QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

INVESTIGATION AND BIOLOGICAL CONTROL OF TOXIGENIC FUNGI AND MYCOTOXINS IN DAIRY CATTLE FEEDS

BY

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A Thesis Submitted to

the Faculty of the College of Arts and Sciences

in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Environmental Sciences

June 2020

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ABSTRACT

ALASMAR, REEM M., Master: January: [2020], Environmental science

Title: Investigation and Biological control of toxigenic fungi and mycotoxins in Dairy Cattle Feeds

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Mycotoxins are secondary metabolites synthesized by mycotoxigenic fungi, contaminating human and animal food and feed. Aflatoxin B1 (AFB1) and ochratoxins (OTA) are frequently detected in animal feed products causing many health issues in humans and animals.

In this M. Sc research work, we evidenced the presence of OTA and AFs below the EU maximum permissible limits. Mycotoxigenic fungal strains have been isolated from the animal feeds. The morphological and molecular identification of these toxigenic fungi allowed the evidence of *A. flavus*, *A. niger*, *A. carbonarius* and *P. verrucosum* that synthesize during their growth mycotoxins compounds (Aflatoxins & Ochratoxins) at levels higher than the detection limits and the corresponding genes were evidenced.

In order to set up safe and efficient biological control strategy of these mycotoxigenic fungi, we decided to use yeasts known to be very safe for such purpose. Thus, in the second part of this Thesis, 14 yeast strains were isolated from different fermented food, dairy and meat products. These strains showed a great antifungal and spectrum of activities through the synthesis of Volatile Organic Compounds (VOCs). Among these yeast strains, a particular strain of *Kluyveromyces marxianus* QKM-4, had the strongest antifungal VOCs that we were able to identify. GC/MS based analysis of yeast VOCs

showed long chain alkanes including nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36) and tetracosane (C24) that can control the mycotoxigenic fungal strains and their mycotoxins synthesis. Testing the mycotoxin binding potential of the live and heat-inactivated QKM-4 cells, showed the reduction of OTA and DON up to 58% and 49%. The findings of the present study clearly demonstrate a strong antifungal potential of *Kluyveromyces marxianus* QKM-4 for its possible application in the agriculture and food industry. In another applied part of the present work, the strain QKM-4 of *Kluyveromyces marxianus* showed a great inhibition potential of the mycotoxigenic fungal growth, spore's germination and 100% protection of tomato and grape fruits from the *in vitro* infection of the latter by mycotoxigenic fungi for more than 10 days.

DEDICATION

This work is dedicated to my father, Eng. Moath Alasmar,

& my Husband, Eng. Mohammad Abdalwahab.

ACKNOWLEDGMENTS

The success and completion of this project required a lot of guidance and assistance from many people that I was very privileged to have their continuous support and supervision throughout the project.

Firstly, I would like to thank and dedicate this project to my father Dr.Eng. Moath Alasmar, mother, and my husband Eng. Moahmmed for their endless support and understanding through the process of working on this project and throughout my university years.

I would like to thank Prof. Samir Jaoua for his continuous and very efficient guidance and supervision during this MSc thesis research work from the beginning till the end.

I am also very thankful to all Biotechnology and Bioremediation laboratory members, especially Dr. Zahoor Ul Hassan and Ms. Randa Zeidan for important support to run these experiments. The MSc was made possible by QU student grant QUST-1-CAS-2020-1 from Qatar University.

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CHAPTER 1: INTRODUCTION

Mycotoxins are the secondary toxigenic compounds synthesized for use in a large variety of foods by specific species of modified fungi. The widely discovered mycotoxins are aflatoxins such as patulin, citrinin, ochratoxin (OTA), and trichothecene including Fumonisins, deoxynivalenol (DON), HT2 toxin (HT2), T2 poison (T2), Zearalenone (ZEN). These metabolites are synthesized in large quantities by fungi from the following genera: *Fusarium, Penicillium, Alternaria* and *Aspergillus* (Medina-Córdova, *et al.,* 2018). Some fungal species can synthesize one type of mycotoxin, while others can produce greater numbers of mycotoxins. Nevertheless, the development of the toxigenic fungi may not really infer mycotoxins formation. Thus, the non-appearance mycotoxins are not always synthesized by active fungi but can also be synthesized by inactive fungi (Sarrocco & Vannacci, 2018).

Mycotoxins production and development can happen at different stages of food processing and production. Specific environmental factors, such as sun, microbial competition, nutrient availability and substratum composition, pH, humidity, water, usage of pesticides and fungicides, and the existence of specific insects generally influence their growth. Consequently, it is hard to portray the suitable environment that will encourage both growth of fungi and formation of mycotoxins (Medina-Córdova, *et al*., 2018).

This M. Sc. Thesis project aims to monitor the presence of fungi and mycotoxins in Dairy Cattle Feeds and set up biological control methods based on local yeast activities. We explored the biocontrol potentialities of different local yeast strains isolated from food products. Furthermore, the antifungal potentials of local yeasts strains to reduce the growth of mycotoxigenic fungal species of different genera such as, *Aspergillus, Fusarium* and *penicillium* and their synthesis of their mycotoxins were

investigated.

1.1 Research objectives:

1. Monitoring the presence of fungi and mycotoxins in Dairy Cattle Feeds.
2. Isolation and Application of local yeasts strains volatile organic compounds in the biological control of toxigenic fungi in Qatar.

CHAPTER 2: LITERATURE REVIEW

2.1 Mycotoxins:

Mycotoxins are a massive and diversified group of toxic secondary metabolites of low molecular weight. There is a well-known mycotoxin group that includes fungi that belong to the genera *Aspergillus, Penicillium, Alternaria* and *Fusarium*. More than 399 mycotoxins have been investigated, identified and published so far (Carolina Santos Pereira, 2019). The widely discovered mycotoxins are aflatoxins such as patulin, citrinin, ochratoxin (OTA), and trichothecene including Fumonisins, deoxynivalenol (DON), HT2 toxin (HT2), T2 poison (T2) and Zearalenone (ZEN). The next part is introducing each one with its associated toxicological effects.

2.1.1 Aflatoxins:

Aflatoxins are generally synthesized by *Aspergillus* species, like *A. flavus* and *A. parasiticus*, However, *A. nomius* & *A. pseudotamarri* synthesize AFs as well. The aflatoxins are divided into many different sub-toxins, but only few kinds are copious such as: B1(AFB1), B2 (AFB2), G1(AFG1) and G2(AFG2) (Pereira, Cunha and Fernandes, 2019). On the other hand, their products are derived from aflatoxins, like aflatoxins AFM1 and AFM2, that are known to be major contaminants. AFs are a group of toxigenic fungi which is of high concern about human toxicity. Moreover, its fungal toxicity affects the human food chain in different ways: firstly, when humans consume crops that are contaminated or processed food products, where the AFs are verystable during foodprocessing. On the other hand, indirectly through tissue, eggs and dairy products of animals fed with AF-contaminated feeds, by excretion of the secondary metabolites of AFB1 and AFM1 hydroxylated derivatives.

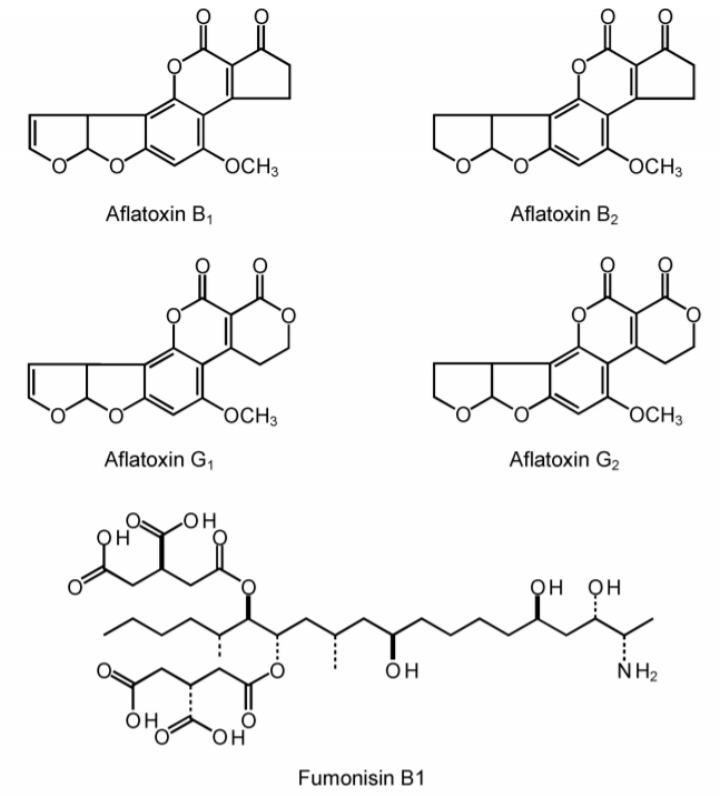

Figure 1. Aflatoxins chemical structures (Palumbo et al. , 2008).

