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Research Article

Molecular Study of Filamentous Fungi in Raw Milk Collected from Industrial Dairy Farms in Alborz and Tehran Provinces of IRAN in 2017

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Abstract

Background and Objective: Milk as a complete food source comes from a variety of sources, including cattle, goats, sheep and buffalo, for human consumption. Knowledge of fungal diversity in the environment is poor compared with bacterial biodiversity. The main objective of this study is to identify the raw milk in healthy conditions.
Method: In this study, milk samples collected from 14 industrial dairy farms in Alborz and Tehran Provinces were examined. 262 milk tank samples were cultured and then DNA of filamentous fungi were extracted and amplified rDNA16s -ITS-1.ITS-4 region was identified using sequencing test.
Results: This review was conducted from February 2017 to January 2018. 11.8% cases were identified with filamentous fungi including the following genera and species. Results demonstrated that the key point is that mycotoxin M1 is reduced in the pasteurization process, and its risk is also reduced but not removed; thus, new techniques and better technology are needed to overcome this problem.
Conclusion: The microbial milk contamination source comes from herd hygiene and health status, mastitis prevalence, production environment, and milking parlor and milk conserving practices in dairy farm. Moreover, these facts are implicated in milk quality and milk spoilage and unsafe dairy products. The milk production system and the dairy plant operations keep track in pasteurized milk and fresh dairy products reviewing the traceability in field situational diagnosis report.

Keywords: Milk, Filamentous fungi, food safety, healthy, microbial milk, health status.

Background and Objectives

Milk is a complete food supply. Today, special attention is being paid to milk, one day of the year (June the first) is called “world milk day”[1]. The consumers prefer a safe and healthy milk product selection, with a great variety and availability in the market. This fact affects the health and nutrition consumer’s information about the milk products made with raw milk[2]. Milk is also an important source of bacterial infection for human health, when milk is consumed without pasteurization[3-6]. Milk is a basic food in the human diet with great value as a nutritious healthy food; in the first years of human life, milk and dairy products are an important nutritional fact in the diet of the adult population[7].

Due to the presence of various nutrients, milk is considered as a complete and suitable food supply for the growth of various species of microorganisms such as bacteria and fungi. In order to standardize the quality of consumable milk, its status has been studied in terms of the microbial load, generally including bacteria.

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Fungi have been less studied in this regard, except where the amount of aflatoxin in milk has been taken into consideration. Aflatoxin is a fungi secondary metabolite. Three genera of filamentous fungi play a greater role in food supplies contamination and poison production including Aspergillus, Fusarium, Penicillium. A. flavus and A. parasiticus are among the common types of aflatoxin producing Aspergillosis .

In addition, A. flavus causes contamination in various types of humans and livestock food[2]. Since milk is used as a complete food supply by different classes of people with different age groups, it is important to check its health, especially for children and the elderly[3]. Milk can be harmful, if it is infected with aflatoxin, as the leading cause of carcinogenicity and mutagenicity as well as immune system suppression. When cows eat the food contaminated with mycotoxin, by passing through the bloodstream, mycotoxin can be excreted through urine, bile, and milk and even contaminate other dairy products such as cheese and yogurt. Aspergillus fungi can approximately produce 20 types of aflatoxin, among which aflatoxin I is of greater importance. Feeding livestock with moldy waste bread and fodder can indirectly contaminate milk. Aflatoxin has been studied in many parts of the world, including North Africa[4], Mexico[5], Korea[6], Portugal[7], and Turkey[8] and also in different cities of Iran. Except for aflatoxin, there is another mycotoxin known as Zearalenone, which is mainly produced by Fusarium in food[9-12]. This toxin has been investigated in various food supplies including buffalo milk in the northwestern region of Iran Among different types of aflatoxin, the prevalence rate of AFM1 (Aflatoxin M1) is high in milk, mainly produced by A. parasiticus and A. flavus more than other fungi[13]. The objective of the dairy industry is to maintain productivity and competitiveness in a growing milk commerce, which is demanding a large volume of milk and a wide range of dairy products in the food market and the preferences of the final food consumer with remarkable differences according to patterns of consumer behavior by demographic categories, culture, and socioeconomic variations in the human population in the food market

Methods

Samples collection:

Raw milk samples were collected from February 2017 to January 2018 from milk collection tanks of 14 farms in two Alborz and Tehran Provinces, Iran, using sterile bottles and then transferred to the laboratory with ice. The samples were tested within 24-48h.

