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ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF MICROFLORA OBTAINED FROM SPICES AND SPICE MIXES.

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ABSTRACT

Food borne disease outbreaks caused by spices have been on the increase in recent years. Spices are used for flavor, color, aroma, taste and preservation of food and beverages but like any other object, they are also not free from microbial association. The present investigation is therefore, designed to throw light on the microbial status of some ground spices and spice mixes including commercial brands. A total of twenty seven (eight local and nineteen commercially packed) samples were collected and analyzed to assess Total Bacterial Count (TBC) and Total Fungal Count (TFC). The TBC varied from the range of 10^4 to 10^7 cfu/gm with the highest of $5.9X10^7$ cfu/gm. The microorganisms were identified through biochemical as well as molecular methods. Most of the isolates belong to the genus *Bacillus. E.coli* and *Staphylococcus* were also isolated from two samples. The total fungal count ranges from 10^2 to 10^6 with the highest of $6.3X10^6$ cfu/gm. The most predominant ones were *Aspergillus flavus* (max.36.36%) and *Mucor spp*. (max. 37.5%).The counts obtained were higher than the maximum acceptable levels provided by the Food and Agriculture Organization (FAO) of the United Nations. It is therefore, recommended that strict hygienic measures should be observed during the mixing of the spice in order to reduce the microbial load to an acceptable level.

Keywords: Spices, Molecular characterization, Contamination, Bacterial count, Fungal count.

INTRODUCTION

In recent years increasing consumer awareness has emphasized the need for microbiologically safe food. Since the human food supply consists basically of plants and animals or products derived from them, it is understandable that our food supply can contain micro-organisms in interaction with the foods. When the micro-organisms involved are pathogenic, their association with our food is critical from public health point of view. Serious health hazards due to the presence of pathogenic microbes in food can lead to food poisoning outbreaks [1]. According to CDC [2],food borne disease outbreaks caused by imports were on the increase between 2009 and 2010 in which fish and spices were reported to be the most common sources. Spices could be defined as the natural vegetable products or mixture thereof, without any extraneous matter that is used for flavoring, seasoning and imparting aroma to foods. Spices like other food substances may carry some bacteria, yeast and mould spores. According to FAO report [3], spices and herbs including agricultural products may be contaminated from different sources and this may occur during storage, distribution or during processing stage of the spices. The microbial flora on many spices is generally dominated by aerobic spore forming and non spore forming bacteria. Some of these bacteria like *Bacillus cereus* and *Clostridium perfringens* are recognized as having potential pathogenicity and have been incriminated in food poisoning [4]. A recent study conducted on the spices used for local meat in Northern Nigeria showed that the spices were heavily contaminated with pathogenic bacteria: *E.coli*, *Salmonella* sp., and *Clostridium* sp. isolated from some of the samples [5].When properly dried and stored, spices are generally resistant to microbial spoilage. However, spices are raw agricultural materials and if the moisture content is too high, toxigenic molds like *Aspergillus spp, Penicillium spp. and Fusurium spp.* [6] may grow offering the opportunity for aflatoxin production [7,8].

The present study aimed to throw light on safety of spices; also the quality of local and commercial brands of spices and spice mixtures was compared by assessing their microbial load and the presence of pathogenic micro-flora.

MATERIALS AND METHODS

Sample Collection

27 samples were collected, of which eight local and nineteen commercial brands of spices and spice mixture were collected from the local market of Hardwar and adjoining areas (Table-I).

Microbial Analysis

The initial bacterial and fungal population in the spices was determined by transferring one gram of sample in sterile test tube containing 10 ml of 0.1% peptone water as diluents. Each tube was shaken on vortex mixer. Serial dilution upto $1:10^7$ were made and 1 ml of aliquot from each dilution was plated on 15 ml of Nutrient Agar Medium (Himedia, India) plate for total bacterial count and on Sabrourd Dextrose Agar medium (Himedia, India) for total fungal count (TFC). All the plates were incubated at 35°C±2 for 48 hours (bacteria) and at 27°C±2 for 5-7 days (for fungi). After incubation, colonies were counted.

Isolation and identification of Isolates

After development of bacterial colony on the Agar surface, colonies were randomly selected and isolated on respective Agar slants. The selected bacterial colonies were studied for various characters viz color, form, elevation etc. following Bergey's Manual of Determinative Bacteriology [9]. The bio-chemical tests of the isolated bacteria were carried out through bio-chemical kit for Gram positive (KB013-Himedia, India) and Gram negative bacteria (KB001- Himedia, India). Also, molecular characterization (16S rDNA sequencing) of two isolates were done in which forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1.

The fungi isolated were characterized based on their macroscopic appearance on the culture medium, microscopic morphology and types of asexual spores produced and identified by reference to the compendium of soil fungi [10].