2.1.2 Ochratoxins:

Ochratoxins A (OTA) and B (OTB) are produced by *Aspergillus* & *Penicillium*, mainly by *A. ochraceus*, *A. Carbonarius*, *P. verrucosum*, and *P. nordicum*. In recent studies, it was noticed that the OTAs are strongly associated with significant nephrotoxic effects especially in animals with more exposure to naturally occurring levels in feeds, and it was concluded that kidneys are the main target organ (Zhao *et al*., 2017). On the other hand, epidemiological studies showed that consuming high doses of OTAs toxins can cause liver damage, in addition to lymphoid and intestinal tissues necrosis (Duarte, Lino and Pena, 2012). In terms of human toxicity, Ochratoxins contributes to fetal kidney disease and are classified in possibly carcinogenic group 2B. Nowadays, the public health has been extremely concerned about the transfer of OTA to animal-derived foods (Denli and Perez, 2010).

2.1.3 Zearalenone:

ZEN is one of the mycotoxins synthesized by *F. graminearum*, *F. culmorum, F. ceralis and F. equiseti.* The ZEN mycotoxins are very similar in structure to the estradiol female hormone and commonly classified as a nonsteroidal estrogen. All these chemical characteristics give to the ZEN toxins the binding capacity to the estrogen receptors, which in turn causes different impacts that are related to reproductive disorders. This happens in humans and breeding animals. ZEN toxins are not classified as carcinogenic to humans and belong to group three regarding IARC (Pereira, Cunha and Fernandes, 2019)

A vast assortment of toxic impacts on animals and people have been seen because of the intake of food that is contaminated with mycotoxins, for example: immunosuppression, cancer-causing, mutagenic, teratogenic and genotoxic impacts (Luísa & Anderson, 2018). Nevertheless, the effect of mycotoxins on health relies upon various variables, including stages of intake, the toxicity quality of the mycotoxin, and the individual's age and weight. There are also other key factors such as existence of different types of mycotoxins, time of exposure, individual's health status and mechanism of action of the compound that determines the effect of mycotoxins on health (Medina-Córdova, *et al.,* 2018).

2.1.4 Deoxynivalenol (DON):

Trichothecenes toxins are mainly produced by the *Fusarium* species. TRCs are one of the broad groups of fungal metabolites with more than 150 structurally-, classified into four forms chemically. B-TRC is represented by DON. It is one of the toxic TRCs and is very frequent in animal husbandry. On the other hand, ingestion of low to moderate amounts of this toxin by animals or humans will lead to high susceptibility to pathogens. According to the IARC, DON was classified by IARC as carcinogenic to humans. Moreover, not all structures of mycotoxins were identified,

because of the change of their main compounds into various shapes (modified mycotoxins), which are not yet recognized by regular strategies and could bring about underreporting. During the mid-1980s, the emerged expression of “masked mycotoxin” was appointed due to instances of mycotoxicosis that did not correspond with mycotoxins identified in the sample analyzed (Luísa & Anderson, 2018).

The high level of toxicity might be due to undetected structures, for example, conjugated mycotoxins (Sarrocco & Vannacci, 2018). All these metabolites, which have been recently named as modified mycotoxins, experience changes in their structure, shape, polarity of these metabolites, molecular mass and solubility. However, modified mycotoxins may be created by fungi that are important in the defense mechanism of the organism affected by the fungi.

Moreover, these metabolites may be synthesized during food production from contaminated crude materials, and sometimes they can be returned to the parent toxin when digested by the affected organism (Luísa & Anderson, 2018).

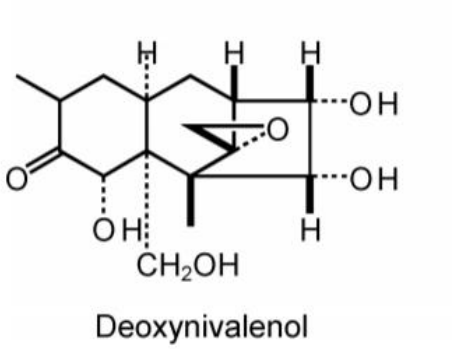

Figure 2. The chemical structure of Deoxynivalenol A addressed in this paper.(Palumbo *et al.* , 2008).

2.2 Modified mycotoxins:

The first modified mycotoxin found, in spite of the fact that the term “masked mycotoxin” was still not appointed was aflatoxin M1, which is produced by the

hydroxylation of aflatoxin type B1 and secreted in the secretions of animals that fed on food such as corn, mize and cereals contaminated with this mycotoxin (Sanzani *et al.,* 2016).

After this discovery, scientists found another substance obtained from ZEN. Notwithstanding, the detection of different types of mycotoxin was massive only lately. This was mostly because of the introduction of liquid chromatography with mass spectrometry, the most fundamental method utilized in the clarification of those mycotoxin compounds (Alberts, *et al*., 2017). Although the classification of various types of mycotoxins is confounding and still requires more examination, the expression “masked mycotoxins” proved to be utilized only for mycotoxins compounds that were synthesized by plants' defense mechanisms.

Nevertheless, it has been discovered that mycotoxin compounds may also be synthesized by different reactions, for example, food processing and animal digestion (Sanzani *et al.,* 2016).

In this manner, the expression “modified mycotoxin” was lately instituted to indicate that all structures were formed from mycotoxins, despite their source. This term incorporates compounds synthesized by processes carried out by microorganisms or synthesized by plants metabolism (Neme & Mohammed, 2017).

In the literature, a few studies tried to classify the modified mycotoxins depending on the way that their parent mycotoxin ties itself to the food molecules. When mycotoxins bind to food substance by covalent bonds, they are classified as "conjugated" compounds. Then again, when the mycotoxins connect to food elements through non-covalent bonds such as physical entrapment, it is designated unseen. Despite that, all structures of mycotoxins are considered "bound" for being connected to the matrix (Medina-Córdova, *et al.,* 2018).

2.3 Prevention of mycotoxins in food chain:

Mycotoxins in cereals:

There are only 15 crop plants that give ninety percent of the food supply worldwide out of the abundant fifty thousand plants species suitable to eat, such as rice, corn and wheat, which make up 66% of this 90% (Saeger, 2011). However, the staple food establishes the most important component of food intake worldwide and supplies most of energy and nutrient needs. These grains are the staple foods of more than four thousand million individuals (Medina-Córdova, *et al.,* 2016). The grains forms 26% of the energy of the primary staple food in Europe, while in Africa it forms 46 % (James & Zikankuba, 2018).

Studies about mycotoxigenic fungi usually focus on cereals because they are found in abundance. This causes extreme crop loss and a drop in the quality of the crops harvest because of the accumulated mycotoxins on crops. Nevertheless, accumulation of mycotoxin remains even after harvest, on account of the development of the inoculum found in caryopses and ill-advised capacity conditions(Greco *et al.*, 2014).

There are many examples which show that introducing beneficial fungi during development can help lessen mycotoxin build up in the food. The subject of several studies was Fusarium Head Blight (FHB) triggered by trichothecene-contaminated wheat due to its severe implications and *Aspergillus* corn infection along with aflatoxin production(Medina-Córdova, *et al*., 2018).

2.4 Reduction of mycotoxins on fruits:

Aspergillus and grape fruits:

As stated by the International Organization for Vine and Wine (OIV), global grape production amounted to 75.1 million tons (MT) in 2014. China is known to be the largest place producer of grape wine with 11.5 MT, led by the USA and Italy with up to 7.0 MT each. The bulk of the grapes produced are used in the wine industry, and a small number of the grapes are often sold worldwide as natural juice or as dried raisins. (Medina-Córdova, *et al*., 2018).

Black *A. niger* and *A. carbonarius* are always present in vineyards. All these fungi are the fundamental toxin producers found in grape and it is derivatives after harvest, while *A. carbonarius* is as the most grounded maker of OTA (Covarell *et al.,* 2015). They are considered as one of the few pre-harvest strategies that reduce the possibility of fungal infection and subsequently, of the contamination by mycotoxin during the process and as end compound (Sarrocco & Vannacci, 2018).

Regardless of the utilization of preventive strategies, other factors like protecting grapes from mechanical and insect destruction, for example, will also play a role in preventing ochratoxins from infecting and accumulating on plants. In addition, the utilization of biological agents is still the best eco-friendly way that guarantees a drop in synthetic contribution for the ecosystem and people's health (Covarelli *et al.,* 2015).

