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Microbial and Fungal Contamination of Wheat Flour, Dough, and Bread Samples Collected from Isfahan, Iran

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THE present investigation was done to evaluate the microbial and fungal contamination and also molecular identification of fungal species in flour, dough and bread samples collected from Isfahan, Iran.Two-hundred and forty different types of cereals including flour, dough and bread were collected. Samples were subjected to microbial and fungal counting and also PCR-based identification of fungal species and sequencing of *Aspergillus* species. Ranges of total bacterial count of flour, dough and bread samples collected from breads were between 2.83±0.99 and 6.43±1.12 log cfu/g. Flour, dough and bread samples collected from bulk breads had the higher counts of total bacteria than those of flat breads. Ranges of mold count of flour, dough and bread samples collected from breads were between 0.00±0.00 and 1.63±0.63 log cfu/g. Flour, dough and bread samples collected from flat breads had the higher counts of mold than those of bulk breads.Distribution of *Penicillium*, *Cladosporium*, *Mucor*, *Aspergillus*, *Alternaria*, *Canninghamella*, *Hyaline*, *Rhizopus*, *Epicoccum* and *Syncephalestrum* amongst detected fungal colonies were 24.40%, 20.10%, 20.10%, 19.00%, 3.80%, 3.80%, 3.80%, 2.70%, 1.00% and 1.00%, respectively. Sequencing of isolated Aspergillus genus revealed that all strains were related to flavus, oryzea, terreus,andnijer species. Detected total count and mold count in studied bread samples were lower than limit standards announced by Institute of Standards and Industrial Research of Iran. However, considering the high consumption rate of these kinds of food samples among Iranian people, severe surveillance programs should perform to inhibit from their bacterial and fungal contamination.

Keywords: Total bacterial count, Fungal species, Identification, Bakery products.

Introduction

Bread is the main part of daily meal of the majority of people in different countries[1]. Per capita annual consumption of bread amongst Iranian people is about 160 kilograms which explicitly confirms its high consumption rate in comparison average global consumption (about 130 kilograms) [2]. Wheat flour is the main ingredient used for preparation of bread and other types of bakery products all-around the world [3, 4]. Wheat flour has a low water activity content and is mainly considered as a safe product[5, 6]. In keeping with this, it is prone to fungal, especially mold contamination[7-9]. Mold contamination of wheat flour and breads are important factors decrease their shelf-life[10].

Mycotoxin-producing fungi such as *Aspergillus*, *Penicillium* and *Fusarium* are the most important groups of molds responsible for contamination of breads and bakery products[7] [11-13]. These fungal species are mainly associated with mastitis in dairy farms and also dangerous abortion in dairy cattle in some parts of the world [9]. Mycotoxins can cause acute or chronic health problems including teratogenicity, carcinogenicity, and immune-

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toxicity[9]. Aflatoxins which are mainly produced by *Aspergillus* species, especially *Aspergillus flavus* (*A. flavus*) and *A. parasiticus* are the most clinicaly important mycotoxins[14]. Aflatoxins are classified as group 1A potential human carcinogens by an International Agency of Research on Cancer[14].Diverse surveys have been conducted on the role of animal species, particularly their resources such as milk and meat as a vector of fungal transmission to human population [9, 14].

Mycotoxins are resistant to high cooking temperatures and unfavorable environmental conditions, and are often found in processed and cooked foods[15-17]which make them important from the public health prospective. Thus, it is important to assess the fungal population of breads and bakery product to ensure their hygienic qualities. Several researches have been conducted on fungal contamination of flour and breads but they have almost been concentrated on fungal counts [18-20]. In the other hand, there were no previously published data on determination of fungal species in flour, bread and dough samples. Conventional identification of fungal species was based on specified and time-consuming morphological characteristics on culture media[21]. However, rapid, safe and sensitive detection of fungal species using polymerase chain reaction (PCR)-based amplification of certain target genes has been developed as accurate and valid alternative method [14, 22].

Institute of Standards and Industrial Research of Iran (ISIRI) has been determined some standard limits for fungal and especially mold contamination of bread and bakery products. Though, several investigations have been reported fungal contamination of flour and bakery products higher than announced standard limits of ISIRI[3, 23-25]. Moreover, higher distribution of *Aspergillus* species has also been reported in Iran[12, 25, 26]. Furthermore, there were no PCR-based identification of fungal population of breads and other bakery product in Iran [23-26].Therefore, the present research was done to assess the fungal population of bread, flour and dough samples collected from two major types of breads (flat and bulk breads) and mold species identification using PCR and sequencing analysis.

