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Comparative Analysis of Simplex and Duplex PCR for Detection of Adulteration of Goat Milk and Its Heat-Treated Products

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ABSTRACT

Introduction: Ensuring the safety and authenticity of milk and technological products of its processing is the primary task of the dairy sector of the industry. Modern molecular genetic technologies make it possible to ensure effective detection of adulterated dairy products, namely to assess the presence of substitution of one type of milk for another. However, there is a significant lack of studies focused on the molecular identification of dairy products that have undergone various thermal processing regimes. In this regard, the influence of milk heating processes on the degradation of nucleic acids and their subsequent analysis using PCR technologies to determine the species composition in the food industry is becoming an actual direction.

Purpose: To conduct a comparative analysis of the effectiveness of simplex and duplex polymerase chain reaction (PCR) methods for determining the origin of milk and milk products subjected to different thermal treatments.

Materials and Methods: The work was carried out in the laboratory of applied microbiology and genomics of microorganisms of the All-Russian Research Institute of Dairy Industry. The objects of the study were raw, pasteurized, sterilized milk, fermented milk products on yogurt starter and binary milk mixtures of cattle and small ruminants obtained on their basis. This study aims to apply PCR technologies to solve the problem of determining the species composition of milk obtained from cow (*Bos taurus*) and goat (*Capra hircus*) and products based on them. Total DNA was extracted from food samples for subsequent analysis by simplex and duplex PCR using a set of species-specific oligonucleotide primers.

Results: The sensitivity of simplex and duplex PCR assays for milk-based products was compared and it was found that the relative detection limit for bovine DNA using duplex PCR was lower than simplex PCR and was 50 % for raw milk, 10 % for pasteurized milk and yoghurt starter sour milk. The sensitivity of detection of goat DNA by duplex and simplex PCR was at the level of 1 % except for sterilized milk mixtures: when duplex PCR was used, the detection limit for goat DNA was lower and amounted to 5 %.

Conclusion: Molecular genetic methods using mitochondrial targets make it possible to determine the origin of milk in dairy products. The possibilities of PCR application in the analysis of heat-treated dairy products are limited by the size of the amplicons obtained. PCR-based test systems provide a wide range of opportunities for composition and adulteration detection in the dairy industry.

Keywords: PCR; species identification; milk adulteration; heat treatment of milk

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Сравнительный анализ симплексной и дуплексной ПЦР для выявления фальсификации козьего молока и продуктов его термической обработки

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АННОТАЦИЯ

Введение: Обеспечение безопасности и подлинности молока и продуктов его переработки — первостепенная задача молочного сектора промышленности. Современные молекулярно-генетические технологии позволяют обеспечить эффективное выявление фальсифицированной молочной продукции, а именно оценить наличие подмены одного вида молока другим. Однако исследований, посвященных молекулярной идентификации молочных продуктов, прошедших различные температурные режимы термической обработки, крайне мало. В связи с этим, актуальным направлением становится изучение влияния процессов нагревания молока на деградацию нуклеиновых кислот и последующий их анализ с помощью ПЦР-технологий для определения видового состава в пищевой промышленности.

Цель: Провести сравнительный анализ эффективности методов симплексной и дуплексной полимеразной цепной реакции (ПЦР) для определения происхождения молока и продуктов его переработки, подвергнутых различной термической обработке.

Материалы и методы: Исследование выполнено в лаборатории прикладной микробиологии и геномики микроорганизмов Всероссийского научно-исследовательского института молочной промышленности. Объектами исследования выступали молоко сырое, пастеризованное, стерилизованное, кисломолочные продукты на йогуртовой закваске и полученные на их основе бинарные молочные смеси крупного и мелкого рогатого скота. Данное исследование направлено на применение ПЦР-технологий для решения проблемы определения видового состава молока, полученного от коровы (*Bos taurus*) и козы (*Capra hircus*) и продуктов на их основе. Из образцов пищевых продуктов выделяли суммарную ДНК для последующего анализа методом симплексной и дуплексной ПЦР с помощью набора видоспецифических олигонуклеотидных праймеров.

Результаты: Было проведено сравнение чувствительности симплексного и дуплексного ПЦР-анализа продуктов на основе молока, в ходе которого было установлено, что относительный предел обнаружения коровьей ДНК при использовании дуплексного ПЦР-анализа ниже, чем симплексного, и составил 50 % для сырого молока, 10 % — для пастеризованного молока и кисломолочного продукта на йогуртовой закваске. Чувствительность обнаружения козьей ДНК при дуплексной и симплексной ПЦР оказалась на уровне 1 % за исключением смесей стерилизованного молока: в случае использования дуплексной ПЦР предел обнаружения козьей ДНК был ниже и составил 5 %.