2.5 The possibility of using Yeast strains for the biological control of Harmful fungi found on food:

Yeasts are considered a beneficial biocontrol agent because they are adaptable organisms which can be used against risk of OTA contamination in grape and against risk of AFs contamination in tomato. This beneficial trait is due to factors that make the yeast fungi unique for use as biocontrol agents. These factors include their simple food prerequisites, their capability of living in various environmental conditions, in

addition to a quick development rate and no formation of lethal substances (Mwakinyali, *et al*., 2019).

Epiphytic yeasts constitute the bulk of the microbes found outside the grapes. They have adapted very fast to their biological and ecological niche and can play an important role as bio-control products against *Aspergillus* spp. for use at the pre-harvest step (Laitila, 2015). *Candida guilliermondii* versus *Aspergillus* spp., *Issatchenkia orientalis* versus *A. carbonarius* and *A. niger, Metschnikowia pulcherrima, Issatchenkia terricola* and *Candida incommunis* versus *A. niger* and *A carbonarius, Aureobasidium pullulans* versus *A. carbonarius* or *Candida magnus* and *Candida purpose* versus *A. tubingensis* are only several instances of fungi which have reduced the aggregation of dangerous fungi and the occurrence of damage to the grapes. (Alberts *et al.,* 2017).

A few researchers have found that the utilization of non-ochratoxigenic *Aspergilli* reduces *A. carbonarius* on grape. *A.carbonarius* atoxigenic strains were uncommon and some non-OTA strains are synthesized by different types of black *Aspergilli* species (Mwakinyali, *et al*., 2019).

However, some isolated strains may be useful when used as agents for food production and as biocontrol agents to minimize the development of OTA in vineyards (Laitila, 2015). In the recent years, scientists reported that *Penicillium adametzioides* acts as a possible bio-control molecules for ochratoxin-delivering *A.carbonarius,* because of its capacity to constrain the development of the harmful fungi and OTA generation. More studies into this field is required to understand the involved mechanisms of OTA control and to understand its behavior and effect on the environment (Medina-Córdova, *et al.,* 2018)**.**

2.6 Yeast Habitats:

Yeasts are microorganisms generally dispersed in various environments such as water and airborne particles, plants, nutrients, and can be found in people digestive system. Moreover, yeasts are overwhelming species in different environmental niches since yeasts are able to quickly colonize nutrient rich substances (Medina-Córdova, *et al.,* 2016). Yeasts are considered as fungi that reproduce by asexual reproduction that causes growth with single cells (Medina-Córdova, *et al.,* 2018). For example, Debaryomyces spp. which belongs to the family *Saccharomycetaceae* have biotechnological potentials. Furthermore, it was originally isolated from water environments. It can also be isolated from many niches with low water motion (Alberts *et al.,* 2017).

Remarkably, these yeast species can be found in food and other natural origins. However, Debaryomyces strains used as biological control agents against fungi cause health problems when separated from fruits, processed meat and dairy products (Medina-Córdova, et al., 2016). Consequently, a different biodiversity of yeasts strains can be isolated from food products and can act as a strong biological control agent against contamination by fungus (Greco et al., 2014)

2.7 Conclusion:

Mycotoxins are the most secondary toxicogenic compounds synthesized by fungi which affect the food chain. They can develop at any level of food processing and manufacturing. Their growth is affected by different environmental factors, including microbial competition, nutrient accessibility and substrate structure, pH and heat.

Mycotoxins contaminate mainly grains and has many health risks on humans and animals including immunosuppression, cancer-causing, genotoxic, teratogenic and

mutagenic. Moreover, modified mycotoxins make more difficult the control of mycotoxins because not all their structures are identified. Therefore, studying mycotoxins to monitor their presence in the food chain is crucial.

Many yeasts strains demonstrated important potentials as biological control agents against toxigenic fungi and mycotoxins in the food chain. On the other hand, bio-prospecting of different yeasts strains should be performed to reduce the growth of toxigenic fungi and mycotoxin to select strong biological control agents.

CHAPTER 3: MATERIAL AND METHODS

3.1 Materials:

3.1.1 Strains:

The following fungal strains were provided by our Italian partner team in Italy, by Prof. Quirico Migheli (Sassari Univ. Italy)

Table *1*: List of fungal strains used in the current project (Reference strains)

Reference Code	Strains
AC82	*Aspergillus carbonarius*
CECT 2687	*Aspergillus flavus*
Af14	*Aspergillus niger*
AN8	*Aspergillus ochraceus*
AF82	*Aspergillus parasiticus*
AW82	*Aspergillus westerdijkiae*
23	*Fusarium anthophilum*
29	*Fusarium chlamodosporum*
16	*Fusarium culmorum*
24	*Fusarium graminearum*
19	*Fusarium oxysporum*
18	*Fusarium subglutinus*
14	*Fusarium prolifiratum*
13	*Fusarium verticilliod*
PC44	*Penicillium camemberti*
PE82	*Penicillium expansum*
PD43	*Penicillium digitatum*
PI48	*Penicillium italicum*

TF11	*Penicillium verrucosum*

Table *2*: Yeast strains isolated in this work from different fermented food samples, dairy products and meat products

Strain	Food sample	Sample ID	Yeast strain no.	Genus & species (this work)
1	Meat	M1	QR1-5/Y1	
QCL#2	Meat	M1	QR1-5/Y2	*Clavispora lusitaniae*
QKS#3	Meat	M1	QR1-5/Y3	*Kazachstania servazzii*
QKM#4	Laban	L2	QR1-5/Y4	*Kluyveromyces marxianus*
QKS#3	Meat	M1	QR1-5/Y5	*Kazachstania servazzii*
QKS#6	Meat	M1	QR1-5/Y6	*Kazachstania servazzii*
QKS#7	Meat	M1	QR1-5/Y7	*Kazachstania servazzii*
QAP#8	Animal feed	SB	QR1-5/F8	*Aureobasidium pullulans*
9	Animal feed	SD	QR1-5/F9	
10	Animal feed	SD	QR1-5/F10	
QC#11	Animal feed	SD	QR1-5/F11	*Cryptococcus*
12	Animal feed	SD	QR1-5/F12	
QKS#13	Animal feed	SD	QR1-5/F13-1	*Kazachstania sp*
QCL#14	Animal feed	SA	QR1-5/F14-2	*Clavispora lusitaniae*
QCC#15	Animal feed	SA	QR1-5/F15	*Cutaneotrichosporon curvatus*

3.1.2 Media preparation:

Potato dextrose agar (PDA): (250 g/L) potatoes infusion from dextrose (20 g/L). 42 g of potato dextrose agar were suspended in 1 L of dH_2O. The sterilization was carried out at 121ºC for 15min.

Dichloran Rose Bengal Chloramphenicol Agar (DRBC): 5 g/L Peptone bacteriological, 10g/L D(+)Glucose, 9 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 15 g/L agar, 25 mg/L rose Bengal (5% w/v in water, 0.5 ml), 2 mg/L dichloran (0.2% w/v in ethanol, 1 ml), 100 mg/L chloramphenicol, 1L water, distilled, The final pH was adjusted in range 5.5–5.8. The sterilization was carried out at 121ºC for 15min.

- **Czapek yeast extract agar (CYA):** 1 g K_2HPO_4, 10 ml czapek concentrate, 1 ml Trace metal solution, 5 g/L Yeast extract, powdered, 30 g/L Sucrose, 15 g/L agar, 1L Water, distilled. The final pH was adjusted to 6.7. The sterilization was carried out at 121ºC for 15min.
- **Czapek concentrate :** 30g/L $NaNO_3$, 5 g/L KCl, 5g/L $MgSO_4.7H_2O$, 0.1 g/L $FeSO_4.7H_2O$, 100 ml Water, distilled.

Malt extract agar (EA): 20 g/L Malt extract, powdered, 1g/L Peptone, 20 g/L Glucose, 20 g/L agar, 1L Water, distilled. The final pH was adjusted to 5.6. The sterilization was carried out at 121ºC for 15min.

25% Glycerol nitrate agar (G25N) : 0.75 g K_2HPO_4, 3.7 g/L Yeast extract, 7.5 ml czapek concentrate, 250 g/L Glycerol, analytical grade, 12 g/L agar, 750 ml Water, distilled. The final pH was adjusted to 7.0. The sterilization was carried out at 121ºC for 15min.

- **Trace metal solution**: 0.5 g/L $CuSO_4.5H_2O$, 1g/L $ZnSO_4.7H_2O$, 100 ml water, distilled

Yeast Extract Peptone Dextrose Agar (YPDA): 20g/L bacteriological peptone, 10 g/L yeast extract, 20 g/L Glucose, 15 g/L agar, 1L water.