Table I List of collected samples

Spice Type(Local)	Scientific Name	Sample No.
Chilli	*Capsicum Annum*	LC1
Chilli	*Capsicum Annum*	LC2
Coriander	*Coriander Sativum*	LCR
Amchur	*Magnifera Indica*	LA1
Amchur	*Magnifera Indica*	LA2
Turmeric	*Curcuma Longa*	LTR
Cumin	*Cuminum Cyminum*	LCU
Garam Masala	-	LCM

Commercial Brands	Brand	Sample No.
Chilli	Brand A	BCA
Garam Masala	Brand A	BGA
Garam Masala	Brand B	BGB
Garam Masala	Brand C	BGC
Garam Masala	Brand D	BGD
Sabzi Masala	Brand A	BSA
Sabzi Masala	Brand B	BSB
Sabzi Masala	Brand D	BSD
Sabzi Masala	Brand E	BSE
Chicken Masala	Brand A	BHA
Chicken Masala	Brand C	BHC
Chicken Masala	Brand D	BHD
Amchur	Brand A	BAA
Amchur	Brand D	BAD
Turmeric	Brand A	BTA
Coriander	Brand B	BOB
Coriander	Brand C	BOC
Chat Masala	Brand C	BCrC
Sambhar	Brand E	BSaE

RESULTS

Microbial load in local spice samples.

Total Bacterial Count: The mean bacterial count in local spice samples was 8.4 X 10^6 cfu/gm. The total viable count varied with different spice samples that ranged from 4.0 X 10^4 to 5.9 X 10^7 cfu/gm (Table-2A).

Total Fungal Count: The mean fungal count in local spice samples was found to be 7.4x10^6 cfu/gm, which ranges from 2.6 X 10^3 to 6.3 X 10^6 cfu/gm (Table-IIA).

Microbial load in commercially packed samples.

Total Bacterial Count: The mean total bacterial count in commercially packed spice sample was 7.84 X 10^5 cfu/gm. The Total Viable Count ranges from 1.1 X 10^4 to 4.2 X 10^6 cfu/gm (Table IIB).

Total Fungal Count: The mean total fungal count in commercially packed sample was 1.7 X 10^5 cfu/gm. The total fungal count ranges from 1.3 X 10^2 to 1.6 X 10^6 (Table IIB).

Table-IIA Microbiological Analysis of local spice samples

Spice Type (Local)	Sample No.	Bacterial Count* (cfu/gm)	Fungal Count* (cfu/gm)
Chilli	LC1	4.1 X 10^5	5.1 X 10^4
Chilli	LC2	5.9 X 10^7	6.3 X 10^6
Coriander	LCR	2.4 X 10^6	3.4 X 10^5
Amchur	LA1	---	4.1 X 10^4
Amchur	LA2	4 X 10^4	3.8 X 10^5
Turmeric	LTR	3.2 X 10^6	2.6 X 10^3
Cumin	LCU	2.1 X 10^6	3.2 X 10^5
Garam Masala	LCM	2.1 X 10^5	5.2 X 10^4

Mean Bacterial count- 8.4 X 10^6

Mean Fungal count- 7.4X10 , *Average of Triplicates

Table-IIB Microbiological Analysis of Branded spice samples.

Commercial Brands	Sample No.	Aerobic Plate Count* (cfu/gm)	Fungal Count* (cfu/gm)
Chilli	BCA	3.1 X 10^4	3.8 X 10^4
Garam Masala	BCB	2.4 X 10^4	2.6 X 10^5
Garam Masala	BCB	3.6 X 10^6	4.0 X 10^4
Garam Masala	BCC	2.2 X 10^4	1.3 X 10^2
Garam Masala	BCD	2.4 X 10^6	2.8 X 10^5
Sabzi Masala	BSA	-	1.1 X 10^3
Sabzi Masala	BSB	5.2 X 10^5	2.4 X 10^5
Sabzi Masala	BSD	3.2 X 10^5	4.3 X 10^4
Sabzi Masala	BSE	1.1 X 10^4	2.1 X 10^3
Chicken Masala	BHA	4.3 X 10^5	3.3 X 10^3
Chicken Masala	BHC	2.1 X 10^5	5.2 X 10^4
Chicken Masala	BHD	2.4 X 10^6	1.6 X 10^6
Amchur	BAA	3.2 X 10^5	2.2 X 10^4
Amchur	BAD	4.2 X 10^6	3.3 X 10^5
Turmeric	BTA	1.5 X 10^4	1.8 X 10^3
Coriander	BOB	3 X 10^4	3.6 X 10^4
Coriander	BOC	1.2 X 10^4	1.4 X 10^2
Chat Masala	BGC	3.4 X 10^5	4.1 X 10^5
Sambhar	BS_AE	1.4 X 10^4	2.8 X 10^3