Выводы: Молекулярно-генетические методы с использованием митохондриальных мишеней позволяют определять происхождение молока в молочной продукции. Возможности применения ПЦР при анализе молочных продуктов, прошедших термическую обработку, ограничены размером получаемых ампликонов. Тест-системы на основе ПЦР предоставляют широкие возможности для определения состава и выявления фальсификации продукции в молочной промышленности.

Ключевые слова: ПЦР; видовая идентификация; фальсификация молока; тепловая обработка молока

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INTRODUCTION

Milk is a highly nutritional product that plays a key role in human nutrition. It provides the body with essential proteins, lipids, minerals, fatty acids, vitamins, and other vital components (Zobkova et al., 2018, Kourkouli et al., 2024). According to the UN Food and Agriculture Organization, milk is one of the animal products that can contribute to the Sustainable Development Goals established by the UN General Assembly[1]. When consumed properly, milk can help combat undernutrition in children under the age of five, low birth weight and stunted growth, anemia in women of reproductive age, as well as obesity and infectious diseases[2]. The recommended daily intake of milk and dairy products is 325 kg per capita per year (Agarkov et al., 2023). Analytical studies predict an annual growth of 7.32 % in the dairy market from 2024 to 2028[3].

Cow and goat milks are among the most popular types of milk in the world (Hazra et al., 2017). In 2023, Russia produced 33.5 million tons of cow milk, an increase of 1.2 million tons from 2021 (Zimnyakov et al., 2023). The annual production of goat milk varies from 236,000 to 255,000 tons[4]. The composition and properties of milk largely depend on its source (Shuvarikov et al., 2018; Barłowska, et al., 2012). Cow milk is the most accessible and affordable type of milk. A cow gives much more milk than other dairy cattle (~15 L/day). Compared to goat milk, cow milk contains more iron, sulfur, zinc, molybdenum, ribonuclease, lipase, alkaline phosphatase, and xanthine oxidase. However, goat milk is higher in calcium, potassium, magnesium, phosphorus, chlorine, and manganese. It is also lower in lactose, which makes it more suitable for people with a lactase deficiency (Merkusheva et al., 2005; Golinelli et al., 2014).

The modern dairy industry is facing a growing trend towards products made from a mixture of cow and goat milk. Such products not only combine the unique nutritional properties of both milks, but also are fairly affordable for the consumer. Goat milk is also used as a fortifying component to create functional products (Meldenberg et al., 2020; Lad et al., 2017).

However, since raw milk has a short shelf life, milk products are often exposed to mechanical (separation, homogenization, etc.) or thermal (pasteurization, sterilization, etc.) treatments (Shegidevich, 2021; Charykov et al., 2017). Moreover, it may be difficult to identify the exact composition of dairy products that have undergone various processes and to establish milk substitution in the products from goat and other types of milk (Lopez-Calleja et al., 2004).

The fight against adulteration of dairy products is a high priority for the global community, since such products are consumed by vulnerable social groups: children, pregnant women, the elderly, and sick people. Adulterated dairy products pose a serious threat to their health and lives due to possible allergic reactions and other negative effects (Gilmanov et al., 2020; Handford, et al., 2016). Infant formulas deserve a special attention since substituting one type of milk for another is totally unacceptable in this product. Many consumers prefer formulas based on goat milk since it has been proven to be better tolerated by the baby's organism (Zakharova et al., 2021). However, despite the growing popularity of these products, there are still no standards to govern the testing of milk for its species specificity.

Molecular genetic methods are widely used to determine the species specificity of dairy products (Gilmanov et al., 2020; Pokorska, et al., 2016). One of them is a simplex polymerase chain reaction (PCR) carried out with one pair of primers (Wang et al., 2020; López-Calleja et al., 2004; Hazra et al., 2017). Simplex PCR can identify the DNA of one or several animal species. Yet, to identify several species, it needs universal primers that can yield PCR products of different lengths after amplification. However, it is not always possible to create universal primers that would produce unambiguous results on the electrophoregram. In other words, it may be difficult to create such primers that would clearly distinguish between different species based on the length of the resulting PCR products. Therefore, an alternative multiplex PCR (M-PCR) is often performed, which requires a simultaneous use of several sets of primers in one reaction.

