The use of PCR method for adulteration detection of goat dairy products manufactured by smallholders

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ABSTRACT

The analysis of food adulteration is an important element of improving health safety. Both accidental and intentional adulteration of food, can cause serious health consequences for humans and can be considered as health hazard. In recent years, molecular biology techniques has been increasingly used in food analysis due to their high precision and the ability to obtain results in a short time, as well as the identification and quantitative determination of food adulterants. The samples tested in the study were goat's milk, yogurts, fresh and ripening cheeses produced by smallholders all over Poland. The analysis included DNA isolation, which was used as template for duplex PCR and detection of goat's and cow's mitochondrial DNA fragments. The obtained results indicate that the applied technique enabled the rapid identification of adulteration in analyzed goat's milk products, among which in only one product out of thirty five the presence of undeclared cow's milk was detected. This indicates good food safety standards applied on small-scale farms.

1. Introduction

Health protection is the main goal of all European Union regulation and standards in the agriculture, animal husbandry and food production sectors (Pettoello-Mantovani and Olivieri, 2022). The European Commission developed an integrated "from farm to fork" approach to food safety covering all sectors of the food chain, including feed production, primary production, food processing, storage, transport and retail sale (Regulation (EC) No 178/2002). EU legislation on food and feed safety also includes the regulations of the need for correct labeling of food products the most important of which is the composition of the product and its authenticity.

The currently observed increasing rate of food production and its high costs might encourage some of food producers to replace the most expensive ingredients with cheaper or more available ones. Also inaccurate or incomplete cleaning of the processing equipment may result in the introduction of an undeclared ingredient into the product (Visciano and Schirone, 2021). Both intentional and accidental food adulteration can be considered as health risk, due to a high incidence of food allergies which are currently considered a significant health problem, especially in industrialized countries (Anagaw et al., 2024). Undeclared allergens present as contaminants in food products constitute the main risk for allergic people. Therefore, reliable and rapid methods for food allergen detection and species identification are necessary to monitor correct

food labeling and improve consumer protection (Fu et al., 2019).

Among food products, cow's milk and its products are a common cause of food allergies. Cow's milk contains about 30 proteins with potentially allergenic properties (Tsabouri et al. 2014). The highest allergenic potential expresses αs1-casein fraction (Schulmeister et al. 2009, Cong et al. 2013) and β-lactoglobulin dominating among milk whey proteins (Wal, 2004, Järvinen et al. 2001), for which there is no equivalent among human milk proteins. It was proved that cow's milk proteins, even at low concentrations, are highly allergenic (Sampson, 2003, Wal, 2004). Clinical symptoms of allergy to milk proteins are diverse and may affect many organs and systems, in extreme cases leading even to anaphylactic shock (Małaczyńska, 2000). In most cases cow's milk can be successfully substituted in diet of an allergic individual with milk of other species:. goats, without causing allergic symptoms (Ensminger et al. 1993). Therefore adulteration of goat's milk or its products with cheaper cow's milk may be considered a health hazard.

Currently used methods for identifying potential allergens in foods are aimed at detecting the allergen itself or its marker indicating its presence in the offending food (Poms et al. 2004). The techniques most commonly used for this purpose are based on the detection of specific proteins or encoding them deoxyribonucleic acids (DNA) fragments. In routine food analysis, the enzyme-linked immunosorbent assay (ELISA) and the polymerase chain reaction (PCR, real-time PCR) are commonly
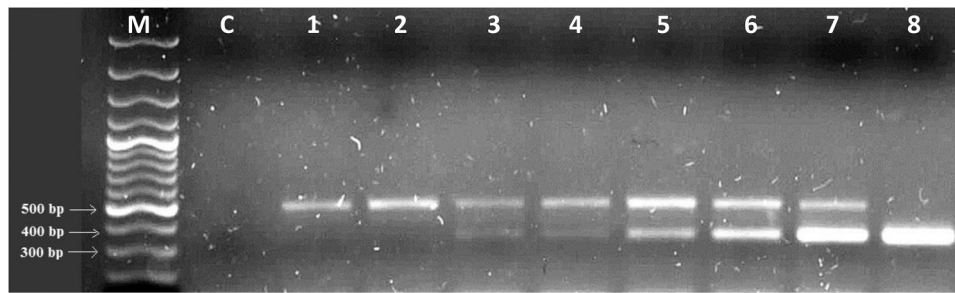
Fig. 1. Electrophoretic separation of PCR products amplified with duplex-PCR for detection of cow's and goat's mitochondrial DNA fragments. Consecutive lanes represent products obtained from: 100 % goat milk/no cow's milk (lane 1) and products obtained from mixtures of 0.1, 0.5, 1, 10, 50, 75,% of cow's milk in goat' milk (lanes 2–7) In lane 8 the amplicon obtained from 100 % cow milk sample was resolved. C- negative control containing no DNA, M - 100 bp DNA ladder (Thermo Fisher Sci).