Mean Bacterial count - 7.84 X 10^5

Mean Fungal count - 1.7 X 10^5

Average of Triplicates

Table IIIA Percentage occurrence of mycoflora obtained from local spices

Spice (Local)	*Asp. flavus*	*Asp. fumigatus*	*Asp. ochraceous*	*Asp. niger*	*Mucor*	*Trichoderma*	*Fusarium*	*Rhizo-pus*	*Penicilliu m*	*Alter-naria*	*Geotrichum*	*Cladosporium*	*Nigrospora*	*Verticilliu m*	Total
Chilli (1)	4(19.04)	2(9.52)	-	2(9.52)	5(23.81)	2(9.52)	1(4.76)	-	2(9.52)	2(9.52)	-	1(4.76)	-	-	21
Chilli (2)	6(33.33)	2(11.11)		1(5.55)	2(11.11)	-	4(22.2)	-	1(5.55)	-	-	-	-	-	18
Coriander	-	-	2(20)	-	2(20)	1(10)	-	1(10)	-	1(10)	2(20)	-	1(10)	-	10
***Amchur* (1)**	5(25)	-	1(05)	2(10)	4(20)	-	-	-	3(15)	-	1(05)	-	-	1(5)	20
Amchur (2)	7(30.43)	3(13.04)	-	3(13.04)	2(8.69)	1(4.34)	-	2(8.69)	-	3(13.4)	-	2(8.69)	2(10)	-	23
Turmeric	2(22.22)	-	-	-	3(33.3)	-	3(33.33)		1(11.11)	-	-	-	-	-	9
Cumin	4(25)	1(6.25)	1(6.25)	-	6(33.5)	-	1(6.25)	2(12.5)	1(6.25)	-	-	-	-	-	16
Garam Masala	5(27.77)	1(5.55)	-	-	3(16.66)	-	3(16.66)	2(11.1)	-	1(5.55)	-	1(5.55)	-	2(11.11)	18

All figures in parentheses represents percentage%

Table IIIB Percentage occurrence of mycoflora obtained from commercially branded spices

Spice	*Asp.flavus*	*Asp. fumigatus*	*Asp. ochraceous*	*Asp. niger*	*Mucor*	*Trichoderma*	*Fusarium*	*Rhizotus*	*Penicillium*	*Alternaria*	*Geotrichum*	*Cladostorium*	*Nigrospora*	*Verticillium*	Total
Chilli	4(18.18)	3(13.63)	-	2(9.09)	5(22.72)	1	2(9.09)	2(9.09)		1(4.54)	1(4.54)	1(4.54)	-	-	22
Garam Masala-A	3(14.28)	1(4.76)	2(9.52)	1(4.76)	4(19.04)	2(9.52)	1(4.76)	3(14.28)	1(4.76)		1(4.76)	-	2(9.52)	-	21
Garam Masala B	5(22.72)	3(13.63)	-	2(9.09)	3(13.63)	1(4.54)	2(9.09)		1(4.54)	2(9.09)	-	-	1(4.54)	2(9.09	22
Garam Masala C	3(13.4)	4(17.39)	1(4.34)	3(13.04)	4(17.39)	-	3(13.04)	1(4.34)	2(8.69)	1(4.34)	-	1(4.34)	-	-	23
Garam Masala D	6(27.27)	2(9.09)	2(9.09)	-	3(13.630	-	1(4.54)	2(9.09)	1(4.54)	2(9.09)	-	1(4.54)	2(9.09)	-	22
Sabzi Masala-A	2(16.66)	-	-	-	4(33.33)	-	3(25)	1(8.33)		2(16.66)	-	-	-	-	12
Sabzi Masala B	4(26.66)	2(13.33)	2(13.33)	1(6.66)	-	2(13.33)	-	-	2(13.33)	-	-	2(13.33)	-	-	15
Sabzi Masala D	5(26.31)	3(15.78)	-	2(10.52)	6(31.57)	1(5.26)	-	1(5.26)	-	-	-	-	-	1(5.26)	19
Sabzi Masala E	3(25.1)	-	-		4(33.33)	-	2(16.66)	1(8.33)	-	2(16.66)	-	-	-	-	12
Chicken Masala- A	4(25)	-	-	1(6.25)	5(31.25)	3(18.75)	1(6.25)	-	2(12.4)	-	-	-	-	-	16
Chicken Masala C	4(36.36)	2(18.18)	-	-	4(36.36)	-	-	-	1(9.09)	-	-	-	-	-	11
Chicken Masala D	3(30)	-	2(20)	3(30)	2(20)	-	-	-	-	-	-	-	-	-	10
Amchur-A	5(23.81)	-	-	-	5(23.81)	-	3(14.28)	2(9.52)	2(9.52)	3(14.28)	-	-	-	1(4.76)	21
AmchurD	6(35.29)	2(11.76)	-	-	4(23.52)	2(11.76)	2(11.76)	-	-	-	-	1(5.88)	-	-	17
TurmericA	2(25)	-	-	-	6(75)	-	-	-	-	-	-	-	-	-	8