Samples culture:

The samples were cultured on the Sabouraud dextrose agar medium plates
with 0.5% chloramphenicol (SC) for the inhibition of the growth of bacteria. The media were placed at 30 °C and examined for one week. The grown colonies were examined microscopically. The samples containing filamentous fungi were examined for further analysis.

Slide culture preparation:

A U-shaped tube and a clean slide were put inside a glass plate and placed inside the autoclave to be sterilized completely (121 °C, 15 min). One piece of gel (1cm2) was cut from the potato agar medium using a sterile scalpel and placed on the slide placed on the U-shaped tube inside the glass plate. Then, using sterile forceps, a piece of fungus colony under study was planted on four sides of the gel, and a sterile coverslip was placed on it. Some water was poured into the plate to prevent the gel from drying, and plate was placed in the laboratory environment for about 20 days. The coverslip on the surface of the gel was taken by sterile forceps and placed on a clean slide, on which a drop of lactophenol cotton blue was added. Then, the slide was passed slowly over the flame one or two times and after a few moments examined by an optical microscope with a magnification of 40 × 10 × (X40) (Zeiss, Germany). The fungi were identified based on the different mycelium forms, including regular, irregular, branched, having a median blade, and lacking a median blade and the type of arrangement of chlamydoconidia, microconidia, and macroconidia .

DNA extraction:

DNA was extracted from filamentous fungal colonies based on the modified method of (14). The quality of extracted DNA was examined with

agarose gel 1%, and its density was measured with nano-drops. Then, DNA amplification was done using the PCR method and sequencing test.

Genome amplification :

For species identification, t he DNA was extracted and examined on agarose gel to assess its quality. PCR was performed to amplify16srDNA ITS-1-ITS-4 region using primer sequences as follows: Sense: ITS-1: 5"TCCGTAGGTGAACCTGCGG3", and Antisense: IT'S-4: 5"TCCTCCGCTTATTGATATGC3" (15.(

The PCR reaction was done as follows: 2.5 µL of each primer was added to a volume of 45 µL containing: 10 × PCR buffer + MgCl2, 10 mM dNTP mix, and 1 unit.µL-1 of Taq DNA polymerase and template (genomic DNA) (Amplicon, Denmark). Thermal cycle reaction was carried out by a thermal cycler (Peqlab. Germany). It was composed of a pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 45 seconds, and a final extension step at 72° C for 75 sec. Then PCR fragment was analyzed by electrophoresis on 1% agarose gel.

PCR product sequencing:

The samples were counted foraerobic mesophilic bacteria, psychotropic, andStaphylococcus aureus. For counting aerobic meso-philic bacteria, the Nutrient Agar and pure platemethods were used. The obtained PCR fragments were transferred for sequencing analysis and the resulted nucleotide sequences were checked for identity in the global Gene data bank (NCBI, NIH.

Results

In this study, cow raw milk samples were collected from 14 farms during one year, and the microbial analysis of milk was performed by studying the filamentous fungi. In this study, of 262 milk samples, 31 cases were identified with filamentous fungi. Of 354 fungal colonies, 11.8% were identified as filamentous fungi, including Aspergillus 22%, Cladosporium 3%, Fusarium 42%, Itersonilia 3%, Mucor 29%, and Penicillium 3 %. The prevalence rate of the identified fungi is shown in (Chart 1))