applied (Chi et al. 2024, Di Pinto et al., 2017, Mafra et al. 2022). It was showed by many authors (Ahsani et al., 2010; Askari et al., 2011) that although ELISA is a specific, quick and economical diagnostic method, PCR is the most modern practical technology in diagnosing as it is more rapid, with results obtained in a few hours, and also more reliable (Ahsani et al., 2011; Mohammadabadi et al., 2011). The analysis based on a more stable marker like DNA, is not dependent on gene expression. Another advantage of genotyping methods is that the discriminatory power of DNA-based methods is generally superior to that of phenotypic methods, therefore the method has a broad application not only for adulteration analysis, but also in microbiological research, for example in studies on population genetics and microbial epidemiology (Ahsani et al., 2011; Gooki et al., 2018; Shahdadnejad et al., 2016).

DNA based methods have also became common, due to the high stability of the molecule under the high temperature, pressure and chemical treatment used in food processing (Lo and Shaw, 2018). For identification, 12S rRNA gene sequences (López-Calleja et al., 2005; Liao et al., 2017), growth hormone gene (Lopparelli, et al. 2007) or mitochondrial genome (Dąbrowska et al. 2010) in single-plex (De et al. 2011), duplex (Rodrigues et al., 2012), and triplex end-point PCR have been used (Hai et al., 2020). The classic PCR allows a rapid screening for adulteration, while real time PCR is a precise tool for determining the amount of adulterant.

In recent years the increasing production of farmer's dairy products manufactured on small scale from the milk of various ruminants has been observed. These products are becoming moreand more popular and are often chosen by consumers as less processed, made from local ingredients and therefore of high beneficial properties. Although those products are subjected to the same law regulation and control procedures as those produced on industrial scale, there is little information on the frequency of their adulteration.

Therefore the aim of the research was to analyze the adulteration frequency of various goat's dairy products (milk, yoghurts and cheeses produced by smallholders) available on the Polish market. To our best knowledge no analysis has been previously carried out the on frequency of adulteration of goat dairy products produced in Poland by smallholders.

2. Materials and methods

The material analyzed in the study were samples of goat's milk and goat's dairy products bought at the local markets and originating from small farms all over Poland. The samples (35 in total, purchased in amount of 100 g or 100 ml) were: milk: 8 samples; yoghurts: 6 samples; cheeses 21 (ripening cheeses: 12 samples, fresh cheeses: 6 samples, mold cheeses: 3 samples). All samples were marked on their labels as products made exclusively from goat's milk. The collected samples were used for DNA extraction and further PCR analyses.

2.1. DNA isolation form food sample

The DNA extraction was made with the use of „GeneMATRIX Food-Extract Kit" (Eurx, Poland) according to manufacturer procedure form 300 mg of homogenized solid and 300 μl liquid samples, isolation was made in 2 ml Eppendorf tubes. DNA concentration was determined with the use of Nano Drop Spectrophotometer (Thermo Fischer Scientific, USA). Each isolation was performed in duplicates. The eluted DNA was used as template for PCR reaction.

2.2. Primers used in the study

The sequences of primers used in the study were synthesized according to Kotowicz et al. 2007. BosD for 5'- CAATAACTCAACACA-GAATTTGC-3'; BosD rev 5'-CGTGATCTAATGGTAAGGAATA-3', amplifying the 300 bp fragment of *Bos taurus* mitochondrial genome: 15 856–16 156 bp (Genebank Acc. no V00654) and Goat D for 5'-CCAA-CATGCGTATCCCGT-3' and GoatD rev 5'-AGCGGATGCATGATGAAATG-3', amplifying the 444 bp fragment of *Capra hiricus* mitochondrial genome (16 043–16 487 bp, Genebank Acc. no AF533441).

