Molecular Characterisation of Lipolytic Fungi Isolated From Meat Products

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Abstract: This work was carried out to evaluate the fungal quality of luncheon, basterma and minced meat sold in different groceries and supermarkets in Gharbia Governorate under different trade names, as well as to evaluate lipolytic activity of isolated fungi and molecular characterization of some of this lipolytic fungi isolated from meat products. The mean total mould counts were $3X10^2 \pm 1.2X10^2$, $2.7X10^2 \pm 3.9X10$ and $5.2X10^3 \pm 1.9X10^3$cfu/g for luncheon, basterma and minced meat, respectively. In the examined samples, 7 mould genera were identified. The isolated mould species were Aspergillus sp, Alternaria sp, Endomyces sp, Euroteium sp, Penicillium sp, Monascas sp and Geotrichum sp were isolated from the examined samples at varying percentages. Aspergillus was represented by 4 species, A. flavus and A. niger were isolated from examined meat product, while A. ochraceous and A. roseoglobulasus were isolated from basterma only. A total of 55 isolates belonging to 17 species were tested for their ability to produce lipolytic enzyme. Four lipolytic A. niger isolates were subjected to PCR identification, and were sequenced in both directions. Sequences were analysed and aligned by Clustal method using the program of DNA star (Laser-gene, Wisconsin, USA).
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1. Introduction

Undoubtedly the main source of animal protein is meat and it's products and it is also a high source of several vitamins and minerals, and so meat products are considered a favourable food as it's easy to buy, fast to cook so it's the first choice for many people to eat. Meat products may contaminate with one of the most dangerous microbial hazards represented in moulds **(Morshdy et al., 2015)**.

Meat products become contaminated during processing and handling operations. **Dijksterhuis and Samson (2002)**

Lipases (triacylglycerol acylhydrolase, EC 3. 1. 1. 3) are hydrolytic enzymes that catalyze the hydrolysis of fats and oils to glycerol and free fatty acids at the oil-water interface **(Jooyandeh et al., 2009).** A considerable number of fungal and bacterial lipases have been commercially produced with the former being preferable, because fungi generally produce extracellular enzymes, Extracellular secretion of lipase enzyme has been well studied for a number of fungi, primarily Zygomycetes, hyphomycetes, and yeasts. In meat, lipid hydrolysis can take place enzymatically or non-enzymatically. The enzymatic hydrolysis of fats is termed lipolysis or fat deterioration and is governed by specific enzymes such as lipases, estarases and phospholipase. Lipolytic enzymes could either be endogenous of the food product (such as milk) or derived from psychrotrophic microorganisms **(Ghaly et al., 2010)**. During lipolysis, lipases split the glycerides forming free fatty acids which are responsible for common off-flavour, frequently referred to as rancidity **(Huis in't Veld, 1996 and FAO, 1986)**. The main enzymes involved in meat lipid hydrolysis are phospholipase A1 and phospholipase A2 **(Toldra, 2006)**. Lipid hydrolysis process is regiospecific and involved three steps of biosynthetic pathway: cleavage of triacylglycerol, acyl migration and cleavage of 1-monoacyl-sn-glycerol **(Belitz et al., 2009; Christie, 2010).**

Internal Transcribed Spacer (ITS) regions have been successfully used to generate specific primers capable of differentiating closely related fungal species. Analysis of the ITS is also sensitive enough to identify intraspecific variation that is not apparent from morphological analysis **(Abd-Elsalam et al., 2004).**

This study was carried out to throw light on the incidence of lipolytic fungi in meat products with molecular characterisation of this lipolytic fungi existing in such products.

2. Material and methods

Collection of samples

A grand total of 60 random samples representing table luncheon (20), basterma (20) and minced meat (20) were collected under different trade names then were kept in sterile polyethylene bags and preserved in an ice box then transferred to the laboratory under complete aseptic condition without undue delay to be examined mycologically.

Food homogenate preparation: according to **APHA (2001).**

Determination of total mould count according to the technique recommended by **(ISO, 2008):**

Identification of mould isolates: according to the technique recommended by **Pitt and Hocking (2009)**, **Frisvad and Samson (2004)**

Detection of lipolytic activity: according to **Sunitha et al., (2013)**

We used the sensitive plate assay for detection of lipase activity in growing cultures (10, 11). Detection of lipase activity was performed on Peptone Agar medium supplemented with Tween 20 separately sterilized and added 1% to the medium. At the end of the incubation period, a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity.