[1] Assembly, G. (2017). Resolution adopted by the General Assembly on 6 July 2017. In Technical Report A/RES/71/313.

[2] FAO. (2023). Contribution of Terrestrial Animal Source Food to Healthy Diets for Improved Nutrition and Health Outcomes–an Evidence and Policy Overview on the State of Knowledge and Gaps.

[3] Statista. (2024). Dairy products & eggs worldwide: Statista's forecast. https://www.statista.com/outlook/cmo/food/dairy-products-eggs/world-wide

[4] Milknews (2023). What is happening in the cow's milk market. https://milknews.ru/longridy/Chto-proishodit-na-rynke-kozego-moloka.html

Modern multiplex PCR systems can identify donkey, camel, horse, goat, and cow milks or dairy products from them (Deng et al., 2020). Previous studies have mainly focused on the detection of species adulteration in raw milk (Kourkouli et al., 2024; Rodrigues et al., 2012) and/or cheese (Golinelli et al., 2014). Very few studies have explored potential problems with identifying dairy products exposed to different heat treatments. Since the range of dairy products is quite diverse, the dairy industry should intensively expand the application of universal methods to detect adulteration in foods regardless of their production methods.

We aimed to compare the effectiveness of simplex and duplex PCR methods in determining the origin of raw milk and its heat-treated products.

MATERIALS AND METHODS

Study Objects

Raw goat and cow milks were purchased at a local market. In a laboratory, they were used to prepare pasteurized and sterilized milk samples, fermented milk products with yoghurt starter, and binary mixtures of milk matrices with different volumes of the milks.

Samples Preparation

Pasteurized and Sterilized Milk

Raw milk samples were pasteurized at 90 ± 2 °C for 5 minutes in a water bath. Raw milk samples were sterilized by autoclaving at 121 ± 2 °C and 0.15 MPa for 3 minutes in an NB-1100 steam sterilizer (N-Biotek, South Korea).

Fermented Milk Products with Yoghurt Starter

Fermented milk products were produced with liquid starters made from sterilized goat and cow milks. To prepare the starters, each milk sample was inoculated with the cultures *Str. thermophilus* (strain 6kb) and *Lactobacillus delbrueckii subsp. bulgaricus* (strain L37/7) from the collection of the All-Russian Research Institute of Dairy Industry (VNIMI). Then, the milk samples were cooled to the fermentation temperature and incubated at 40 ± 2 °C in a thermostat overnight.

To produce fermented milk products, 5 % liquid yogurt starter was added to the pre-pasteurized and cooled milk samples. The mixtures were incubated again at 40 ± 2 °C for 4 hours. Then, the fermented milk products were cooled to 25–30 °C, thoroughly mixed, and kept in a refrigeration chamber at 4 ± 2 °C overnight.

Binary Mixtures

Milk mixtures with different ratios of goat and cow milks were prepared from the obtained heat-treated products, with goat milk proportions of 100, 99, 98, 95, 90, 85, 80, 75, 70, 60, 50, 40, 30, 25, 20, 10, 5, 2, 1 and 0 %.

DNA Extraction

Milk product samples of 1 ml (liquid) or 0.5 ml (yoghurt) were centrifuged at 10,000 g for 5 minutes. The supernatant was removed and the resulting sediment was used to extract nucleic acids with a DNA-Sorb-S-M kits (Central Research Institute of Epidemiology, Russian Federal Service for the Oversight of Consumer Protection and Welfare) following the manufacturer's instructions.

Oligonucleotide Primers

Oligonucleotide primer sequences were adopted from Deng et al., 2020 (Table 1).

Table 1

Characteristics of Oligonucleotide Primers

Gene	Primer	Sequence	Size of Product	Localization
16S rRNA	BT-F	5′-ACCCTCTCGACTAAACAACCAAGATAG-3′	583 bp	NC_006853.1
	BT-R	5′- TGGGGCTAGGAGTTAATCATTTGTTG -3′		
D-loop	CH-F	5′- ACTCCACAAGCTTACAGACATGCCA -3′	184 bp	NC_005044.2
	CH-R	5′- GAAGGCTGTATGTCCGCGTTATATG -3′		

Simplex and Duplex PCR Amplification

A specific region of the 16S rRNA gene of *Bos taurus* was amplified by PCR using the species-specific primers BT-F and BT-R at an annealing temperature of 57 °C. PCR screening was performed in 25 µl of the reaction mixture consisting of 5 µl of 5xScreen Mix (Evrogen, Russia), 1 µl of 10 mM BT-F primer, 1 µl of 10 mM BT-R primer, 1 µl of a DNA sample, and 17 µl of deionized water (Evrogen, Russia).