Material and Methods

Sampling

The present descriptive cross-sectional study was carried out from October 2017 to March 2018in the Isfahan city, Iran. Two-hundred and forty bakery samples including flour(n=80), dough(n=80), and bread(n=80) were randomly collected from 20 local bakeries of the Isfahan city, Iran. Samples were collected from two major types of breads (flat and bulk breads). Samples were immediately transferred to Food Safety Research Center of the Isfahan University of Medical Sciences, Isfahan, Iranin cooler with ice-packs.

Mold count

Ten grams of each sample was added to 90 ml of Ringer's solution and shaken for approximately 10 min. Then, serial dilution was done to -1000 fold. Aliquots consisting of 1 ml of each dilution were spilled into the plate and poured by yeast-extract-glucose chloramphenicol agar(YGCA, Merck, Darmstadt, Germany, pH=6.6±0.2). The arrangement of medium was (g/liter): yeast extract 5.0, D (+) glucose 20.0, chloramphenicol 0.1, agar-agar 14.9. After 5 days incubation at 25°C, mold colonies were counted according to the method described previously [27].

Total count

Ten grams of each sample was added to 90 ml of Ringer's solution and shaken for approximately 10 min. Then, serial dilution was done to -1000 fold. Aliquots consisting of 1 ml of each dilution were spilled into the plate and poured by plate count agar(PCA, Merck, Darmstadt, Germany, pH=7.0±0.2). The composition of medium was (g/liter): peptone from casein 5.0, yeast extract 2.5, D(+)glucose 1.0, agar-agar 14.0. After 3 days incubation at 30°C, colonies were counted according to the method described previously [27].

Isolation and Identification of fungi

Species of fungal strains were further identified using the Malt Extract Agar (MEA, Merck, Darmstadt, Germany) media. Cultures were incubated at 25°C for 7 days and then the individual genera of molds were taxonomically identified on the basis of their macroscopic and microscopic morphology according to method described previously[28-30].

DNA extraction

Genomic DNA was extracted from the fungal colonies using the phenol-chloroform DNA extraction protocol. Briefly, 10 µL spores of moldes were added to 300 µl of lysis buffer(100 mmol Tris, 20 mmol EDTA,100 mmol NaCl,2% SDS). After grinding for 1 minute, 300 µl of phenol-chloroform solution was added and centrifuged at 5000 g for 5 min. The aqueous phase was moved to a new tube and 300 µl phenol-chloroform was added and centrifuged at 5000 g for 5 min. Nucleic acids in the aqueous phases were precipitated with 0.1 volume of sodium acetate 3 M and equal volume of isopropanol, incubating at -20 °C for 10 min. After centrifugation at 10000g for 10 min, 300 µl of 70% ethanol was added to the precipitates and centrifuged for 5 min. To conclude with the supernatant discarded and 50 µl of deionized distilled water were added to the precipitate and the pellets of DNA were kept at -20 °C[31]. Extracted DNA samples were subjected to quantification by NanoDrop device (NanoDrop, Thermo Scientific, Waltham, USA), qualification (2% agarose gel) and purity checking (A260/A280).

The universal fungal primers

Bt2α (5'GGTAACCAAATCGGTGCTGCT TTC3') and Bt2b (5'ACCCTCAGTGTA GTGA CCCTTGGC 3') (650 bp) primers were used for PCR amplification of Beta-tubulin gene (Kamari et al., 2017)[31].PCR mixture contained 5 Ml of extracted DNA, 25 pmol of each Bt2α and Bt2b primers, 400 µmol, dNTPs, 2.5 µl buffer, 1.5 mmol mgcl2, 2.5 U of Taq polymerase in a final volume of 25 µl.The PCR amplification was done as follow: denaturation of DNA at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension phase at 72°C for 6 min [31]. Seven microliters of each PCR product with three microliters of loading buffer were run onto 1.5% agarose gel and electrophoresed in Tris-borate-EDTA buffer.Gels have been moved to the transilluminator machine and taken photos of bands.

Sequencing

The amplicons were purified and cycle sequencing reactions were performed in forwarding direction. The raw nucleotide sequencing was analyzed with MEGA 4 software(MEGA version 4,. Resulting sequences of isolates were evaluated using NCBI BLAST searches for fungal sequences existing in DNA databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Data obtained from the experiments were transferred to Excel software. SPSS/21.0 software was applied for statistical analysis. Data were analyzed using Univariate Analysis Of Variance test (ANOVA) and one sample T-test. Numerical meaning was regarded at a P value < 0.0005.