Fungal Mycelium Preparation and DNA Isolation: Fungal mycelium was prepared from pure culture using 50 ml of SDA broth in 100 ml conical flasks and was incubated at 25±1°C for 7 days. Mycelia from 50 ml broth were harvested by filtration through Whatman sterile filter paper and the fungal genomic DNA was isolated **(Rohlf, 1998).** After DNA extraction, lipase-producing fungi were identified via ITS-PCR of rDNA region with ITS1 and ITS4 primers. PCR amplification conditions for A. niger were: 5 min initial step followed by 38 cycles at 94 °C for 1 min, 5C for 1 min and 72 °C for 1.5 min and a final extension step at 72 °C for 5 min. Amplification products were electrophoresed in agarose gels (3% w/v) (Agarose, Sigma, USA). And stained with ethidium bromide using Gene Ruler 100bp DNA Ladder (Fermentas Company, Cat.No.SM0243, USA). Finally, we made sequencing to the PCR product on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new 454 technology, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. In addition, considerable cost advantages now make genome sequencing with the 454-technology accessible to the research community. Sequences were analyzed and aligned by Clustal method using the program DNA star (Laser-gene, Wisconsin, USA).

3. Results and Discussion

Meat is preferred by millions of people all over the world as a major source of animal protein. But meat is considered a favourable media for growth of various pathogens, so could be spoiled rapidly. The decomposition of different constituents of meat including protein and fat resulting in bad odours and other unfavourable changes. Therefore, spoilage of meat and meat products should be limited and controlled to protect the human consumers from infection and allergy **(Aksoy et al., 2014)**. In the present study, the mean mould count was $3X10^2 \pm 1.2X10^2$, $2.7X10^2 \pm 3.9X10$ and $5.2X10^3 \pm 1.9X10^3$cfu/g in luncheon, basterma and minced meat, respectively as described in Table (1). The results of luncheon samples nearly similar to those obtained by **Shaltout and Salem (2000) and Elsayed et al (2018)** reported that the mean mould count in luncheon and minced meat was1.3x102±2.1x10 cfu/g and 2.8x102±4.3x10cfu /g, respectively**.** while higher prevalence was recorded by **El-Tabiy (2006)** who reported that the mean mould count in luncheon and minced meat were $1.3x\ l0^4 \pm 9.3\ x10^3$ and $2.1x\ l0^6 \pm 6.1x10^5$ cfu/g, respectively. **Hussein (2008)** who reported that mean mould count of sausage ($2.26\times10^2 \pm 0.58\times10^2$ cfu/g).

Table (1): Incidence of mould isolated from meat products (No=20 for each product):

Meat products	No of examined samples	No of positive mould samples	Min.	Max.	Mean ± SE
Luncheon	20	9	3X10	$8.5X10^2$	$3X10^2 \pm 1.2X10^2$
Basterma	20	20	2X10	$5.2X10^2$	$2.7X10^2 \pm 3.9X10$
Minced meat	20	14	$3.3X10^2$	$7.8X10^3$	$5.2X10^3 \pm 1.9X10^3$

Mould contamination of meat and meat products may occur during slaughtering of the animals, transportation, or during processing of meat products through the use of contaminated equipments or contaminated additives and spices which considered the most important source of mould contamination in meat products **(Jay et al., 2005).**

The data obtained from table (2) declared that 7 mould genera could be isolated and identified from the examined meat samples. The identified mould genera were; Aspergillus, Alternaria, Endomyces spp, Penicillium spp, Monascas spp Geotrichum spp. In addition, that 4 Aspergillus species could be identified from meat product samples. The most predominant

species were; Aspergillus flavus and Aspergillus niger which were present in 4 (20%), 4 (20%), 2(10 %), 2 (10 %),4 (20%) and 3(15%), respectively, in Luncheon, Basterma and Minced meat. A. flavus and A. niger were present in all examined meat samples. A. ochraceous and A. roseoglobulasus were only present in Basterma. Incidence of Penicillium species isolated from examined meat samples revealed that P. citreonigrum, P. glabrum P. polonicum, P.citrinum and P.simplicissim were the most predominant species isolated from Luncheon with frequency of 1(5%). In addition, the result achieved in table (2) it is obvious that P. crusteoum, P. paneum and P. brevicompactum were isolated from the Basterma samples only.