A specific region of the D-loop gene of *Capra hircus* was amplified by PCR using the species-specific primers CH-F and CH-R. PCR screening was performed in 25 µl of the reaction mixture consisting of 5 µl of 5xScreen Mix (Evrogen, Russia), 1 µl of 10 mM CH-F primer, 1 µl of 10 mM CH-R primer, 1 µl of a DNA sample, and 17 µl of deionized water (Evrogen, Russia).

The reaction mixture for a simultaneous duplex PCR amplification of specific fragments of the mitochondrial genomes of cows and goats consisted of 5 µl of 5xScreen Mix (Evrogen, Russia), 1 µl of 10 mM BT-F primer, 1 µl of 10 mM BT-R primer, 1 µl of 10 mM CH-F primer, 1 µl of 10 mM CH-R primer, 1 µl of a DNA sample, and 15 µl of deionized water (Evrogen, Russia).

The amplification was performed on a MiniAmp thermal cycler (ThermoFisher Scientific, USA) in the following stages: 1) primary denaturation at 95 °C for 5 minutes; 2) 35 cycles of denaturation at 95 °C for 15 seconds, primer annealing at 57 °C for 15 seconds, chain elongation at 72 °C for 30 seconds, and final chain elongation at 72 °C for 10 minutes. The theoretically expected lengths of the amplicons for *Bos taurus* and *Capra hircus* were 583 bp and 184 bp, respectively.

The controls for PCR analyses included a negative control with water added to the reaction mixture instead of DNA, a control with a cow's pure genomic DNA, and a control with a goat's genomic DNA, both isolated from the ear notches of the animals.

Analytical Electrophoresis

The PCR results were analyzed by separating the amplicons in a 2 % agarose I gel (VWR International, USA) stained with an ethidium bromide solution at an electric field voltage of 7 V/cm of the gel. The 100+ bp DNA Ladder (Evrogen, Russia) was used as a DNA fragment length marker. The gel electrophoresis results were visualized using a Vilber E-Box-CX5.TS gel documentation system (Vilber, France) on a Vilber Super-Bright transilluminator (Vilber, France) at 312 nm.

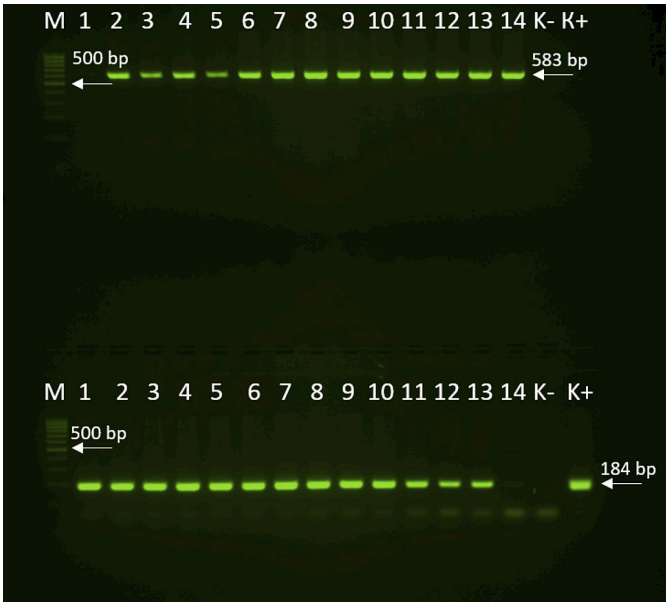
RESULTS

Analysis of Binary Milk Matrices by Simplex PCR

First, we tested the BT-F and BT-R primers for their ability to detect the target sequence of ribosomal 16S RNA of the *Bos taurus* (cow) genome by simplex PCR in the model mixtures of goat and cow milks simulating different levels of milk adulteration. Similarly, we detected the presence of the goat DNA in the above-described binary milk mixtures using the CH-F and CH-R primers complementary to the sequences of the *Capra hircus* (goat) mitochondrial genome. The milk matrices contained 1–99 % of cow milk admixture (v/v). According to the electrophoregrams, the BT-F and BT-R primers could effectively detect cow milk admixtures (from 1 %) in both raw (Figure 1) and