2.3. PCR conditions

The reaction mix contained $1\times$polymerase buffer, 20 nmole of BosD primers and 5 nmole GoatD primers (Genomed, Warsaw, Poland; Table 1), 200 μM of each deoxinucleotide triphosphate (Thermo Fisher Scientific, USA), 1,5 mM $MgCl_2$ (Thermo Fisher Scientific, USA), 1 U of Taq DNA polymerase (Thermo Fisher Scientific, USA) and 10 ng of isolated DNA as a template.

The PCR conditions were: (95°C for 30 s, 52°C for 30 s and 72°C for 2 min) $\times$ 35 cycles, followed by 72 °C for 5 min. The reaction was performed using the T3 thermal cycler (Biometra, Germany). Each PCR was performed in duplicates. The PCR products were resolved in 1.5 % TAE-agarose gel at 100 V. Their images were taken and documented with a charge-coupled device camera system (Vilber-Lourmat, France).

3. Results

The sensitivity of the method was assessed using the method of Kotowicz et. al (2007) by preparing the PCR standardization curve (Fig. 1). DNA isolated from mixtures of cow's and goat's milk with varying amounts of cow's milk (0, 0.1, 0.5, 1, 10, 50, 75, 100 %), was used as template for duplex PCR. Sample with no addition of cow's milk (100 % goat milk) was assigned as "0" was sample labeled: 100 % was pure cow's milk. In each PCR analysis negative control containing no DNA was included to exclude reagents contamination.

That approach allowed to estimate the detection level at 0.5 % addition of cow's milk. In the sample to which 0.1 % cow's milk was added no 300 bp band representing cow's mitochondrial DNA was
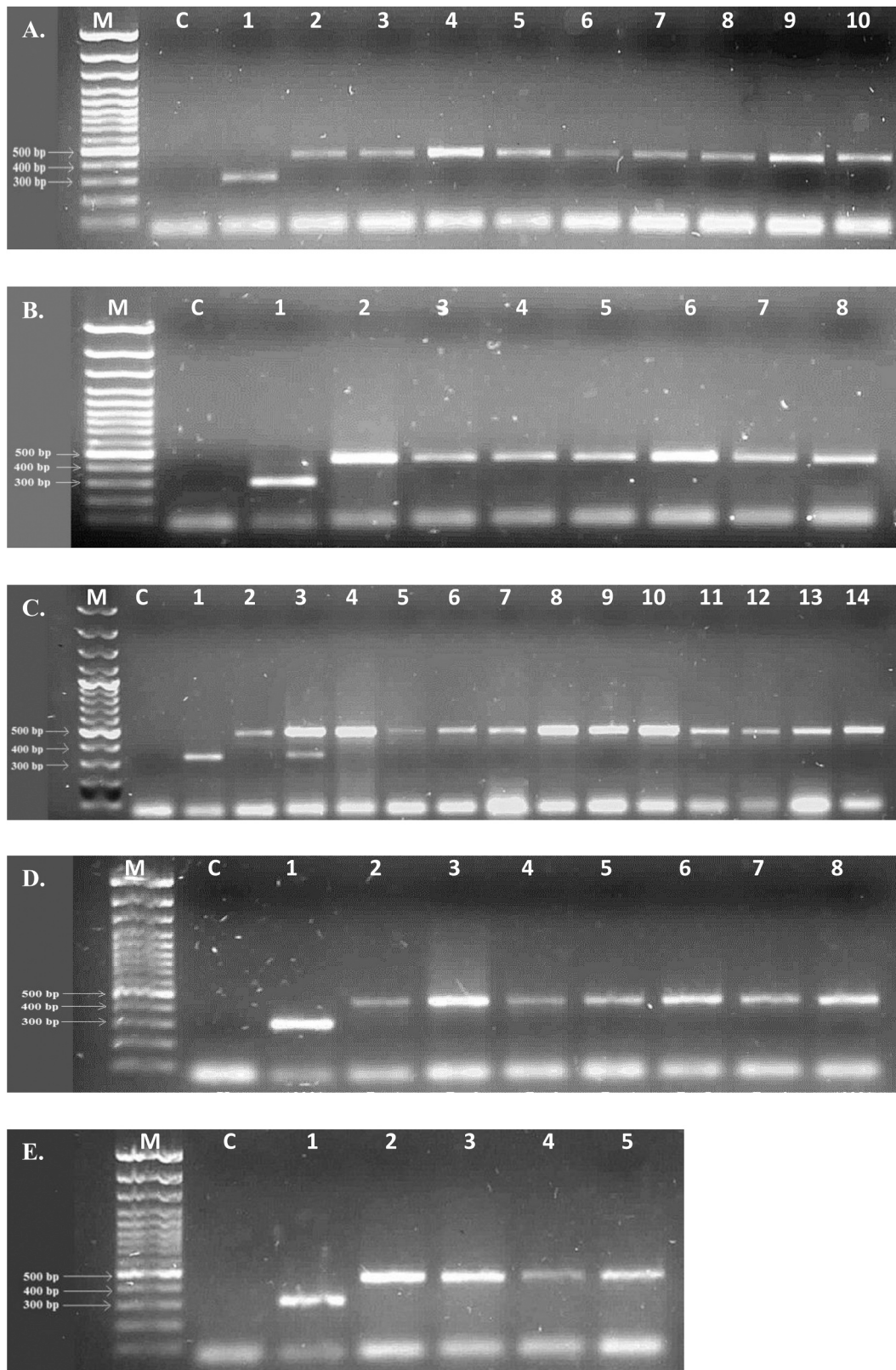
Fig. 2. Electrophoretic separation of PCR products amplified with duplex-PCR for detection of cow's and goat's mitochondrial DNA fragments. DNA used as a template was isolated form goat: [A]. milk, [B]. yoghurts, [C]. ripening cheeses, [D]. fresh cheeses, [E]. mold cheeses. Lane no 1 at Fig A-E represents 100 % cow's milk, consecutive lanes represent products obtained from: analyzed samples (lanes 2–9 on Fig. 2A; lanes 2–7 on Fig. 2B; lanes 2–13 on Fog. 2 C; lanes 2–7 on Fig. 2D and lanes 2–4 on Fig. 2E). On all pictures last lane represents the amplicon obtained from 100 % goat milk sample as positive control. C- negative control sample containing no DNA, M - 100 bp DNA ladder (Thermo Fisher Sci).