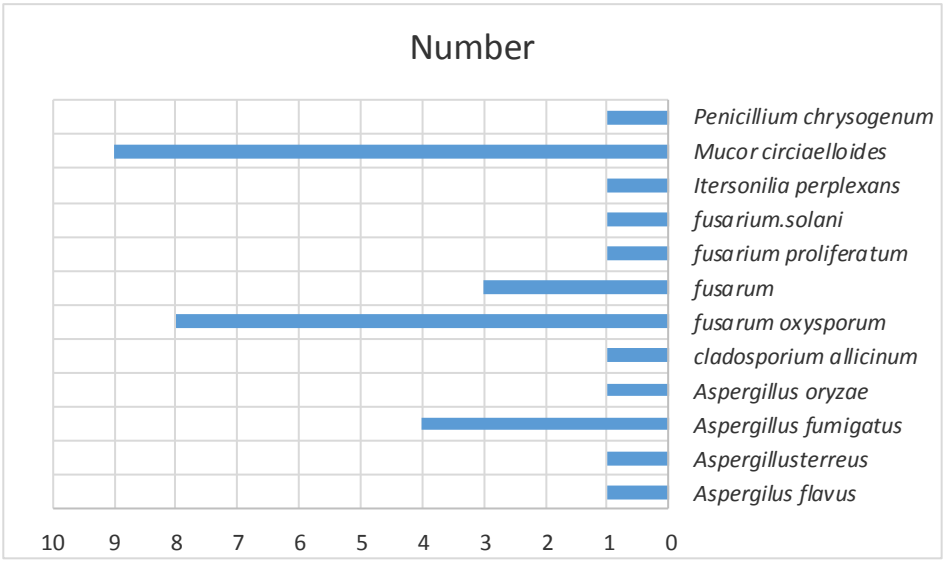


Chart 1. The prevalence rate of identified filamentous fungi in milk

The samples were cultured, and the results of slide culture are shown (Figure 1 and 2).

Fig. 1. Slide culture

Fig. 2. Slid with optical microscope

Ribosomal DNA was amplified by 16s rDNA ITS -1-ITS-4 primers and further for sequencing (Figure 3).

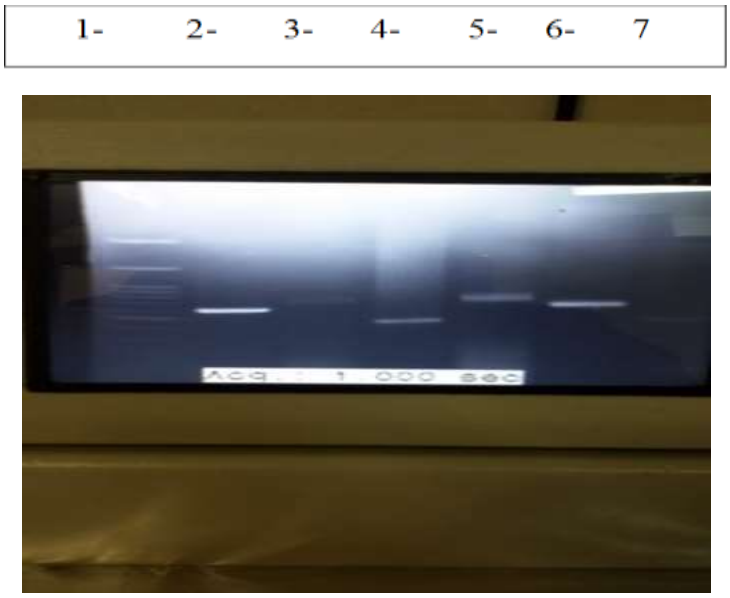


Fig. 3. Filamentous fungi PCR products

Lane1: size marker 100bp, Lane 2-7: filamentous fungi

This research was done in four seasons; the frequency and type of the resulting filamentous in spring, indicated Mucor as almost the highest rate of presence among other fungi. However, in summer, the frequency of F. oxysporum was more than the other fungi. Furthermore, in autumn, A. fumigatus was the most prevalent fungus and the results for winter, indicated that the frequency of Mucor was more than others (chart 2 to 5)