3.1.3 Kits:

ELISA kits for the determination of aflatoxins and ochratoxin A were obtained from R-BioPharm, Germany. Its components are: 96 microtiter plate, standards (1.3 mL), wash buffer salt tween, conjugate (6 mL), antibody (6 mL), substrate/chromogen (10 mL), stop solution (14 mL) and Buffer solution at pH 7 were prepared in the lab. The kit provides six standards for mycotoxins, which are all listed in table below for standards concentrations:

Table *3*: Elisa kit standards concentrations:

No. of standards	AFs (µg/L)	OTA (µg/L)
1	0	0
2	1	50
3	5	100
4	10	300
5	20	900
6	50	1800

3.1.4 Equipment and machines:

All equipment's and machines used in current project:

- Autoclave machine.
- Centrifuge: Thermo SCIENTIFIC (Germany).
- Fridge: (4 C°), (-20 C°) & (-80 C °).
- Laminar: LABCONCO (USA)
- Microscopes: Light Compound microscope (Leica, China)

- Oven
- Shaking incubator: (SHEL LAB, USA)
- Sonication machine: (BANDELIN SONOREX, W. Germany)
- Incubator: BINDER (Germany).
- Distilled water system.

3.1.5 Solutions and Buffers:

Normal saline water 0.9%

0.9 g of NaCl (BDH, England) was dissolved in 100 mL sterile dH2O.

Chloramphenicol (100 mg/mL)

The chloramphenicol is prepared by suspending 1g of chloramphenicol powder (SIGMA-ALDRICH, UK) in 10 ml absolute ethanol. Before use, the solution was filtered using 0.2 µm syringe filter (Acrodisc, USA) and kept at -20ºC.

0.13 M Sodium Bicarbonate Buffer (pH 8.2):

8.401 g Sodium Bicarbonate (NaHCO3) is added in 1 L Distilled Water, pH (8.2).

Aflatoxin & Ochratoxin extraction solvents:

1 mL of formic acid was dissolved in 99 mL of Methanol-dichloromethane-ethylacetate (1:2:3), 0.13 M Sodium Bicarbonate Buffer, DCM (dichloromethane (SIGMA-ALDRICH)), Methanol (50%).

3.2 Methods:

3.2.1 Isolation and morphological identification of toxigenic fungi from Dairy Cattle Feeds:

Toxigenic fungi were isolated from dairy cattle feeds. Dairy Cattle feeds were aseptically collected from Qatari market and Baladna Company. After grinding and diluting in sterile distilled water, samples were placed on Dichloran-Rose bengal-Chloramphenicol agar (Faucet-Marquis *et al.*, 2016). After 3 days of fungal incubation,

developed colonies were transferred to specific media for identification like malt extract agar, czpeck yeast extract agar and glycerol nitrate agar.

According to morphological characters, all fungal isolates were identified morphologically depending on their macroscopic and microscopic features. Furthermore, macroscopic identification was based on what was observed, such as the colony color, size of the fungal spores, their texture and pattern. On the other hand, microscopic characteristics include elevation of the philiades, the size of the conidiophores and the last features protrusion of the hyphae, which can be noticed clearly by a microscope level. The isolates identified such as *Fusarium, Penicillium, Alternaria, Aspergillus flavus and Aspergillus parasiticus* were kept at -80 °C for future studies coming.

3.2.2 Exploration of mycotoxin synthesis potential of fungi (in vitro):

Mycotoxins were extracted by adding a volume of 10 ml of 80% methanol extraction solvent to the supernatant. It was important to shake the contents well by hand or vortex to make sure the extraction solvent and the samples are thoroughly mixed. Mycotoxin were extracted by shaking at 225 rpm for 10 minutes at 25 °C in an orbital shaker. Finally, the mycotoxin extract was filtered by 24 cm Vicam filter paper, and later the filtrate collected was put in a clean 15 ml falcon tube for mycotoxin concentration determination using ELISA RIDA Screen ELISA kits (R-Biopharm, Darmstad, Germany) (UlHassan *et al*. 2018).

Exploration of OTA:

To determine ochratoxins A producing potential of fungal isolates that were identified on morphological basis, such as *Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus niger, Aspergillus westerdijkiae, Aspergillus steynii, Penicillium nordicum* and *Penicillium verrucosum,* fungal isolates were plated on

YES media and were used for the estimation of ochratoxin A production potential using culture plug method described by Bragulat *et al*. (2001) with few modifications (Bragulat *et al*., 2001).

Starting by inoculated fungi on (YESA) at the middle of 90 mm petri plate, 20ml of the media were placed in each plate and incubated at 27°C for 7 days. After the incubation time, three agar plugs of size 5 or 7 mm were removed from the center of each colony to estimate exactly the production of toxins.

The plugs were weighed and added in falcon tubes containing 0.5 ml methanol and covered with aluminum foil, because of the sensitivity of toxins for light. After shaking for 60 mins in the shaker at 30°C, 100 µl of samples were taken in another vial, and dried for 15 mins making it ready to reconstitute again in 100 µl of sodium hydrogen carbonate (0.13 M) buffer and vortexing it well. Then, the samples were mixed for 3 min, using 50 µl in ELISA assay. All the samples were diluted for 10-1, 10-2, 10-3, 10-4, because the toxins levels were above the detection limit of ELISA.

Exploration for AFs:

To determine aflatoxin synthesis potential of fungi, *Aspergillus flavus* and *Aspergillus parasiticus* were used for testing their potential for the synthesis of aflatoxins using the following protocol: The fungal spores were inoculated on (YESA) media and incubated for 7 days at 27 ^{O}C. After 3 days, 3 plugs of 7 mm diameter were excised from each sample, from the center, rim and around the colony, as shown below in figure 3. Then, the plugs were transferred to covered falcon tubes that contained 1ml chloroform and 500 µl of methanol-water (35:65). After mixing it for 3 min, the samples were incubated in Sonicator for 60 mins, which was used to extract the mycotoxins using waves. From each sample, 0.5 ml was transferred to eppendrof

tube and dried using SpeedVac. Finally, the residues were re-suspended with 0.5 ml of 50% methanol. For ELISA assay, 50 µl of re-suspended extract were used.

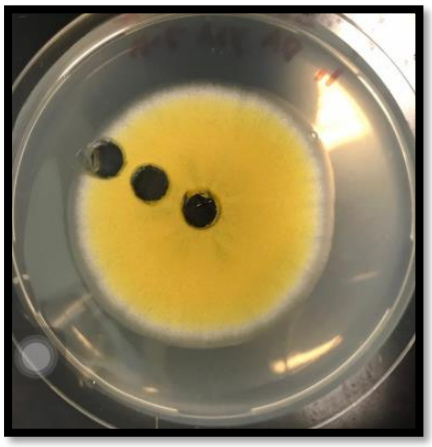

Figure 3. Plugs size of 5mm cut from *Aspergillus flavus* plates.

3.2.3 Determination of Mycotoxins concentrations:

3.2.3.1 Determination of (ochratoxins A) levels synthesis by isolated toxigenic fungi:

Using an ELISA kit for determination of ochratoxin A concentration, the following protocol was followed.

Sample preparation:

From the previous task, the ochratoxins A were extracted and ready to use. 50 µl per well were used in the assay. All the ELISA kit reagents were kept at room temperature (20-25 °C) before use. The ochratoxin (A) enzyme conjugate was given with the kit as concentrate and the conjugate diluted enzyme solution has a limited stability and it should be freshly prepared. Only the calculated required amount was reconstituted and shaken well before usage. The conjugate concentration was prepared by diluting 1:11(1+10) in conjugate dilution buffer for example (200 ul concentrate + 2ml buffer, ready to use and it was enough for 4 microtiter strips.

In addition, washing the PBS tween buffer was needed and the salt buffer was given

with the kit. The entire buffer salts were dissolved in one liter of D.H2O making it ready to use.

Assay procedure:

The assay protocol is summarized in the following steps:

1. 50 µL of the 6 standard solution (ready to use given with the kit) were transferred to separate duplicated wells.
2. 50 µL of extracted samples (Ochratoxin A) were added to separate wells as duplicate.
3. 50 µL diluted enzyme conjugate were transferred carefully to each well.
4. The plates were mixed well by shaking slowly, and then it was incubated for 40 min at RT covered with aluminum foil.
5. After 30 min of incubation, the liquid was discarded from the wells, and the microwells holder was tapped upside down forcefully against absorbent paper to make sure all the liquid is removed from the wells from the wells.
6. 250 µL of washing buffer were transferred to the wells and poured out again. This step was repeated two to three times.
7. 100 ul of the substrate/chromogen were added to each well. The plate was mixed slowly by manual shaking and incubated for 15 min at room temperature in the dark.
8. 100 µL of the stop solution were transferred to each well. The plate was mixed carefully three times and the absorbance was read within 30min following the addition of the stop solutions.
9. A specific software reader was used, the RIDA®SOFT Win (Art. No. Z9999), for the calculation of the OTA levels synthesized by the isolated fungi

3.2.3.2 Determination of aflatoxin levels synthesis by isolated toxigenic fungi:

Using an ELISA kit for the determination of aflatoxin concentration, the following

protocol was followed.