The results illustrated in table (3) showed A.niger, A. flavus, A.ochraceus, P. citreonigresum, P. glabrum P.cirtinun, P. crusteoum, P. paneum, P. brevicompactum and P. simplicissimum were having lipolytic activity with a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity. These results nearly similar to those obtained by **Nasser (2002), Shaltout (2002), Subash, et al. (2005), Soliman and Shalaby (2008), Korashy and Wahbba (2008), El-Diasty and Salem (2009) Ouf et al. (2010) and Shaltout et al. (2014).**

Table (2): Incidence of isolated mould from meat products (No=20 for each product):

Isolated mould genera	Luncheon		Basterma		Minced meat	
	No	%	No	%	No	%
Aspergillus spp	6	30	14	70	5	25
A. flavus	4	20	4	20	2	20
A. niger	2	10	4	20	3	15
A. ochraceous	-	-	5	25	-	-
A.roseoglobulasus	-	-	1	5	-	-
Alternaria spp	1	5	2	10	-	-
Endomyces spp	-	-	-	-	7	35
Eurotium spp	2	10	-	-	-	-
Penicillium spp	5	25	6	30	-	-
P.citreonigrum	1	5	-	-	-	-
P.glabrum	1	5	-	-	-	-
P.polonicum	1	5	-	-	-	-
P.citrinum	1	5	-	-	-	-
P.crusteoum	-	-	2	10	-	-
P.paneum	-	-	2	10	-	-
P.brevicompactum	-	-	2	10	-	-
P.simplicissim	1	5	-	-	-	-
Monascas spp	1	5	1	5	-	-
Geotrichum spp	1	5	-	-	4	20

These PCR systems described to date are useful only in identifying the genus Aspergillus as a whole or the single species. The ITS region contains variable elements that allow for sequence-based identification of Aspergillus species **(Iwen et al., 2002)**; therefore, the region offers a possible template for design of species-specific primers for identification of the major pathogenic species. Most previous publications on PCR-based detection or identification systems for Aspergillus spp. were based on using 18S or 28S rDNA as target DNA. However, the sequences in these regions are conserved across a wide range of fungi; it is therefore difficult to design truly species-specific primers. As reported previously, the more variable ITS regions have proven more useful for identification of fungal species **Makimura (2001).**

Polymerase Chain Reaction (PCR) amplification of ITS region of rRNA genes with ITS1 and ITS 4 primers yielded distinct DNA bands for all representative isolates investigated DNA sequences of Aspergillus niger identified by similarity searches in the GenBank gave.

Molecular biological identification systems for pathogenic aspergilli have been suggested as a solution to this problem: for example, a PCR based diagnostic method for detecting the genus Aspergillus using 18S rDNA has been designed **(Makimura et al., 1994 and Yamakami et al., 1996)**. Systems have also been described for specific detection of Aspergillus niger with primers based on regions of the 28S rDNA **(Henry et al., 2000 and Zhao et al., 2001)** or of the internal transcribed spacer (ITS) 1 and 2 regions of ribosomal DNA (rDNA). These PCR systems

described to date are useful only in identifying the genus Aspergillus as a whole or the single species A. niger. The ITS region contains variable elements that allow for sequence-based identification of Aspergillus species.

Table (3): Lipolytic activity of mould on tween 80 media:

strains	No of examined strains	positive strains		No of negative strains
		No of positive strains	Diameter of inhibition zone (mm)	
A.niger	15	15	16-17	-
A.flavus	16	9	10	7
A.ochraceus	5	5	19	-
A.roseoglobulasus	1	-	-	1
P.citreonigresum	4	4	9	-
P.glabrum	2	2	7	-
P.polonicum	1	-	-	1
P.cirtinun	1	1	12	-
P.crusteoum	2	2	17	-
P.paneum	3	3	12-13	-
P.brevicompactum	2	2	10-11	-
P.simpliccissim	3	3	7	-
Alternaria sp	3	-	-	3
Endomyces sp	7	-	-	7
Euroteium spp	2	2	6	-
Monoscasp spp	2	-	-	2
Geotrichum spp	5	5	13-14	-