observed (Fig. 1; lane 2). In the remaining samples a distinct band of 300 bp was visible the intensity of which correlated with the increaseing amount of cow's milk added (Fig. 1, lanes: 3–7).

PCR detection of cow mitochondrial DNA was performed on 35 goat dairy products purchased from various small farm producers (Fig. 2). All products were classified as products made exclusively from goat milk. Among the products 8 milk samples (Fig. 2A, lanes 2–9), 6 yoghurts samples (Fig. 2B, lanes 2–7); 21 cheeses samples (12 samples of ripening cheeses: Fig. 2C: lanes 2–13, 6 samples of fresh cheeses: Fig. 2D: lanes 2–7 and 3 samples of mold cheeses: Fig. 2E: lanes 2–4) were analyzed. In each PCR the negative (no DNA) and positive controls (DNA isolated from 100 % cow milk -lanes 1, and DNA isolated from 100 % goat's milk) and were added.

In all analyzed product groups the negative controls used did not show the presence of contaminating DNA, what proves that the reaction was carried out in compliance with high laboratory quality standards. Among all samples the presence of cow mitochondrial DNA was detected in one sample of ripening cheese. Although classic PCR is not a quantitative method, the intensity of 300 bp band in the adulterated sample may suggest that the adulteration level was rather low and reached the value up to 10 %.