Sample preparation:

From the previous task, the aflatoxin was extracted and ready to use. 50 µl per well were used in the assay. All the ELISA kit reagents were kept at room temperature (20-25 °C) before usage. In addition, washing the PBS tween buffer was needed and the salt buffer was given with kit. The entire buffer salts were dissolved in one liter of D.H2O making it ready to use.

The assay protocol is summarized in the following steps:

Assay procedure:

1. 50 µL of the 6 standard solution (ready to use given with the kit) were added to separate duplicated wells.
2. 50 µL of the extracted samples (Aflatoxin) were added to separate wells as duplicate.
3. 50 µL of enzyme conjugate were added carefully to each well.
4. 50 µL of the antibody were added to each well, mixed well and then incubated for 30 min at room temperature in the dark.
5. After 30 min of incubation, the liquid was poured out of the wells, and the microwells holder was tapped upside down vigorously against absorbent paper for complete removal of liquid from the wells.
6. All wells were filled with 250 µl of washing buffer and the liquid was poured out again. The washing procedure was repeated two to three times.
7. 100 µL of the substrate/chromogen were transferred to each well. The plate was shaked slowly by manual shaking and incubated for 15 min at room temp in the dark.
8. 100 µL of the stop solution were added to each well. The plate was mixed carefully three times and the absorbance was read within 30 min after the

addition of the stop solutions.

9. A specific software reader was used, the RIDA®SOFT Win (Art. No. Z9999), for the calculation of the Aflatoxins levels synthesized by the isolated fungi.

3.2.4 DNA extraction:

PureLink® Plant Total DNA Purification Kit was used for the DNA purification of of fungal strains (Catalog number k1830-01).

Sample preparation protocol summarized in the following steps:

1. Fungi were transferred from -80 C° to a fresh PDA medium.
2. After incubation for 3 days of at 30 C°, the fungi spores were transferred from PDA solid media to PDB (approximately 20 ml in 50 ml falcon tube).
3. The samples were incubated at 30 C for 3 days in shaker at 140 rpm for 48 hrs.
4. Then, the fungi samples were filtered using filter papers.
5. The samples were then washed 3 times with saline water 0.9%.
6. They were then grinded into a powder under Liquid nitrogen.
7. Samples were preserved at -80 C°.

Kit extraction procedure summarized in the following steps:

1. 250 ul of the resuspension buffer (R2) were added to 100 mg of fungi.
2. Samples were vortexed for 3 min, then 15 ul of 20% SDS and 15 ul of RNase A (20 mg/ml) were added.
3. The lysate was incubated for 15 min at 55 C to complete lysis.
4. The lysate was centrifuged at high speed for 5 min to discard any insoluble materials.
5. The supernatant was transferred to sterile tubes, and 100 µl of N_2 were added to the clear lysate.
6. The samples were mixed well and incubated on ice for 5 min.
7. Then, samples were centrifuged at maximum speed in a micro centrifuge for

5 min at room temperature.

8. 250 µL of clear lysate was transferred to a new tube.
9. 375 µL of Binding buffer (B4) and ethanol were added to lysate.
10. The purification procedure was proceed in 8 steps:

- Samples from step 9 were placed in PureLink Spin Cartridge in a collection tube (with the kit).
- The cartridge was centrifuge at 10,000 x g for 2 min at room temperature. Then, the flow was discarded, and the column was placed into the Wash tube, which was given with the kit.
- 500 µL of the washing buffer (W4) were added to the spin column.
- The Cartridge was centrifuged at 10,000 x g for 2 min at RT again.
- The flow was discarded. The column was put in the tube and 500 ul of the washing buffer (W5) mixed with ethanol were added.
- Then, the column was centrifuged twice at 10,000 x g for 2 to 5 min to remove any residual Wash buffer (W5).
- Using sterile DNase-free, the spin column was put in a 1.5 ml centrifuge tube
- Using elution buffer (provide with kit), 100 ul were added and the samples was incubated for 10 to 15 min.

1. The second elution step was necessary to obtain more DNA.
2. The purified DNA should be preserved at -20 C°.

3.2.5 MinElute PCR Purification

The protocol is summarized in the following steps:

1. 5 volumes of PB buffer are added to 1 volume of the PCR mix. They were then mixed well by vortex.

2. The samples were transferred in 2 ml collection tubes in MinElute column.
3. Samples were centrifuged for 1 min and then the flow was discarded. The MinElute column was put back into the same collection tube.
4. 750 µL of buffer PE were transferred to the MinElute column and centrifuged for 3 min and then the flow was discarded. The MinElute column was transferred back into the same collection tube.
5. The MinElute spin was placed on a new clean 1.5 ml micro centrifuge tube, and then a 10 µL of the elution buffer (EB) were added in the center of the membrane.
6. The purified DNA was analyzed on a gel by transferring 1 volume of loading Dye to 5 volumes of the purified DNA.

3.2.6 Molecular identification of toxigenic fungi:

1. PCR amplification using Different specific primers

Isolated and morphologically identified toxigenic fungi species, including *Fusarium, Penicillium, Alternaria, Aspergillus flavus and Aspergillus parasiticus* were identified at molecular level. The fungi underwent DNA amplification by Polymerase Chain Reaction (PCR) technique through a 25 µl reaction mixture. The reagents were used for preparation of master mix for PCR reaction shown in Table 1.

Table 4: Reaction mix for PCR reaction.

no.	Reagent	Volume
1	PCR Master mix	12.5 µl
2	PCR water	9.5 µl
4	Forward primers	1 p moles/µl
5	Reverse primers	1 p moles/µl
6	Template DNA	1 ng/µl

The amplification process can be summarized into three steps with a total of 40 cycles. In each cycle, the DNA is desaturated at 94 C° for 5 minutes. In the next step, denaturation was done at 94 C° for 20 seconds. This was followed by annealing at 55 C° for 20 seconds and extended for 60 seconds. Then, the final extension was at 72 C for 30 min.

The PCR reaction was carried out using the GeneAmp® PCR system 9700 series thermal cycler from Applied Biosystems. Additionally, the DNA samples from the fungal isolates were subjected to amplification using ITS1 (foreword primers) and ITS4 (reverse primers) as universal primers. The primer ITS1/NIG was specific for *A. niger* DNA. The primer Carb1/2 was specific for *A. carbonarius.* The primer FLA1/2 was specific for *A. flavus* DNA. The primer PAR1/PAR2 was specific for *A. parasiticus* DNA.

Table 5: PCR oligonucleotides

Name	Sequence	Amplicon size (bp)	Target	Reference
ITS1	TCC GTA GGT GAA CCT GCG G	550	ITS region	(Henry *et al.*, 2006)
ITS4	TCC TCC GCT TAT TGA TAT GC			
PAR1	GTCATGGCCGCCGGGGGCGTC	430	*A. parasiticus* (specie specific)	(Sardiñas *et al.*, 2010)
PAR2	CCTGGAAAAAATGGTTGTTTTGCG			
FLA1	GTAGGGTTCCTAGCGAGCC	500	*A. flavus*	(González-

FLA2	GGAAAAAGATTGATTTGCGTC		(specie specific)	Salgado *et al.*, 2005)
OMT-208	GGCCCGGTTCCTTGGCTCCT AAGC	1024	*omt-1* gene (aflatoxin synthesis)	Shapira *et al.*, 1996 (*Mycotechnology: Present Status and Future Prospects - Mahendra Rai - Google Books*, no date)
OMT-1232	CGCCCCAGTGA GACCCTTCCTCG			
VER-496	ATGTCGGATAATCACCGTTTAGAT GGC	895	*ver-1* gene (aflatoxin synthesis)	Shapira *et al.*, 1996
VER-1391	CGAAAAGCGCCA CCATCCACCCCAATG			
APA-450	TATCTCCCCCCGGGCATCTCCCGG	1032	*apa-2* gene (aflatoxin synthesis)	Shapira *et al.*, 1996
APA-1482	CCGTCAGACAGCCACTGGACACG G			
CAR1	GCATCTCTGCCCCTCGG	420	*A. carbonarius* (specie specific)	(Patiño *et al.*, 2005)
CAR2	GGTTGGAGTTGTCGGCAG			

ITS1	TCCGTAGGTGAACCTGCGG	420	*A. niger* (specie specific) *pks gene* (ochratoxin synthesis in *Aspergillus)*	(González-Salgado *et al*., 2005)
NIG	CCGGAGAGAGGGGACGGC			
AoOTA-L	CATCCTGCCGCAACGCTCTATCTTTC	490		(Huy, Mathieu and Lebrihi, 2005)
AoOTA-R	CAATCACCCGAGGTCCAAGAGCCTCG			

2. Gel electrophoresis:

The PCR products were migrated in a 1 % agarose gels in TBE buffer and stained by utilizing Ethidium bromide. The bands were seen using GEL doc 2000 trans illuminator.

