believed to stem from various factors such as the diversity of samples collected from the market, the analytical methods employed, and the sensitivity of these methods. Furthermore, this situation can also result from improper or insufficient cleaning of processing equipment, as well as the introduction of unregistered ingredients into the final product (Dąbrowska et al., 2010).

The results of this study showed that there is a high level of adulteration in cheeses claimed to contain pure goat milk. Therefore, it was revealed that the production and sales stages of these cheeses should be continuously and carefully observed. A fast and accurate diagnostic method is needed for detailed follow-up. The RT-PCR method used in this study supports the previous study (Tuncay and Sancak, 2022). This method stands out as a useful, fast, accurate and simple method with the ability to detect the presence of sheep and cow milk in goat cheeses even in low amounts. Therefore, it is recommended that regulatory authorities use this method and increase inspections in order to prevent unfair competition and reassure consumers about correct labeling. In addition, cow's milk and dairy products are one of the potential food allergies even at low concentrations. In order to prevent this situation that will lead to health problems, species determination will also help to prevent possible health risks.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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