Figure 1

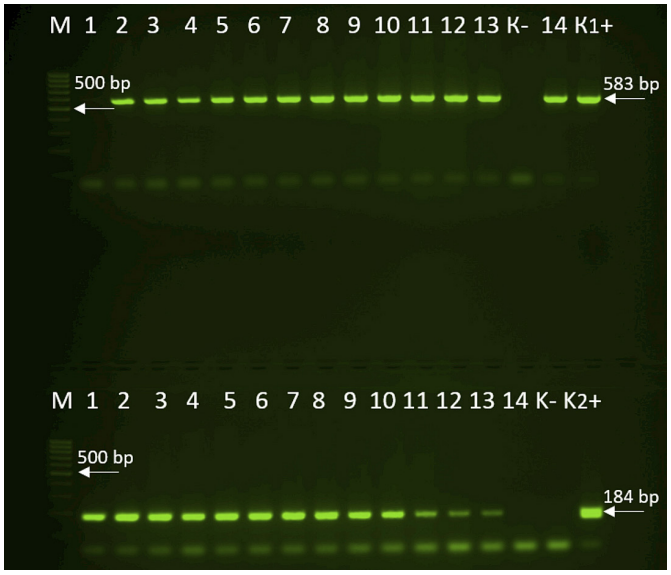
Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle (Top Row) and D-Loop in Goats (Bottom Row) During Simplex PCR (Raw Milk)



Note. 1–14 — DNA samples extracted from raw goat and cow milk mixtures in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K+" — goat DNA; M — DNA length marker "100 + bp DNA Ladder."

Figure 2

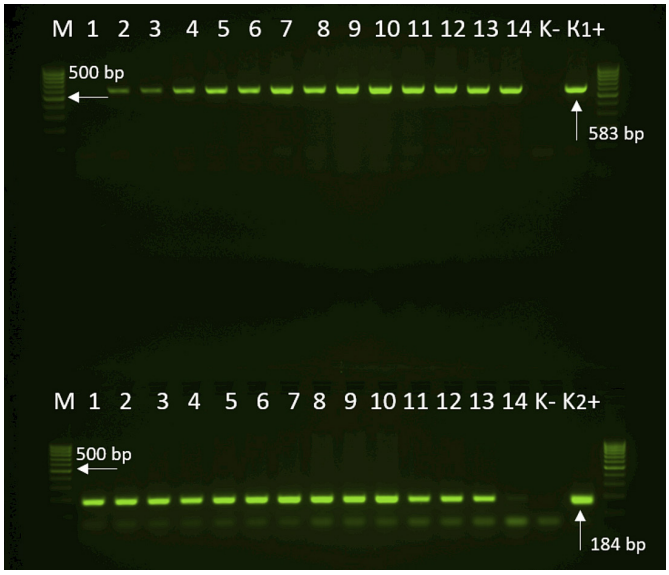
Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle (Top Row) and D-Loop in Goats (Bottom Row) During Simplex PCR (Pasteurized Milk)



Note. 1–14 — DNA samples extracted from mixtures of pasteurized goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K1+" — cow DNA; "K2+" — goat DNA; M — DNA length marker "100 + bp DNA Ladder."

Figure 3

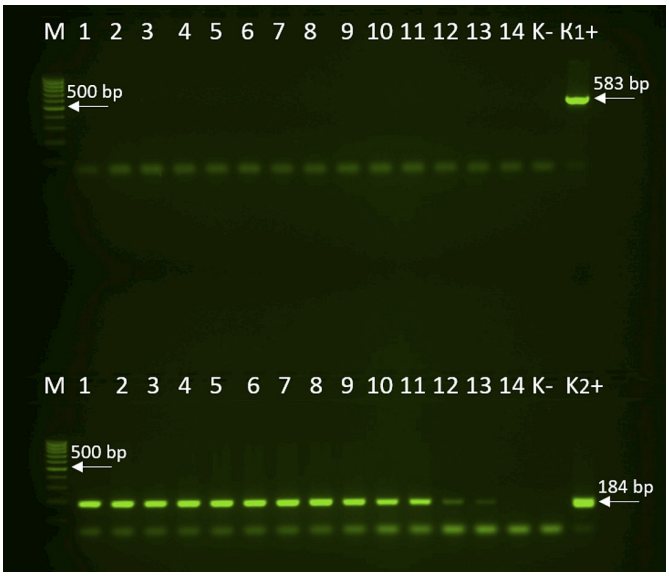
Electrophoregram of PCR Amplification Products of 16S rRNA Gene Fragments in Cattle (Top Row) and D-Loop in Goats (Bottom Row) (Fermented Milk Products)



Note. 1–14 — DNA samples extracted from fermented milk products made with yogurt starter cultures from mixtures of pasteurized goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K+" — goat DNA; M — DNA length marker "100 + bp DNA Ladder."