Results

A total of 240 cereal samples including flour, dough, and bread were collected from flat and bulk breads and studied for microbial and fungal contamination and identification of fungal species. Table 1 represents the total and mold counts of flour, dough, and bread samples collected from flat and bulk breads. Total bacterial count of flour, dough and bread samples collected from flat breads had ranges between 2.8328±0.9892 and 6.2153±0.7817 log cfu/g. Additionally, Total bacterial count of flour, dough and bread samples collected from bulk breads had ranges

TABLE 1. Total and mold counts of flour, dough, and bread samples collected from flat and bulk breads.

Types of b read samples		Mean microbial counts (Log cfu/g ± standard deviation (SD))	
		Total count in PCA	Mold count in YGC
Flat bread	Flour	*3.8623±1.0556a	1.6271±0.6283a
	Dough	6.2153±0.7817b	0.0000±0.00000b
	Bread	2.8328±0.9892c	0.1010±0.4459b
Bulk bread	Flour	4.0659±0.7594a	1.5596±0.9203a
	Dough	6.4335±1.1192b	0.0000±0.0000b
	Bread	3.1478±1.0132c	0.0000±0.0000b

*Dissimilar letters in each column shows statistically significant differences about P<0.0005.

between 3.1478±1.0132 and 6.4335±1.1192 log cfu/g. Flour, dough and bread samples collected from bulk breads had the higher counts of total bacteria than those of flat breads. Otherwise, dough samples in both types of breads had the higher rate of total bacterial contamination, while bread had the lowest. Mold count of flour, dough and bread samples collected from flat breads had ranges between 0.0000±0.00000 and 1.6271±0.6283 log cfu/g. Moreover, mold count of flour, dough and bread samples collected from bulk breads had ranges between 0.0000±0.0000 and 1.5596±0.9203 log cfu/g. Flour, dough and bread samples collected from flat breads had the higher counts of mold than those of bulk breads. Otherwise, flour samples in both types of breads had the higher rate of mold contamination, while dough had the lowest. Statistically significant differences were found between the total bacterial count and mold count of bread samples of flat breads and bulk breads (P<0.0005).

Table 2 represents the distribution of different species of fungi amongst the colonies detected in flour, dough and bread samples collected from flat and bulky breads. All of the identified fungi species were found in flour samples except *Epicoccum* which was isolated from colonies detected in bread samples. *Penicillium* had the highest distribution (24.29%) amongst studied colonies, while *Epicoccum* had the lowest (1.43%). Additionally, 19.29% of detected fungal colonies were related to *Aspergillus* species.

Sequencing analysis of agarose gel electrophoresis of each PCR product in comparison with fungal sequences existing in DNA databases showed that *A. flavus*, *A. oryzea*, *A. terreus,* and *A. nijer* were the main genus amongst all isolated *Aspergillus* isolates. Total incidence of *A. flavus*, *A. oryzea*, *A. nijer* and *A. terreus* species amongst the studied flour samples were55.55%, 18.52%, 18.52% and 7.41%, respectively.

Discussion

Due to the low content of activated water in cereals, breads and other types of bakery products, their contamination throughout the harvesting, storage and preparation were accompanied with fungi and especially molds. Some types of these mold are responsible for production of important mycotoxins with important complications on human health. Thus, it is important to assess the fungal population and mold species identification in the bread, flour and dough samples as a highly consumed food stuffs not only among Iranian people but also among people of all sites of the world.

The present research was done to assess the fungal population and species identification of molds in bread, flour and dough samples collected from two different types of breads (flat and bulk). According to the data, 35.80% of samples had a total count of microorganisms higher than the maximum accepted levels announced by ISIRI (standard level=5 $\log_{10}$cfu/g). Additionally, all of

TABLE 2. Distribution of fungal species amongst the detected colonies in flour, dough and bread samples collected from flat and bulky breads.

Mold species	Source	n	(%) Distribution
Penicillium	Flour	34	24.29
Cladosporium	Flour	28	20
Mucor	Flour	28	20
Aspergillus	Flour	27	19.29
Alternaria	Flour	5	3.57
Canninghamella	Flour	5	3.57
Hyaline	Flour	5	3.57
Rhizopus	Flour	3	2.14
Epicoccum	Bread	2	1.43
Syncephalestrum	Flour	3	2.14
Total		140	100

our studied samples had acceptance counts of fungi compared to limit standards announced by the ISIRI (standard level=3.698 $\log_{10}$cfu/g). However, several investigations were reported fungal contamination higher than permissible maximum levels[23-25].Total mold count obtained in the present research was similar to the those reported by previous Iranian investigation conducted on northwest of Iran[3].Our findings showed that there were no detectable counts for molds in dough samples. This finding is mainly due to the higher activity of yeast in dough samples which caused inhibition from the growth of molds. The highest mean amounts of total count was found in dough samples, while the highest mean amounts of mold count was found in flour samples. Thus, our finding showed that thermal processing decreased the microbial population of bakery products. This finding was similar to the results of previously published data[32-34]. Therefore, the microbial contamination of bread samples may occur after the baking procedure of breads. It may be occur due to the hand manipulation, cutting with a knife and even unfavorable keeping conditions[30]. No statistically significant difference was obtained for the numbers of molds and also total counts between flat and bulk bread samples.