3.2.7 Isolation of yeast strains from fermented food, dairy products and meat products:

Biological control of toxigenic fungi was mainly achieved using non-pathogenic bacteria and yeast spps. (Zeidan *et al*., 2018, Farbo *et al*., 2018). In this research, fermented food, dairy products and meat products were purchased from the local markets and were used for the isolation of yeast strains. Direct or dilution plate inoculation methods were used for the isolation of yeast spp. on yeast extract peptone agar (YPDA). The isolates were purified by sub-culturing on the other YPDA plates.

Figure 4. Yeast strain locally isolates from fermented food, dairy products and meat and streaked on YPDA plate.

3.2.8 Screening of yeasts for antifungal activity and their molecular identification:

For the screening of antifungal activities of yeast species, co-culture method described by Farbo *et al*., 2018 was followed. For this purpose, yeast cells were spread on YPDA plates. After 24 hours incubation, the plate was sealed with PDA plates having inoculated fungal spores. Sealed plates were incubated for 3 to 7 days to note the activity of yeast volatiles against the fungal vegetative growth and sporulation. For the control, fungal plates were incubated after sealing with PDA plates alone. Selected toxigenic fungal spps. from genus *Aspergillus*, *Penicillium* and *Fusarium* were used in the first trail in screening studies. Then, 19 fungal reference strains were also used in screening studies.

The yeast spps. showing significant inhibitory effect on the fungal growth and sporulation, was subjected to molecular identification. DNA was extracted using commercial yeast DNA extraction kits and ITS regions were amplified using ITS1-ITS4 primers. The amplified segments were sequenced and blasts with NCBI database.

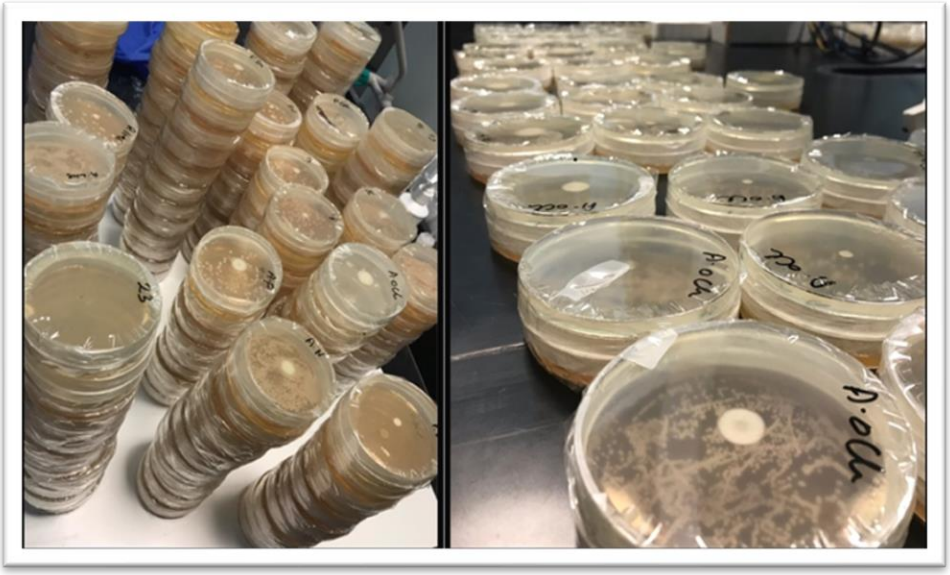

Figure 5. Estimation of the effect of yeasts VOCs on the mycotoxins Synthesis screening of yeasts for antifungal activity against 17 mycotoxigenic fungi reference strains.

3.2.9 Application of yeasts volatiles against toxigenic fungi:

Effect of yeasts VOCs on the fungal vegetative growth:

The antifungal activities of the yeast VOCs against fungal vegetative growth and sporulation of *F. graminearum*, *P. verrucosum and A. parasiticus*, and *A. carbonarius* were experimented with yeast and fungal co-culture experiment. The experimental design avoided contact between yeast and fungal vegetative colony. This was done using yeast extract peptone dextrose agar plates (Yeast extract,10 g; peptone, 20 g; dextrose, 20 g and agar:,15 g for 1 L of medium) and inoculated into 100 µl yeast cell suspensions (10^{-3} cells/mL), which was incubated for 48 h at 25 °C. The cover of the plate was changed with another petri plate for point inoculation into 10 µl of *P. verrucosum* (10^{-3} spores/mL), *F. graminearum* (10^{-3} spores/mL), *A. parasiticus* (10^{-3} spores/mL) and *A. carbonarius* (10^{-3} spores/mL). All plates were covered tightly with parafilm and a second layer of adhesive tape to prevent any

leakage of VOCs. More YPDA plates were prepared as negative controls of fungi without yeast cells. During 3 days of incubation, the sample size, sporulation and morphology was noticed (Zeidan, Ul-Hassan et al., 2018).

Effect of yeasts VOCs on the Mycotoxins Synthesis :

Detection of Ochratoxin concentrations in A. carbonarius and P. verrucosum:

The isolates yeast strain was streaked on YPDA media plates. After 48 h incubation, A yeast pre-culture was prepared by transferring single loop to 30 ml YPDB in a 50 mL tube, and the tube was incubated at 27.5C°/100 rpm for 24 hours. A suspension of the isolates yeast cells having a concentration 10^{-3} cells/mL was prepared from the 24 h yeast broth culture, and from pre-culture, 10 µL was transferred to 990 µL of 0.9% saline water. From the yeast suspension, 100 µL of the yeast cells suspension were transferred to the YPDA media plates and were spread on media by using sterile glass spreaders, The YPDA plates were incubated at 27.5C° for 48 h. On other hands, the fungus spores of *P. verrucosum* and *A. carbonarius* were inoculum on PDA plates and incubated for 5 days at 27.5C°. The spore's suspension was prepared by transferred loop of spores to 0.9% saline water into sterile Eppendrof tubes 1mL. From the spore's suspension; 3µL was transferred to the midle of plates containing PDA and those plates were sealed to the 48 h yeast YPDA plates by using 2 layers of Parafilm and normal tape. All sealed plates were incubated at 27.5C° for 10 days.

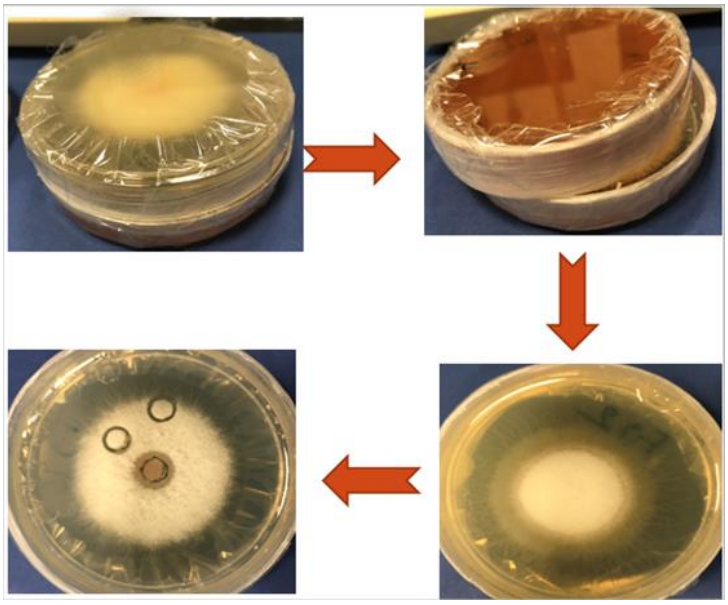

Figure 6. Experimental design used in plates sealing Steps after sealing plates and opened after 10 days incubation to remove 5-6 mm plugs from fungi colony to estimate effect of yeasts VOCs on the mycotoxins Synthesis.