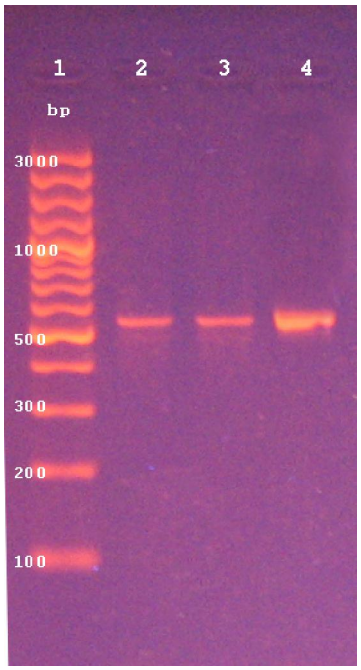

Figure (1): 1.5 % of agarose gel electrophoresis of PCR product with ITS for A.niger

Lane1 Molecular weight marker, Lane2-4 A. niger.

Three A.niger isolates has lipolytic activity on peptone agar medium supplemented with tween 20 were examined by molecular methods polymerase chain reaction (PCR) with using ITS primer. PCR products of A.niger strains were positive on agarose gel electrophoresis of PCR amplification products **(Figure 1).**

The PCR products of the one A.niger isolate was sequenced and aligned with references in the NCBI database. The sequences had 98.1% identity with Aspergillus spp. sequences deposited in the NCBI database. Nucleotide analysis in figure (2) showed greater than 98% similarity for the entire sequence of the one sequenced strain A.niger-AAE-EG017 in comparison of 10 strains of Aspergillus Spp. The sequence similarity for the strains of A.niger-SAM6, A.niger-36, A.tubingensis-ND9, A.niger-129B, A.niger-FIS17, A.tubingensis-G4-14, A.tubingensis-F43-01, A.niger-AL-29, A.awamori-ND6 and A.niger-Egypt-BSU-5 were100 %,100%,100%, 100%,99.7%, 99.4 %,99.7%, 99.7% and 99.7 % **(figure 2)**.

Phylogenetic analysis showed that A.niger-AAE-EG017 accession no. **(MH590624)** was isolated but in different branches, away from A.niger-SAM6, A. niger-AL-29, A.niger-36, A.niger-129B,A.niger-FIS1 and A.niger-Egypt-BSU-5 but remained in the same cluster. While A.niger-AAE-EG017 accession no. (**MH590624**) still in the same group of A.tubingensis-G4-14 **(figure 3)**

Of the strain of A. niger studied, ITS could be amplified from three strains. The A.niger strain is readily distinguished from those of the other species by the 17 base insertion (TCCAGCATCGAATGGG) at nucleotides 1–17, and their distinctive nucleotide changes (AGGA) at the following positions: 4-15-16-17. Some of these variations result in nucleotide lead to amino acid changes **(Figure 4).**

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		98.1	98.1	98.1	98.1	98.1	98.3	98.3	98.1	97.8	98.0	1	A.nig-AAE-EG017
2	2.0		100.0	100.0	100.0	100.0	99.7	99.7	100.0	99.7	100.0	2	A.niger-SAM6
3	2.0	0.0		100.0	100.0	100.0	99.7	99.7	100.0	99.7	100.0	3	A.niger-36
4	2.0	0.0	0.0		100.0	100.0	99.7	99.7	100.0	99.7	100.0	4	A.tubingensis-ND9
5	2.0	0.0	0.0	0.0		100.0	99.7	99.7	100.0	99.7	100.0	5	A.niger-129B
6	2.0	0.0	0.0	0.0	0.0		99.7	99.7	100.0	99.7	100.0	6	A.niger-FIS17
7	1.7	0.3	0.3	0.3	0.3	0.3		99.4	99.7	99.4	99.7	7	A.tubingensis-G4-14
8	1.4	0.0	0.0	0.0	0.0	0.0	0.3		99.7	99.4	99.7	8	A.tubingensis-F43-01
9	2.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0		99.7	100.0	9	A.niger-AL-29
10	2.3	0.3	0.3	0.3	0.3	0.3	0.6	0.3	0.3		99.7	10	A.awamori-ND6
11	2.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.3		11	A.niger-Egypt-BSU-5
	1	2	3	4	5	6	7	8	9	10	11		

Figure (2): Nucleotides identity of sequences of different Aspergillus spp.