Figure 4

Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle (Top Row) and D-Loop in Goats (Bottom Row) During Simplex PCR (Sterilized Milk)



Note. 1–14 — DNA samples extracted from mixtures of sterilized goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K1+" — cow DNA; "K2+" — goat DNA; M — DNA length marker "100 + bp DNA Ladder."

pasteurized (Figure 2) goat milk. Notably, all the samples with the cow DNA showed an amplicon with a theoretically expected size of 583 bp.

In addition, the simplex PCR assay with the CH-F and CH-R primers was highly sensitive to the goat DNA and was able to detect the presence of goat milk (≥1 %) in various binary milk mixtures (Figures 1–4), since all the samples containing the goat DNA had a species-specific amplification product of 184 bp.

Similar results were shown by simplex PCR to detect cow milk in the fermented milk products prepared with yoghurt starter. The detection limit for the cow DNA was 1 % (Figure 3).

However, no reaction products were observed when we attempted to amplify a 583 bp fragment of the bovine 16S rRNA gene on a total DNA matrix isolated from sterilized milk (Figure 4). This might be due to severe damage caused to DNA molecules by the sterilization of raw milk, resulting in fragments whose length was insufficient for the synthesis of amplicons of the required length. Nevertheless, we detected amplified fragments of the goat DNA genes, which confirms the possibility of identifying PCR products of shorter length.

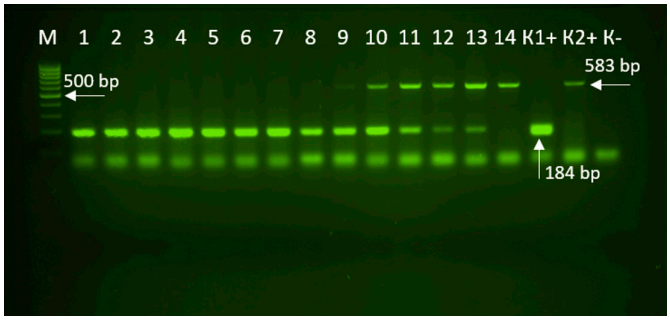
Analysis of Binary Milk Matrices by Duplex PCR

Duplex PCR was performed with two pairs of primers in one tube to determine the relative sensitivity of the assay. The mixtures with a minimal content of cow milk showed no products of 583 bp, but there were bands corresponding to the target product from the goat genome. However, the number of amplified products from the cow genome increased in the samples with higher contents of the target *Bos taurus* DNA template. This resulted in fragments of the theoretically expected length, starting with sample 9 corresponding to a 50 % cow milk admixture (Figure 5).

Duplex PCR analysis of the pasteurized milk mixtures had a relative detection limit of 10 % for cow milk, which was lower than that of simplex PCR (Figure 6). In contrast, the amplicons obtained from the goat mitochondrial DNA were visually distinguishable in samples 1–10 in the electrophoregrams of all binary matrices (Figures 5–8). This indicated high sensitivity of duplex PCR in amplifying the goat DNA, whose lowest limit was 5 % for sterilized milk and 1 % for the other products (Figures 5–7).

Figure 5

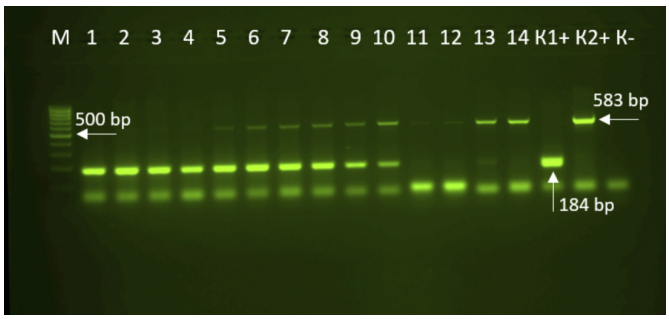
Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle and D-Loop During Duplex PCR (Raw Milk)



Note. 1–14 — DNA samples extracted from mixtures of raw goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K1+" — goat DNA; "K2+" — cow DNA; M — DNA length marker "100 + bp DNA Ladder."

