Research Progress on Mold Detection Technology in Milk and Dairy Products

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Abstract This paper reviews the research progress on mold detection technologies in milk and dairy products, including rapid test sheet methods, molecular biological detection techniques, metabolomics detection techniques, enzyme-linked immunosorbent assay (ELISA), and microbial rapid photoelectric detection systems, aiming to provide optimal choices for mold detection.

Key words Dairy products, Mold, Detection technology

0 Introduction

Molds are widely distributed in natural environments such as air, soil, and water bodies. Some molds often produce toxic and structurally stable secondary metabolites during their growth and reproduction, including aflatoxins, zearalenone, ochratoxins, patulin, fumonisins, *etc.*[1-2]. When dairy cows consume feed contaminated with molds, it can not only cause poisoning in the cows but also lead to the presence of mycotoxins in their milk. Human ingestion of contaminated milk and dairy products may result in poisoning, carcinogenesis, teratogenesis, and even life-threatening consequences in severe cases.

With the annual increase in the consumption of milk and dairy products, China's milk production and dairy product output have also shown a rapid growth trend. From 2013 to 2023, China's milk production achieved continuous growth, increasing from 30.01 million t to 41.97 million t, representing a growth of 39.8% over ten years. From 2018 to 2023, China's dairy product output showed slow growth, reaching 41.97 million t by 2023. In addition to the increase in domestic production, China's dairy product imports have also consistently remained above 3 million t in recent years. Meanwhile, China's per capita total dairy consumption is 43 kg, which, although far below the world average, is also growing steadily. Due to their richness in nutrients such as protein, vitamins, and minerals, dairy products are susceptible to microbial contamination by molds, yeasts, bacteria, *etc.*, which can lead to food safety incidents[3-6]. Xiao Dinghan *et al.*[7] collected milk samples from five dairy farms in Beijing and conducted microbiological testing. The results showed that out of 128 milk samples, 42 contained fungi, indicating a fungal contamination rate as high as 32.8%. Among these, molds accounted for 19%, primarily belonging to the genera *Aspergillus* and *Mucor*. Griffin *et al.*[8] also found that 27% of hard sheep cheese and 17% of soft sheep cheese were contaminated with *Penicillium* molds. Xu Pei *et al.*[9] also found that mold contamination in fruit-flavored fermented milk was significantly higher than in plain fermented milk. This may be due to the higher sugar content in the fruit juice making it more susceptible to microbial contamination. Besides being isolated from dairy products such as yogurt, cheese, and butter[10-12], molds have also been isolated from samples of both healthy milk and clinical mastitis milk[13].

Mold detection is one of the mandatory items in routine testing of dairy products. Currently, the plate count method specified in the GB 4789.15-2016 standard is primarily used. Although this method is simple to operate and yields accurate results, it is time-consuming, requiring 5 d of incubation to obtain results. With the increasing consumer demand for high-quality dairy products characterized by freshness and short shelf life, higher requirements are being placed on mold detection methods. This paper focuses on elaborating the applications of rapid test sheet methods, molecular biological detection techniques, metabolomics detection techniques, enzyme-linked immunosorbent assay (ELISA), and microbial rapid photoelectric detection systems in mold detection.

1 Rapid test sheet methods

Rapid test sheet methods involve attaching specific culture media and chromogenic substances to carriers such as paper sheets, filter paper, or non-woven fabrics, which serve as the substrate. Target microorganisms are identified based on their growth, metabolism, and color development on these carriers. The principle is that target microorganisms metabolically produce specific enzymes. These enzymes break down specific substrates in the culture medium, causing the release of chromogenic substances and imparting a specific color to the microbial colonies. The microbial species can then be identified based on this color reaction. Commonly used chromogenic substances include 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 2-(2-methoxyphenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride[14], hexosamine, VLPA-GLc-

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NAC, *etc.* Although rapid test sheet methods reduce the time required for mold detection and are easy to store, transport, and carry, the spreading growth of hyphae in some molds can make colonies uncountable. Additionally, specific chromogenic agents can only indicate specific types of molds and cannot indicate all mold species. Wang Wei *et al.* [15] simultaneously used the Petrifilm™ Rapid Yeast and Mold Count Plate method and the GB 4789.15-2016 national standard method to detect total mold counts in mold quality control samples, standard strains, and yogurt samples. The results showed that the detection results from both methods were consistent, with no statistically significant difference. Furthermore, the Petrifilm™ Rapid Yeast and Mold Count Plate demonstrated high specificity. Zhang Rong[16], Zhao Hongyang[17], and Vlaemynck *et al.* [18] also reached the same conclusion.