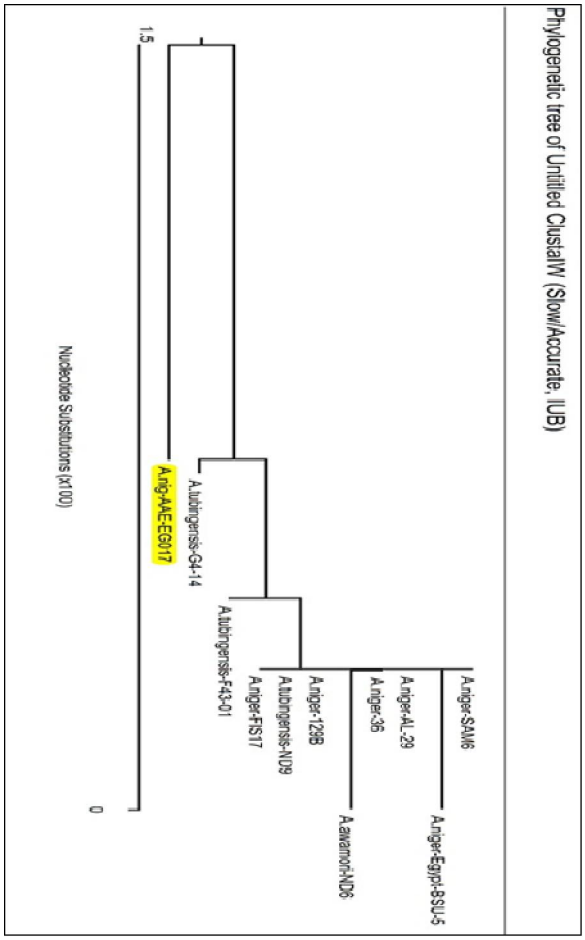

Figure (3): Phylogenetic tree of **Nucleotides** sequences of different Aspergillus spp.