Figure 6

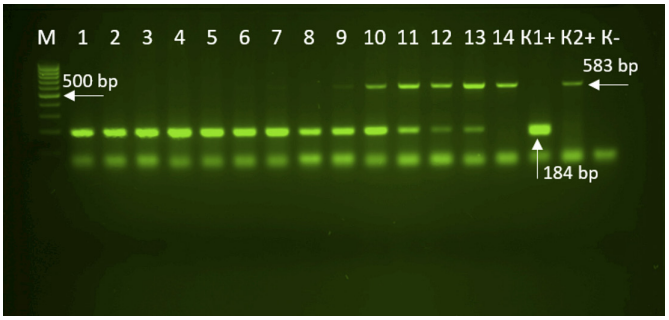
Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle and D-Loop During Duplex PCR (Pasteurized Milk)



Note. 1–14 — DNA samples extracted from mixtures of pasteurized goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K1+" — goat DNA; "K2+" — cow DNA; M — DNA length marker "100 + bp DNA Ladder."

Figure 7

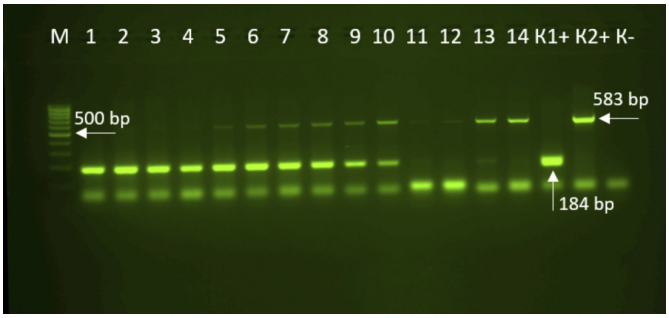
Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle and D-Loop During Duplex PCR (Fermented Milk Products)



Note. 1–14 — DNA samples extracted from fermented milk products made with yogurt starter cultures from mixtures of goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K1+" — goat DNA; "K2+" — cow DNA; M — DNA length marker "100 + bp DNA Ladder."

Figure 8

Electrophoregram of Amplification Products of 16S rRNA Gene Fragments in Cattle and D-Loop During Duplex PCR (Sterilized Milk)



Note. 1–14 — DNA samples extracted from mixtures of sterilized goat and cow milk in the following ratios: 100, 99, 98, 95, 90, 85, 80, 75, 50, 25, 5, 2, 1, and 0 % (goat milk); "K-" — negative control; "K1+" — goat DNA; "K2+" — cow DNA; M — DNA length marker "100 + bp DNA Ladder."

Compared to PCR with one pair of primers, PCR with two pairs of primers demonstrated lower sensitivity on fermented milk products made with yoghurt starter from mixtures of goat and cow milk. The detection limit was 10 % for a cow milk admixture.

Duplex PCR also showed the specific amplification of only the *C. hircus* D-loop fragment (Figure 8).

When conducting duplex PCR on the DNA mixtures of sterilized goat and cow milk, we expected the sensitivity of the goat DNA detection to remain 1 %, as in simplex PCR. However, it turned out to be lower, amounting to 5 %. This decrease may be due to the presence of four primers in the PCR reaction, which had a negative effect on its efficiency. The amplification results of the bovine DNA were similar to those for the PCR with one pair of primers.

DISCUSSION

In this study, we aimed to compare the efficiency of modern molecular genetic methods (simplex and multiplex PCR) for the species identification of milk and milk-based products exposed to different heat treatments. We performed simplex and multiplex PCR analyses of the milk matrix samples using primers for C. hircus and B. taurus. Thermal cycling conditions were identical for all PCRs. The amplicons were then separated in gel to identify the most optimal PCR method. Our results showed higher efficiency of simplex PCR in identifying dairy products compared to duplex PCR. We found that the use of one or two pairs of oligonucleotide primers had a significant effect on the sensitivity of the molecular biology methods. Simplex PCR proved reliable due to its ability to detect even trace amounts of milk adulteration, which is critical when testing dairy products. This is probably because competing processes occur with oligonucleotides in the reaction mixture, decreasing the accumulation of target amplification products during multiplex PCR (Kalle, et al., 2014). There is a risk of non-specific binding of single-stranded DNAs, which can lead to amplification of primer dimers and a decrease in sensitivity. For example, Hird et al. (2006) assessed the relationship between the amplification rate and the size of amplicons obtained from food matrices. The authors found that the small size of amplicons increased the probability of amplification and, at the same time, made non-specific reaction products more likely to appear. Their results confirmed our assumption that the molecular genetic methods are optimal for detecting

specific genetic loci in milk products. Of particular interest was the PCR analysis of sterilized milk, where only short DNA fragments were amplified. The influence of milk heating processes on the degradation of nucleic acids needs to be studied further.