3.2.10 Exploration of the *In-Vitro* effect of yeast's VOCs on *F. oxysporum* contaminating tomato fruit:

In-vitro, the activity of yeast's VOCs against *F. oxysporum* growth was tested on the surface of tomatoes. The tomatoes were washed with tap water, dH_2O, 70% alcohol and dH_2O again. All the tomatoes were inoculated on the surface with 5 ul of spores, suspension in tween 80. Ten out of twenty tomato fruits had almost the same size (6-8 g) and were inoculated with *F. oxysporum* spores. Five tomatoes were placed in an autoclaved glass box (Tupperware) having a plate of YPDA with (48 h) streaked yeast strain 4 (experimental box), while the other five tomatoes were inoculated with *F. oxysporum* spores. They were also placed in another autoclaved glass box having a YPDA plate only (negative control box) .

The other ten tomatoes were divided as more controls: five tomatoes were washed with saline water and placed in an autoclaved glass box with YPDB plate streaked with yeast strain 4, while the remaining five tomatoes were placed in an autoclaved glass box with

empty petri dish. All the petri dishes' were of small in size (60 mm x 15 mm) in order to fit inside the glass boxes. Around each petri dish, small pieces of polystyrene were placed to partially elevate the lid from the other petri dish. The big petri dish was placed to serve as a base for the tomatoes to be placed on. All boxes were covered with their lid tightly. The experiment was performed in aseptic conditions and the boxes were then incubated at 26ºC for 25 days.

Figure 7. Tomato fruits used in biocontrol experiment (A) Organic tomato fruits selected for this experiment, size of tomatoes ranged between (6-8 g). (B) Tomato fruits washed in dH2O & 70% Alcohol.

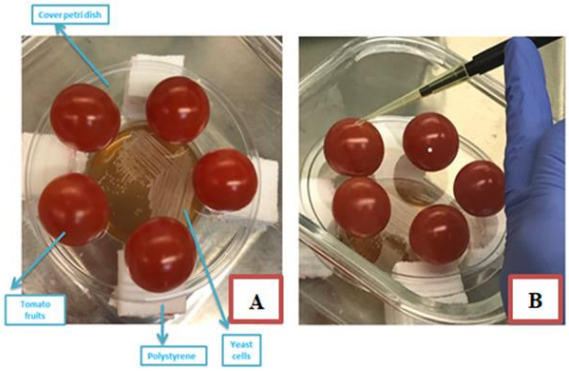

Figure 8. Tomato fruits used in biocontrol experiment (A) Tomato fruits and yeast preserved in the glass sealed box, (B) 5 µL of Fungi spores inoculated on the tomato surfaces.

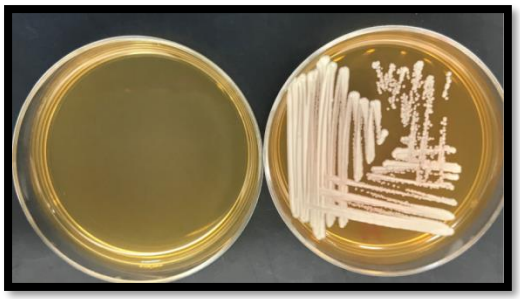

Figure 9. Kluyveromyces marxianus QKM#4 cells streaked on YPDB plates. The petri dishes were small in size 15mm)

3.2.11 Exploration of the *In-Vitro* effect of yeast's VOCs on *A. carbonarius* contaminating grape:

In-vitro, the yeast's VOCs effect on *A. carbonarius* growth was tested on the surface of grapes. The grapes were washed with tap water, dH_2O, 70% alcohol and dH_2O again. All the grapes were inoculated on the surface with 5 ul of spores, suspension in tween 80. Ten out of twenty grapes had almost the same size (2-3 g) and were inoculated with *A. carbonarius* spores. Five grapes were placed in an autoclaved glass box (Tupperware) having a plate of YPDA with (48 h) streaked yeast strain 4 (experimental box), while the other five grapes were inoculated with *A. carbonarius* spores. They were also placed in another autoclaved glass box having a YPDA plate only (Negative control box).

The other ten grapes were divided as more controls: five grapes were washed with saline water and placed in an autoclaved glass box with YPDA plate streaked with yeast strain 4, while the remaining five grape were placed in an autoclaved glass box with empty petri dish. All the petri dishes were small in size (60mm x 15 mm) in order to fit inside the glass boxes. Around each petri dish, small pieces of polystyrene were added to partially elevate the lid from other petri dish. The big petri dish was used to serve as a base for the grapes to be placed on. All boxes were covered with their lid tightly. The experiment was performed in aseptic conditions and the boxes were then

incubated at 26ºC for 25 days.

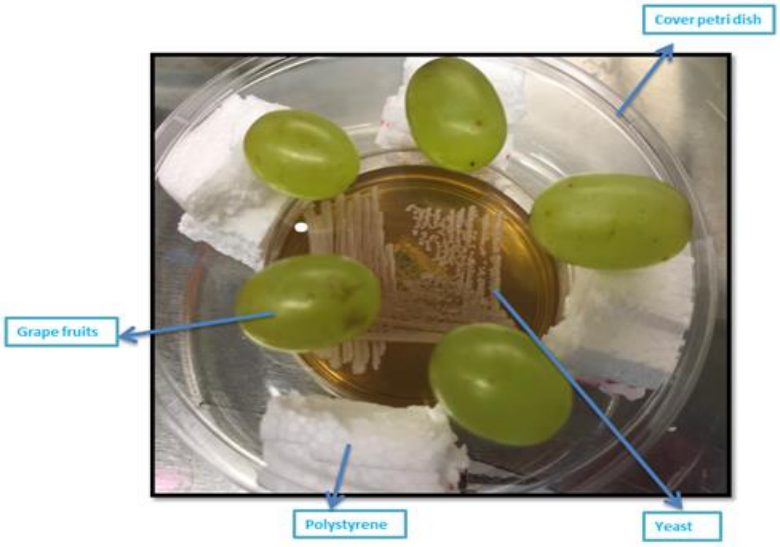

Figure 10. Grape fruits used in biocontrol experiment with yeast Kluyveromyces marxianus QKM#4 Grape fruits and yeast preserved in the glass sealed box.

3.2.12. Investigation volatile organic compounds (VOCs) molecules synthesized by yeast strains:

Many of the VOCs molecules synthesized by yeast strains were locally isolated from fermented food, dairy product and meat. They were then investigated and identified by (GC-MS). The VOCs synthesized by yeast were absorbed on activated charcoal and eluted with dichloromethane, and the elution was subjected to GC analysis. Fresh yeast cells (48 h) in 200 ml (in 500 ml conical flasks) of YPDB were shake-incubated. The conical flask was fitted with two-rubber corks which passed through it with two glass tubes.

One glass tube acted as a supply channel for nitrogen, while the other end was attached to flask and positioned only 1 cm above the yeast culture to eliminate volatile head area. The second glass tube was fitted with flask tube and the other end attached to a VOCs trap. The trap was a glass tube of 6 cm long and 5 mm diameter and filled

with 400 mg of activated charcoal. Before this step, the charcoal was activated by wrapping it in aluminum paper and placing it in the oven at 300°C for 48 h for sterilization and reactivation for 48 h. All the junctions pass through the tightly sealed flask by parafilm and tape to avoid any contamination and to prevent leakage of the VOCs. All the control flasks contained YPDB only and the experiment flask contained yeast culture, placed in a shaking water bath at 30 °C for 48 h. The charcoal absorbed all VOCs that was pumped by nitrogen gas for 50 mins, and the activated charcoals eluted by adding 1 ml of dichloromethane into glass vials, and was kept for 30 mins for more elution. The liquid was collected in vials for the purpose of volatiles analysis using gas chromatography (GC/MS) amended with an MSD detector. The samples were separated on fused silica column (0.32 mm i.d., 30 m length, 0.5 µm film thickness). The column's temperature was set at 50°C for 5 min and it increases to 250°C by 5 °C each minute. In split-less mode the injection port was worked. The MS (EI mode) parameters used were as follows: ionization voltage, 70 eV; mass scale, 30-550 *m / z;* source ion temperature, 180 ° C.

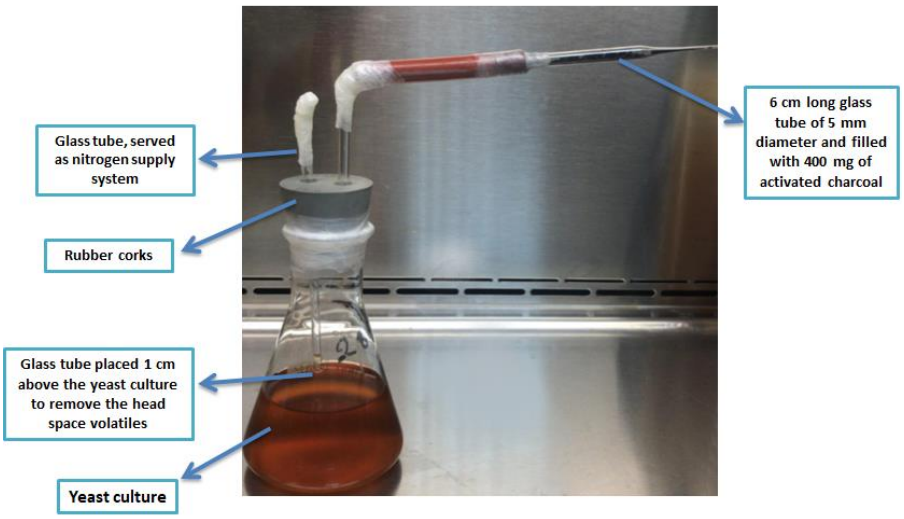

Figure 11. Experimental design used in the identification of Kluyveromyces marxianus QKM#4 VOCs molecules. The conical flask was filled with yeast culture

and was equipped with two-way rubber corks that went into it through two glass tubes. One glass tube acted as a supply system for oxygen, and another tube loaded with 400 mg of activated gas.