4. Discussion

One of the key issues in the protection of dairy products produced from the milk of variuos ruminants are their traceability and authenticity. Clear labeling is strongly recommended to enable consumers made their conscious choices, avoid ethical or cultural concerns and help meet legal requirements. Mislabeling of goat's milk products may be health harmful, especially for individuals allergic to cow's milk protein, who often consume goat's milk as asubstitute. Adulterated products also generate large economic losses as goat's milk is approximately several times more expensive than cow's milk. Over the last twenty years, mislabelling of dairy products, has been reported in several countries (Bottero et al., 2003, Di Pinto et al., 2004, Feligini et al. 2005,; Maskova, Paulickova, 2006; López-Calleja et al., 2007; Mafra et al., 2007; Dąbrowska et al. 2010, Cunha et al., 2016, Di Domenic et al., 2017, Deng et al. 2020). A growing demand for transparency is also observed especially for PDO (Protected Designation of Origin) products manufactured with the use of strictly defined raw materials, like buffalo milk for Mozzarella cheese production, and according to traditional recipe. Their analysis to ensure authenticity and identifiy adulterations has become of great importance (Bonizzi et al., 2006; Diaz et al., 2007; Loparelli et al., 2007).

In the study the prepared PCR standardization curve showed a robust detection ability, with a detection threshold at 0.5 % cow milk addition. This was evidenced by the clear visibility of a 300 bp band representing cow mitochondrial DNA in samples with 0.5 % or higher cow's milk content, (Fig. 1; lane 3). Sensitivity is important in practical applications, as it allows for the reliable detection of even minimal adulterations, which is crucial to maintaining the authenticity and quality of goat milk products. The method used in the study allowed to confirm the detection limit of bovine milk in goat milk products at the level of 0.5 %, which is consistent with the results obtained by Kotowicz et al. (2007) and Rodriguez et al. (2012) who used the same primers set. The application of multiplex PCR assay for detection of 0.5 % addition of bovine milk in ovine, caprine and goat milk in such mixtures was presented also by other authors (Bottero et al., 2003; Golinelli et. al. 2014; Gigliotti et al. 2022), while Bai et al. (2009), Tortorici et al. (2016) and Deng et al. (2020) presented a lower analytical sensitivity: 0.1 % of bovine milk using a uniplex PCR assay in camel, horse, goat and yak milk. Although milk is generally a good source of DNA, variations in PCR detection limits have been reported and may be explained by the wide physiological range of somatic cells, epithelial cells and leucocytes in milk in which DNA is present (Bottero et al., 2003, Dąbrowska et al., 2010, De et al., 2011, Baptista et al. 2021).

In the practical application of this method to 35 collected goat dairy products purchased from small farm producers, only one of the 35 goat's products (a ripening cheese) showed presence of cow's milk. This proves ther generally high level of authenticity of the analysed products. The presence of bovine mitochondrial DNA in this single sample suggests an adulteration level of approximately 10 %, as inferred from comparison of the band intensity to the standardization curve, although the quantative result should be precisely determined by real time PCR, as the classical PCR provides qualitative detection, not a quantitative analysis.

The low incidence of adulteration detections (1 in 35 samples) suggests that most smallholders maintain the integrity of their goat's milk products. However, the detection of adulteration in even one sample highlights the need for routine monitoring and stringent quality control measures. Ensuring the authenticity of goat's milk products is critical to consumer confidence and regulatory compliance therefore the ability to detect even low levels of adulteration helps to ensure compliance with standards and protects consumers from fraudulent practices. It is also important for protecting public health as goat's milk is often chosen for its unique properties and potential health benefits, especially for people with cow's milk allergy or intolerance. The conducted research shows that the duplex PCR method used is sensitive and reliable in quick detection of the presence of cow's milk in goat's milk products. The inclusion of a negative control (no DNA) and a positive control (DNA from 100 % cow and goat milk) in each PCR analysis confirmed the absence of contaminants and the effectiveness of the PCR reaction

5. Conclusions

In recent years, the European Commission has recognized food fraud as an important area of action. Information about the incidence of food fraud in different EU countries can help create an analytical approach to this problem, especially since there is little information about its occurrence in the EU. The growing number of small farmers producing dairy products from the milk of various ruminants creates the need to carefully monitor this type of production. The study provides new data on the incidence of adulteration in small-scale milk production, which could support the application of best practices in the identification and prevention of food fraud in the EU and enable the implementation and maintenance of high standards in the areas of authenticity of dairy products.

The results obtained in this study indicate that the use of the classic PCR technique allowed for quick detection of adulteration of goat dairy products produced by small farmers. Of the analyzed products, only one out of thirty-five contained undeclared cow's milk. This indicates good food safety standards applied by small farmers, their responsible approach and awareness, which will increase consumer confidence in this type of production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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