Several studies have focused on modern molecular analysis methods for testing the authenticity of consumer goods (Lopez-Calleja et al., 2004; Golinelli et al., 2014; Galal-Khallaf et al., 2021). The most widely used of such methods are those based on the polymerase chain reaction with one or more pairs of oligonucleotide primers (simplex, duplex, and multiplex PCR), which allow scientists to amplify target genetic markers (De et al., 2011; Guo et al., 2018; Lopez-Calleja et al., 2004; Galal-Khallaf et al., 2021). The dairy and meat industries commonly use ribosomal genes, such as 12S and 16S rRNA, which play a key role in cellular transcription processes (Tuncay et al., 2022). The use of the 16S rRNA gene is particularly common in phylogenetic studies due to its presence in all cellular forms, high conservation of its functions, alternation of conserved and variable regions within the gene, and the absence of its horizontal transfer from organism to organism (Stackebrandt, 2009). Mitochondrial DNA, including the cytB and D-loop genes, is also frequently used in the studies on milk authenticity. The D-loop is of particular value for these studies due to its high variability (Putri et al., 2019). For example, Deng et al. (2020) used mitochondrial targets such as 16S rRNA and D-loop to study adulteration of different types of raw milk and its heat-treated products. Their experiments involved pasteurization at 62–65 °C for 30 minutes and sterilization at 135–150 °C for 2–6 seconds. The temperature conditions in our study were different, namely pasteurization at 90 ± 2 °C for 5 minutes and sterilization at 121 ± 2 °C for 3 minutes. In addition, we analyzed the fermented milk products exposed to the above heat treatments.

Our results showed that when designing oligonucleotide primers to amplify fragments of species-specific molecular markers, we should pay attention to the size of amplicons in the test systems for identifying the composition of heat-treated dairy products. Our data also emphasized the importance of combined processing of raw milk in case of process disruption, since high-temperature treatment can have a negative effect on nucleic acids. To make duplex PCR more sensitive and as efficient as simplex PCR, a primer panel needs to be developed to amplify 100 bp sections of mitochondrial genomes of ruminants. Furthermore, special consideration should be given to various heat treatment parameters for dairy raw materials, including the temperature range, exposure and cooling time, etc.

One of the key limitations in our study was a narrow range of temperature and time parameters for the production of dairy products. Moreover, the pairs of primers we used for molecular testing were capable of amplifying only 184 bp or 583 bp PCR products. This did not allow us to assess potential risks of false-negative or false-positive PCR results when fragments of greater or lesser length were synthesized. Therefore, raw milk needs to be tested with those oligonucleotide primers which can synthesize fragments of other lengths. Despite these limitations, our

results demonstrate the main problems of analyzing heat-treated milk products and ways to overcome them.

CONCLUSION

Our study confirmed the efficiency of using molecular genetic methods (particularly, simplex and duplex PCR) for the species identification of milk products exposed to various heat treatments. Due to its higher sensitivity, simplex PCR was better at detecting small amounts of milk admixtures. Noteworthily, sterilized milk samples require more robust analysis methods since sterilization significantly destroys the milk's DNA and complicates the amplification of larger gene fragments. This notwithstanding, the proposed molecular genetic methods can effectively detect adulteration in dairy products, which is an important step in protecting consumer rights and ensuring product authenticity. These methods can be used to control the production of dairy products or develop new standards for species identification in the dairy industry.

The above methods can be made more accurate and reliable by developing more sensitive test systems with primer panels capable of amplifying short DNA fragments. Further research should also focus on the influence of various process parameters on DNA fragmentation. This will improve the existing methods for monitoring the authenticity of dairy products and expand their applications in the industry.

AUTHOR CONTRIBUTIONS

Alexey V. Khan: overall supervision and study design; manuscript preparation and development; conducting experimental research; data collection and analysis.

Daria D. Koval: manuscript preparation and development; conducting experimental research; data collection and analysis.

Ekaterina G. Lazareva: visualization; formulation of research objectives and tasks; data collection and analysis.

Oleg Yu. Fomenko: overall supervision and study design; data curation; editing, and approval of the final version of the article.

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