Majority of identified fungal species had zoonotic importance and can transfer from infected animals and even birds into the human population. Thus, they mainly have veterinary impacts. Additionally, some kinds of studied cereals, especially corrupted bread samples, were mainly used for animal feeding. Unfortunately, ranchers were used from these kinds of cereals for feeding of dairy cows, sheep and goats in some parts of Iran. Thus, fungal contaminants will transfer to human population through resources such as milk and meat to human population. Thus, detection of the above mentioned fungal species in bread samples has veterinary-based importance. Feeding of animal species with fungal contaminated bread may cause severe abortion and also mastitic in animal species, especially dairy livestock.

Different species of mold such as Penicillium, *Cladosporium*, *Mucor*, *Aspergillus*, *Alternaria*, *Canninghamella*, *Hyaline*, *Rhizopus*, *Epicoccum* and *Syncephalestrum* were found in studied samples of our research. Penicillium was the most frequently identified mold in studied samples. This finding is also similar to results of previous investigations[35]. Similar fungal population was also reported in some previously reported papers[21, 23-26, 36, 37]. Otherwise, high prevalence of Fusarium species has been reported in cereals and wheat flour [5, 12, 35, 38, 39]which was dissimilar to our results. The differences found in the types of molds reported in various investigations is may be due to the differences in types of samples, method of sampling and weather and climate of sampling places. Detected fungi in the present investigation were reported previously as the most important causes of severe clinical implications[40, 41]. Alternaria is a ubiquitous pathogen with high ability to produce toxin [42-45]. However, it had a low frequency in the samples of our investigation.The existence of Syncephalestrum amongst the identified molds in studied samples is so important due to its ability to produce particular mycotoxins. This finding is in agreement with the results of fungal contamination of wheat reported by recent survey[24]. Mucor is another important identified fungi in studied samples. It is responsible for stone formation in human urinary tract by synthesize oxalic acid and then binding of oxalates to calcium and other minerals[46]. Thus, further studies should address in order to control the presence of Mucor in bakery products.

Our results in PCR sequencing of fungal strains is similar to those reported by Riba et al conducted on Algerian wheat [39].*A. flavus*, *A. niger*and *A. terreus*are common fungi in studied bread samples. *A. flavus* can produce aflatoxin [47, 48]. Additionally, *A. niger* can produce ochratoxin [49, 50]. Furthermore, *A. terreus* is resistant to antifungal drugs [51]. Thus, their high prevalence in the fungal population of bread samples collected from Isfahan, Iran pose an important public health threat regarding the consumption of these products[52].Foodborne pathogens have boost clinical and microbial importance in Iran. Thus, detection of fungal species in cereals samples has a high health-related and food hygienic importance regarding the consumption of this foodstuffs by human and in some cases like corrupted bread samples by animal species.

Conclusions

Inconclusion, high microbial and mold counts in the flour, dough and bread samples collected from Isfahan city, Iran were found. Bulk breads

had the higher counts of total bacteria than flat breads. Dough samples had the higher rate of total bacterial contamination, while bread had the lowest. Flat breads had the higher counts of mold than bulk breads. Otherwise, flour samples had the highest rate of mold contamination, while dough had the lowest. All studied bread samples had the lower mold count than accepted levels announced by ISIRI, while 35.80% of samples had a total count of microorganisms higher than the maximum accepted levels.*Penicillium*, *Cladosporium*, *Mucor* and *Aspergillus*were the most commonly identified fungi amongst the bread samples. Considering the high ability of some of the identified fungi in mycotoxin production, several surveillance programs should perform to prevent from fungal contamination of bread and bakery products. In keeping with the high distribution of total microorganisms and molds in bread samples, further researches should perform to found the exact routes of contamination and also other microbiological and health related aspects of presence of fungi in bread and its products.

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Ethical consideration

The research was permitted by the Moral Council of Research of the Isfahan University of Medical Sciences, Isfahan, Iran (96-2020). Confirmation of the current investigation and the certificates associated with sampling procedure were permitted by Dr. Masoud Sami and Dr. Maryam Mirlohi.

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