CorianderB	3(21.42)			1(7.14)	3(21.42)	2	3(21.42)	-	-	2(14.28)	-	-	-	-	14
CorianderC	3(33.33)		1(11.11)				4(44.44)	-	1(11.11)	-	-	-	-	-	9
Chat Masala-C	4(33.33)	1(7.69)		2(15.38)	2(15.38)	-	1(7.69)	-	-	-	2(15.38)	-	-	-	12
Sambhar Masala-E	4(33.33)	3(25)	1(8.33)		4(33.33)		-	-	-	-	-	-	-	-	12

All figures in parentheses represents percentage %

Identification of Isolates

Bacteria: The bacterial isolates were identified through bio-chemical test including bio-chemical kit for Gram positive (KB013,Himedia Mumbai) and Gram negative bacteria (KB001,Himedia, Mumbai). Most of the isolates belongs to the genus *Bacillus* including *Bacillus Cereus, Bacillus licheniformis, Bacillus thuringiensis. E. coli* (2 samples), *Enterobacter* and *Kliebsiella* (1 sample) were also identified. *Staphylococcus spp*. Including *Staphylococcus aureus, equorum* and *haemolyticus* were also found. Molecular characterization of two isolates were also done (from Xcelris labs ltd., Ahmedabad) in which forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 The first culture was similar to *Bacillus* sp. *S10,* GenBank Accession Number: **KC466241.1** and the other was similar to *Escherichia coli strain DL5.3* ,GenBank Accession Number: **JQ9125398.1**

Fungi : Both the local spice and commercially packed spices collectively harbored sixteen fungal species in which *Aspergillus spp.*(*flavus, fumigatus, ochraceous,niger*) and *Mucor spp*. were predominant ones. Apart from these *Fusarium spp., Trichoderma* sp., *Penicillum* sp., *Alternaria* sp., *Geotrichum* sp., *Cladosporium* sp., *Nigrospora* sp.and *Verticillium* were also identified. The percentage occurrence is reported in Table IIIA and IIIB.

DISCUSSION

The mean values obtained from the bacterial mean count, 10^7 cfu/g and fungal count, 10^5cfu/g were higher than the maximum acceptable levels provided by the Food and Agriculture Organization of the United Nations, FAO [3] ,according to which aerobic plate count and fungal count of spices should not be greater than 10^6 and 10^4 cfu/g respectively. It was observed that total mean count (8.4×10^6 cfu/g) of local spice samples was higher than the commercially packed samples i.e 7.8×10^5 cfu/g. Although four commercially packed samples were also found to have $>10^6$ cfu/g mean count which is an indication of unhygienic practices during their preparation. Freire and Offord [11] reported that most of the bacteria present in spices are aerobic sporeformer. In the present work also most of the isolates found were aerobic sporeformer and rod shaped, among them *Bacillus cereus* is known for food intoxication. Also the presence of coliform is not favorable for consumption and the identified coliforms were *E.coli*, *Klebsiella* and *Enterobacter*, which is again an indication of improper and inadequate hygienic practices as all of them are pathogenic.

The survival of fungal species on dehydrated products is well known. Though the population of resident mycoflora was low, there were other fungal species of pathological importance present in all the samples. *Aspergillus spp. (Aspergillus flavus, Aspergillus fumigatus, Aspergillus ochraceous* and *Aspergillus niger), Penicillium sp.,Mucor spp*. and other species of pathological importance were encountered in the samples tested. *Aspergillus flavus* , a fungus with the ability to produce aflatoxin, was the most frequently isolated with the maximum percentage occurrence in *chicken masala* (36.36%).Other mycotoxigenic fungi such as *Mucor, Penicillium and Fusarium* were also isolated. The isolation of these mycotoxigenic fungi agrees with the work done by Painstil [12] and Addo [13].

The microbiological load in the spices examined was high which represents the unhygienic practices. Contaminating microorganisms might have come from hands of handlers [14].Other practices like harvesting, handling and packing cause additional contamination. Price and Schweigert [15] reported that unless spices are treated to reduce their microbial content, they may add high numbers and undesirable kind of microorganisms to food in which they are used.

CONCLUSION

It was concluded that due to microbial contamination, spices are considered as high risk products and negligence in this area may result in serious contamination that ultimately represents a low quality product to the consumers. As these unwanted and unhygienic conditions are usually due to the lack of knowledge and unawareness of the fundamental sanitary principles, it is preventable by proper training and monitoring. It is recommended that spices should be produced under strict hygienic measures and should be subjected to treatment that would reduce their microbial load.

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