2 Molecular biological detection techniques

2.1 Reverse transcription PCR (RT-PCR) Reverse transcription PCR (RT-PCR) is a molecular biological detection technique that combines the polymerase chain reaction (PCR) with the reverse transcription of RNA. The principle involves using cellular mRNA as a template. Under the action of reverse transcriptase, random hexamers, oligo(dT) primers, or specific primers are used to synthesize a complementary single-stranded DNA (cDNA) strand to the mRNA. This cDNA is then amplified into the target gene through catalysis by DNA polymerase. Vaitilingom *et al.* [19] successfully detected molds and yeasts in ultra-high temperature (UHT) treated milk within 4 h using RT-PCR with the elongation factor EF-1α as the specific primer, achieving a detection limit of 10 cells/mL. The study also showed that this method could be effectively used for detecting molds in yogurt. Bleve *et al.* [20] employed an optimized RT-PCR method that effectively detected fungi in food. In broth culture, fungi could be detected at concentrations as low as 10 CFU/mL. However, in artificially contaminated food, sensitivity decreased due to factors such as food pH, moisture content, and organic acid content. The detection limit in a yogurt matrix was 10^3 CFU/mL.

2.2 Real-time fluorescent quantitative PCR Real-time fluorescent quantitative PCR (qPCR) is a molecular biological technique that measures the total amount of PCR amplification product during the DNA amplification process. This is achieved using fluorescent probes or dyes such as TaqMan, LightCycler, molecular beacons, or SYBR Green. Specific DNA sequences in the test sample are quantitatively analyzed using either an endogenous reference method or an exogenous reference method. Its principle relies on monitoring the entire amplification process of the target DNA in real-time by measuring the accumulation of fluorescent signals. Finally, the target DNA is quantified using a standard curve. Real-time fluorescent quantitative PCR (qPCR) is applicable not only for the quantitative detection of molds and yeasts in milk, cream, whey, and milk powder[21], but also for the determination of fungi in yogurt and cheese[22]. Bleve *et al.* [23] developed a novel real-time fluorescent quantitative PCR (qPCR) technique. This technique achieved a regression coefficient (R^2) of 0.877 and could complete the quantitative detection of fungi in yogurt within 10 h. Dréan GL *et al.* [24] developed a real-time fluorescent quantitative PCR (qPCR) technique. This technique not only specifically and quantitatively detected *Penicillium camemberti* and *Penicillium roqueforti* in curd cheese, but also provided a theoretical basis for the growth kinetic models of *P. camemberti* and *P. roqueforti* in cheese. Compared with traditional detection methods, real-time fluorescent quantitative PCR (qPCR) can complete the quantitative analysis of molds in yogurt within 5 h[25]. Su Huiyu *et al.* [26] employed immunomagnetic beads combined with real-time fluorescent quantitative PCR (qPCR) to detect *Aspergillus flavus* in food, achieving a detection limit of 2 CFU/mL. Moreover, this technique removed PCR inhibitors, enhancing the anti-interference capability of the detection. Real-time fluorescent quantitative PCR (qPCR) can also be combined with isothermal amplification techniques. This combination lowers the amplification temperature, eliminates reliance on expensive detection equipment, and improves the flexibility and convenience of detection[27].

2.3 Multiplex PCR technology Multiplex PCR technology refers to the amplification of multiple target genes by adding several different primers in the same amplification reaction system. This technique not only reduces the requirement for sample volume but also enables the detection and identification of multiple microorganisms in a single test. Xiong Suyue *et al.* [28] designed five pairs of primers based on specific genes of four bacteria (*Staphylococcus aureus*, *Salmonella*, *Escherichia coli* O157∶H7, *Listeria monocytogenes*) and the fungal 18S rRNA gene. Using multiplex PCR technology, they achieved simultaneous detection of these four pathogenic microorganisms and four molds (*Penicillium nalgiovense*, *Aspergillus flavus*, *Penicillium islandicum*, *Aspergillus nidulans*) in food, with a detection limit as low as 1 CFU/25 g. Zhang Qing[29] developed a duplex droplet digital PCR (ddPCR) based on real-time fluorescent PCR. This achieved quantitative detection of *Aspergillus ochraceus*, *Penicillium* spp., aflatoxin-producing fungi, and *Fusarium* spp. Compared with real-time fluorescent PCR, the detection limit was significantly improved by approximately 3 orders of magnitude. Furthermore, the quantitative range for the specific target genes of both aflatoxin-producing fungi/*A. ochraceus* and *Penicillium* spp./*Fusarium* spp. was 2×10^{-7} to 2×10^{-3} ng/μL.