Chart 2. The frequency and type of identified filamentous fungi in spring

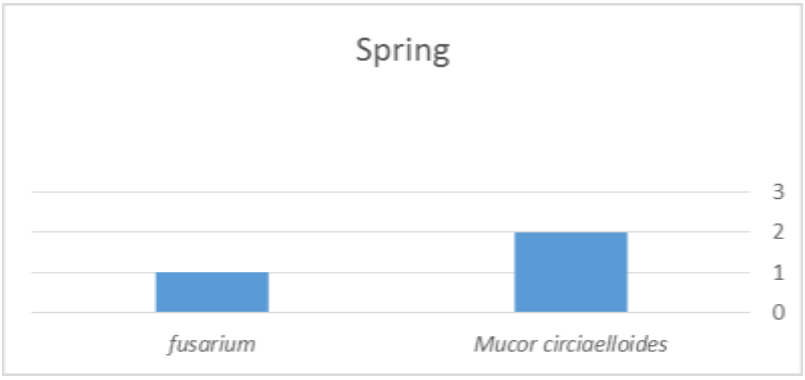


Chart 3. The frequency and type of identified filamentous fungi in summer

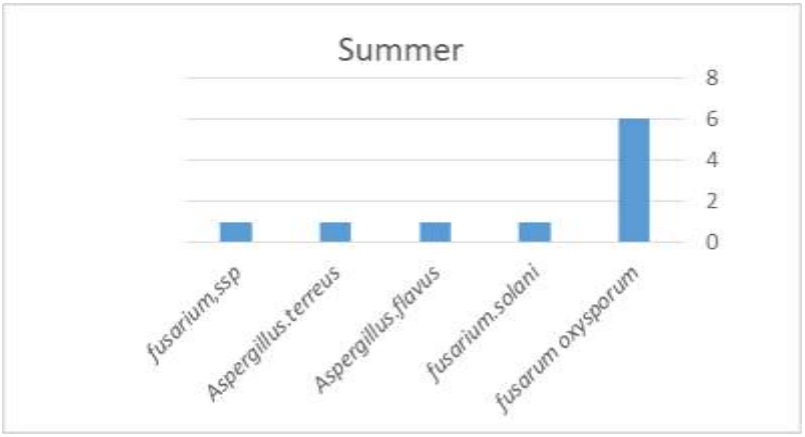


chart 4. The frequency and type of identified filamentous fungi in autumn

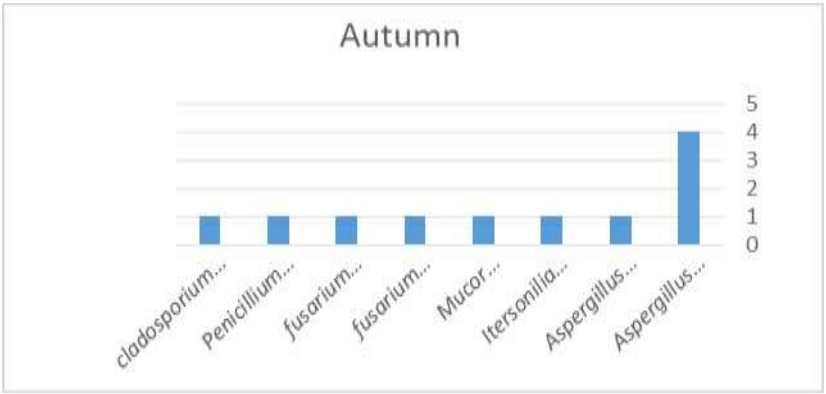
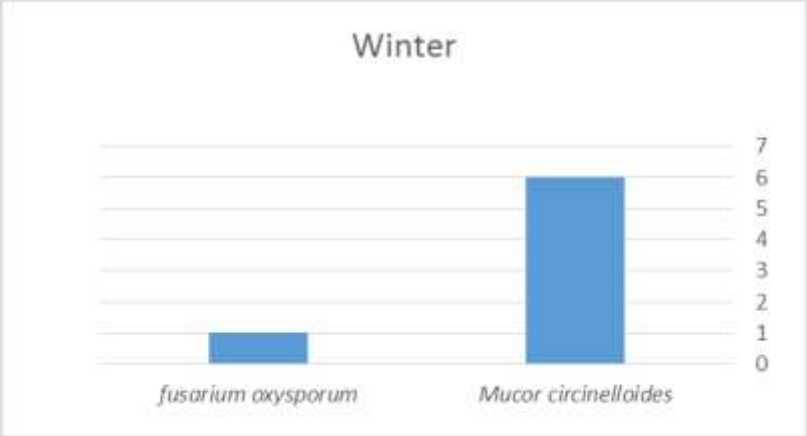