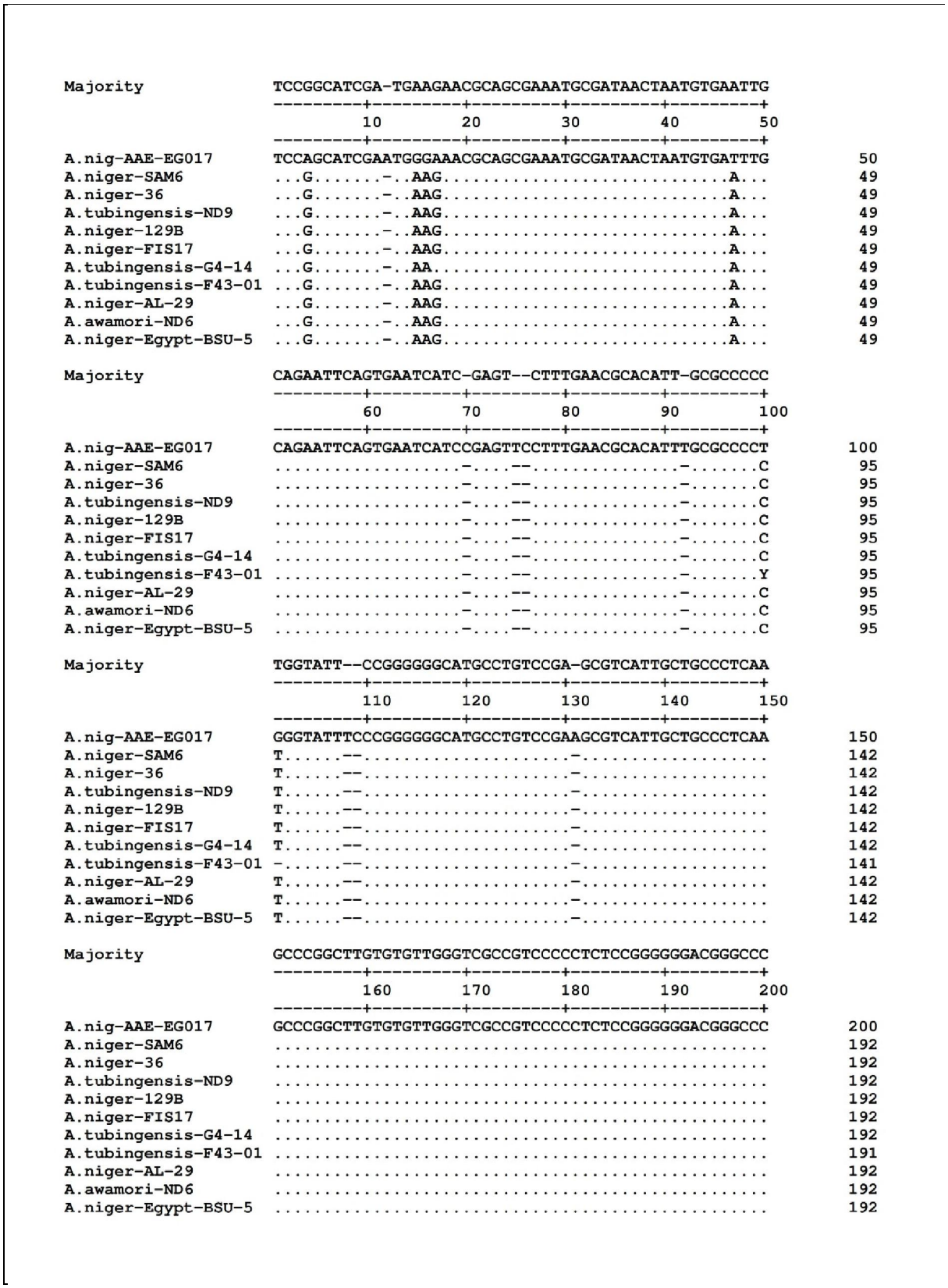

Figure (4): Clustal W Alignment of Nucleotides sequences of A.niger-AAE-EG017compared to different Aspergillus spp.

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Majority            GAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGT
                    ---------+---------+---------+---------+---------+
                             210       220       230       240       250
                    ---------+---------+---------+---------+---------+
A.nig-AAE-EG017     GAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGT      250
A.niger-SAM6        .................................................       242
A.niger-36          .................................................       242
A.tubingensis-ND9   .................................................       242
A.niger-129B        .................................................       242
A.niger-FIS17       .................................................       242
A.tubingensis-G4-14 .................................................       242
A.tubingensis-F43-01 ................................................       241
A.niger-AL-29       .................................................       242
A.awamori-ND6       .................................................       242
A.niger-Egypt-BSU-5 .................................................       242

Majority            CACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTT
                    ---------+---------+---------+---------+---------+
                             260       270       280       290       300
                    ---------+---------+---------+---------+---------+
A.nig-AAE-EG017     CACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTT      300
A.niger-SAM6        .................................................       292
A.niger-36          .................................................       292
A.tubingensis-ND9   .................................................       292
A.niger-129B        .................................................       292
A.niger-FIS17       .................................................       292
A.tubingensis-G4-14 .................................................       292
A.tubingensis-F43-01 ................................................       291
A.niger-AL-29       .................................................       292
A.awamori-ND6       ................................................C      292
A.niger-Egypt-BSU-5 .................................................       292

Majority            TTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
                    ---------+---------+---------+---------+---------+
                             310       320       330       340       350
                    ---------+---------+---------+---------+---------+
A.nig-AAE-EG017     TTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT      350
A.niger-SAM6        .................................................       342
A.niger-36          .................................................       342
A.tubingensis-ND9   .................................................       342
A.niger-129B        .................................................       342
A.niger-FIS17       .................................................       342
A.tubingensis-G4-14 .................................................       342
A.tubingensis-F43-01 ................................................       341
A.niger-AL-29       .................................................       342
A.awamori-ND6       .................................................       342
A.niger-Egypt-BSU-5 .................................................       342

Majority            ATCAATAAGGCGGAGGAAXXX
                    ---------+---------+-
                             360       370
                    ---------+---------+-
A.nig-AAE-EG017     ATCAATAAGGCGGAGGAAAAA                                   371
A.niger-SAM6        ...................                                     361
A.niger-36          ..................                                      360
A.tubingensis-ND9   ..................                                      360
A.niger-129B        ..................                                      360
A.niger-FIS17       .........-..........                                    362
A.tubingensis-G4-14 .........-........                                      359
A.tubingensis-F43-01 .........-.........                                    360
A.niger-AL-29       .........-........                                      359
A.awamori-ND6       ..................                                      360
A.niger-Egypt-BSU-5 ..............                                          356
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Conclusion

This investigation has shown that all meat products Are important Contributors to the transmission of Aspergillus spp (A. niger) which had lipolytic activity and extract ochratoxin A which cause economic loss and toxicity.

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