3 Metabolomics detection techniques

3.1 ATP bioluminescence technology ATP bioluminescence technology is based on the chemical reaction between adenosine triphosphate (ATP) and luciferin, catalyzed by luciferase, which releases light (bioluminescence). A specific detection system is used to measure the light intensity. Within a certain concentration range, the light intensity is proportional to the ATP concentration. As the source of energy for all life activities in living cells, ATP is present in all cells, its content is relatively constant, and it is pro-

portional to the number of cells. Therefore, the number of microorganisms in a test sample can be calculated by detecting the light intensity. This method is fast, typically completing detection within 5 min; the detection is not affected by the physical state of the sample (both liquid and solid can be tested), and the detection process does not alter the original structure or chemical state of the sample[30-32].

3.2 Impedance detection technology Impedance detection technology is a method to determine the number of microorganisms in a test sample by detecting changes in the electrical conductivity of the microbial culture medium. The principle is that microbial growth and metabolism convert electroinert substances added to the medium into electroactive substances. This causes an increase in the conductivity of the medium and a decrease in its impedance. The number of microorganisms is then calculated by measuring the time required for the impedance change to exceed a predetermined threshold value[33]. This method can detect test samples with viable bacterial counts below 10 CFU/mL[34].

4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) utilizes antigens or antibodies adsorbed onto a solid-phase carrier. These bind specifically to enzyme-labeled antibodies or antigens, forming immune complexes. When combined with the test sample, a color reaction occurs. The extent of this color reaction is used for the qualitative or quantitative analysis of the test sample[35]. This technique can be used not only for detecting common foodborne pathogens but also for detecting molds such as *Aspergillus*, *Penicillium*, and *Mucor* in dairy products[36]. Tsai *et al.*[37] developed a double-antibody sandwich ELISA method that could detect *Aspergillus* versicolor, *Cladosporium* spp., *Geotrichum candidum*, *Mucor* spp., and *Penicillium chrysogenum* in Cheddar cheese, soft cheese, and soft yogurt within 2 d. This significantly shortened the detection time compared to national standard methods. Its sensitivity was 1 ng/mL to 1 μg/mL.

5 Microbial rapid photoelectric detection system

The microbial rapid photoelectric detection system is a detection technology based on changes in the color of an indicator in the culture medium caused by the cultivation and metabolism of microorganisms, used to determine the number of fungi in the test item[38]. Common microbial rapid photoelectric detection systems include the BioLumix Microbial Fluorescence Photoelectric Detection System, the Soleris Real-Time Photoelectric Microbial Rapid Detection System, and the BioFirst Real-Time Multi-Temperature Zone Microbial Rapid Photoelectric Detection System[39], among others. All these systems can complete the detection of molds in samples within 48 hours.

Yuan Quan *et al.*[40] diluted yogurt samples and directly incubated and detected them using the BioLumix Microbial Fluorescence Photoelectric Detection System. The results showed that the system could confirm negative results within 48 h, and the concordance rate between its results and those of the national standard method reached 100%. Zhang Cuifen investigated the rapid detection efficacy of the Soleris rapid detection system for microbial indicators in different types of dairy products. The results indicated that the Soleris system could detect molds and yeasts in various dairy products within 48 h. Its detection results showed good correlation with the national standard plate count method. Furthermore, the detection time was negatively correlated with the microbial count in the test sample[41]. Fan Yunxiu *et al.*[42] spiked commercially available yogurt samples and simultaneously tested them using both the Soleris Real-Time Photoelectric Microbial Detection System and the national standard method. The results demonstrated that when the total count of molds and yeasts ranged from 10 to 3.5×10^5 CFU/mL, the Soleris system completed detection within 1.8 to 33 h, and its results showed good parallelism with those obtained by the national standard method. Microbial rapid photoelectric detection systems are particularly suitable for high-throughput sample testing. They offer shorter detection times and avoid the counting difficulties caused by hyphal spreading in molds encountered in the national standard method.

6 Conclusion

In recent years, the frequent occurrence of safety incidents involving milk and dairy products caused by molds has not only heightened public awareness of food safety but has also accelerated the rapid development of mold detection technologies. Traditional mold detection methods based on the national standard plate count method and novel detection technologies each have their own advantages and disadvantages. Although the national standard plate count method requires a longer detection time, it serves as the indispensable gold standard for product testing. In contrast, novel detection technologies offer significant advantages in terms of sensitivity, time consumption, batch sample processing, and on-site rapid testing. The mutual integration and complementarity of novel detection technologies with the national standard detection methods will provide a solid foundation for mold detection in milk and dairy products and for ensuring food safety.

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