chart 5. The frequency and type of identified filamentous fungi in winter



Discussion

Milk is a nutritious food source, and its health is of particular importance in relation to human health[16]. Most studies have focused on the microbial load of milk, especially on bacteria, and less have focused on fungi, especially filamentous types. While most studies have been conducted on aflatoxins in milk, filamentous types have been less investigated. In this study, the presence of filamentous fungi was investigated in milk, regardless of the presence and amount of aflatoxins. In a research conducted in Hamedan, Iran, livestock food was studied, and the type and amount of fungi found in animal feed were determined. It was also determined that the aflatoxins entered the livestock body through the gastrointestinal tract along with the food and into their milk[15-17].

In a study by Lavoie et al. (2012) conducted in Quebec, Canada, the prevalence rate of filamentous fungi was 33% in milk, belonging to 33 species and 25 genera[18]. In another study conducted in Slovenia, the prevalence rate of filamentous fungi was reported as 63%. Of course, in this study, Geotrichum was considered to be a branch of fungi, accounting for 51.5% of the cases[19-22]. In this research, milk samples were directly examined because the goal was to identify the types and amount of filamentous fungi. By examining forage alone, it was not possible to draw an accurate conclusion because even if the forage materials were kept in a standard condition, contamination could be transferred through the environment and air.

The fungi identified in this study were as follows: A. flavus, A. terreus, A. fumigatus, A. oryzae, I. perplexans, P. chrysogenum, C. allicin, F. oxysporum, F. proliferatum, F. solani, M. circinelloides, and Fusarium ssp.

The key point is that mycotoxin M1 is reduced in the pasteurization process, and its risk is also reduced but not removed; thus, new techniques and better technology are needed to overcome this problem.

In various studies, the prevalence rate of fungi was high, which is consistent with the studies conducted on aflatoxins in different seasons. Kind of nutrition used in farms is an effective factor as well. For example, in a research conducted in Chaloos, Mazandaran Province, Northern Iran, the type of nutrition in the target farms was natural grazing in the summer, and the prevalence rate of fungi in this season was lower than in cold seasons. However, in the

present research that was conducted in industrial dairy farms in which natural grazing was impossible and the prevalence rate was high. The result of our study also showed that the prevalence of fungi was higher in autumn, followed by summer and it was less in spring and winter. While it is important to ensure that the quality of milk is maintained at high levels, producers of traditionally manufactured raw milk cheese should be aware that certain farming practices may negatively impact on distinctive flavours and aromas as a consequence of limiting the numbers of specific microorganisms and may need to compensate through the introduction of starters and adjunct strains.

Conclusion

In the present study, the prevalence rate of identified fungi was as follows: Aspergillus 22%, Fusarium 42%, Cladosporium 3%, Itersonilia 3%, Penicillium 3%, and Mucor 29%. In Quebec study, the prevalence rate of Aspergillus and Mucor was reported as 33.8 and 5.9%, respectively. These results are consistent with Slovenia results, in which the prevalence rate of fungi was reported as follows: Aspergillus 33.8%, Mucor 5.9%, Fusarium 2.9 %, and Penicillium 2.9 %. In aforementioned studies, Mucor was reported to be more prevalent in both spring and winter. The identified fungi in this study are found not only in milk but also in other dairy products such as yogurt; as in a study conducted in Bulgaria, the presence of Penicillium in yogurt was reported. The interesting point in our study is that M. circinelloides found in samples under study can be among the pathogens transferred through food, including milk; for example, in a study conducted in the United States, yogurt was reported to be contaminated with this fungus, and people consuming it had symptoms such as nausea, vomiting, and diarrhea. This agent is associated with pathogenicity in humans. Therefore, it is recommended that further studies be conducted to obtain accurate results. It is worth noting that the primary health of raw milk is very important. In a study by Alinia in Taiwan, the level of aflatoxin was reported to be at a permissible limit. Finally, it should be noted that the amount of identified fungi was the highest in summer and autumn.

Competing Interests

There is no conflict of interests.

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