3.2.13 Mycotoxins Binding onto Yeast Cell Wall (YCW):

Preparation of inactive yeast cell wall:

The yeast cell culture was incubated overnight at 26 °C in a shaking incubator at YPDB to prepare yeast cell walls (inactivated yeast). The yeast colony was autoclaved after 48 h and centrifuged quite quickly at 5000x g. The pellet was suspended and washed twice in saline water after the supernatant was drained, then centrifuged again. The pellets were dried overnight in an oven at 60 °C before grinding them into powder (Bzducha-Wróbel *et al.*, 2014).

Preparation of living yeast:

The Yeast culture was prepared from colonies that were streaked on solid YPDA, this was done by transferring a single loop of 100 mL YPDB in 500 mL flasks. The flask was incubated at 26C° /100 rpm for 48 h. A 20 µL of yeast culture was used for the adsorption assay experiment.

Preparation for mycotoxin doses:

5 mg of YCW powder and 20 µl of living yeast cells suspension were mixed with 0.99 ml (YCW) for a total of 5 minutes. An additional 970 µl (living cell) of each buffer were added. To each tube, 10 µl of mycotoxin solution were added, to have a concentration of 1.8 and 0.9 µg/kg of OTA, 0.4 and 0.2 µg/kg of AF, and 80 and 40 µg/kg of DON.

Table *6*: Pure Mycotoxin concentrations used in Mycotoxins adsorption assay

Mycotoxin	High dose (µg/L)	Low dose (µg/L)
AFs	**0.2**	**0.4**
DON	**40**	**80**
OTA	**0.9**	**1.8**

3.2.14 Adsorption to living yeast cell wall:

Yeast cells prepared in the previous section were used to test the potential of different toxins such as AFs, DON and OTA to adsorb to the yeast's cell wall. The adsorption was conducted in two buffers solutions with pH 5 (acetate). From each buffer solution, 970 µL were transferred to 1 mL tubes, and then completed with 20 µL of the yeast cells stock solution. The tube was vortexed very well, then the tube was inoculated with 10 µL of higher and lower mycotoxin doses in different tubes. The tubes were shaken and incubated end-to-end at 37 C/100 rpm for 60 min. Then, they were directly centrifuged at maximum speed for 5 min. The supernatant was separated from the pellet and transferred to other tubes. Both tubes (pellet and supernatant) were kept in Speed Vac. for 30 min. The pellet and supernatant were re-suspended in 10 % methanol for AFs, dH_2O for DON and 0.13 M sodium hydrogen carbonate solution for the OTA to detected all mycotoxins.

3.2.15 Adsorption to inactive yeast cell wall:

The adsorption procedure of inactivating yeast cells to mycotoxins was done in a similar manner to the living yeast cell walls, with the difference being the addition of 5 mg of ground yeast cells powder to the 990 µL buffer solutions (5 pH). The tube was

vortexed well, and then the it was inoculated with 10 µL of higher and lower mycotoxin doses in different tubes. Next, the tube was shaken and incubated end-to-end at 37 C/100 rpm for 60 min. It was then centrifuged at maximum speed for 5 min. The supernatant was separated from the pellet and transferred to other tubes. Both tubes (pellet and supernatant) were kept in Speed Vac. For 30 min. The pellet and supernatant were re-suspended in 10 % methanol for AFs, dH_2O for DON and 0.13 M sodium hydrogen carbonate solution for the OTA to detect all mycotoxins.

Determination of Yeast's adsorption potential using ELSA kits:

The kits were used to determine the adsorption potential of mycotoxins to cells in the pellet and the supernatant of the viable and non-viable yeast cell walls incubated in buffer with pH 5. For the ELISA kit, 50 µL were transferred to the wells from pellet and the supernatant tubes of all three mycotoxins. The percentage of removed mycotoxin from the buffer was determined for the three mycotoxins.

However, the removal percentage of mycotoxins also determined by detected concentration to the original mycotoxin concentration added to the buffer as explained in the equation below.

Percentage of mycotoxin removal

= Original concentration - detected concentration ÷ original concentration *100%

CHAPTER 4: MONITORING THE PRESENCE OF FUNGI AND MYCOTOXINS IN DAIRY CATTLE FEEDS

4.1 Introduction:

Many mycotoxins contaminate agronomic crops worldwide, including aflatoxins, fumonisins, trichothecenes and ochratoxins. These toxins cause both health concerns and economic losses. The *Aspergillus, Fusariu, and Penicillium* species are responsible of most of the agriculture contamination by mycotoxins. These filamentous fungi produce all types of polyketide-derived mycotoxins. The mycotoxigenic fungus induces several forms of crop diseases, such as maize ear rots induced by *Aspergillus* and *Fusarium* species, according to the host crop and fungal strains. These plant diseases caused in crop loss and economic impacts.

Antagonistic microorganisms have the potential to act as biological control agents in many crop systems and therefore to be used as alternatives to chemical antifungal agents for controlling mycotoxins contamination. Many recent studies have reported several bacterial, yeast and fungal isolates that can be applied for the reduction of crop contamination by mycotoxigenic fungi.

In the first part of this chapter, the presence of mycotoxins was monitored in dairy animal feed samples and showed low levels of mycotoxins. However, several strains of toxigenic fungi were isolated from these same dairy animal feed samples. These fungal strains were identified based on their morphology and by molecular ribotyping.. On the other hand, several mycotoxin coding genes were evidenced and mycotoxin synthesis was estimated.

4.2. Monitoring the presence of mycotoxins.in the cow feed samples:

Management of animal nutrition's is crucial for the dairy products' quality. Feed treatment can be applied to both nutritional content and consistency, since other compounds such as cereals; cotton seeds, corn and cabbage are commonly used in cow diets in lactation. Actually, these ingredients very easy and susceptible to contamination by toxigenic fungi and corresponding mycotoxins such as aflatoxins and ochratoxins . Seven mixed dairy cow feed samples were tested for presence of aflatoxins and ochratoxins. None of the samples showed the presence of aflatoxins and ochratoxins in all animal feed samples at the detectible limits (Table 7).

Table *7*: Estimation of the concentrations of aflatoxins and ochratoxins in mixed dairy cow feed samples for dairy cows collected from supermarket in the Qatar

Ochratoxin S.ID	Animal feed Concentration (µg/kg)	Sample type	Company	Origin	Aflatoxin	Animal feed Concentration (µg/kg)	Sample type	Company	Origin
A	< 50.00	Cow feed	Aalaf	QATAR	A	< 50.00	Cow feed	Aalaf	QATAR
B	< 50.00	Cow feed	Aalaf	QATAR	B	< 50.00	Cow feed	Aalaf	QATAR
C	< 50.00	Cow feed	Aalaf	QATAR	C	< 50.00	Cow feed	Aalaf	QATAR
D	< 50.00	Cow feed	Aalaf	QATAR	D	< 50.00	Cow feed	Aalaf	QATAR
E	< 50.00	Cow feed	Aalaf	QATAR	E	< 50.00	Cow feed	Aalaf	QATAR
F	< 50.00	Cow feed	Aalaf	QATAR	F	< 50.00	Cow feed	Aalaf	QATAR
G	< 50.00	Cow feed	Aalaf	QATAR	G	< 50.00	Cow feed	Aalaf	QATAR

4.3. Monitoring the presence of fungi in Dairy Cattle Feeds, and morphological identification of toxigenic fungi from Dairy Cattle Feeds:

These results presented in the previous paragraph are encouraging in term of safety of the animal feed and consequently the dairy products. However, the exploration

of toxigenic fungi in the same product was necessary to predict possible future feed contamination by corresponding mycotoxins.

4.3.1 Evidence and Morphological identification of aflatoxigenic and ochratoxigenic fungi:

Animal feed samples were explored for the presence of mycotoxigenic fungi. The results showed that a large percentage of the animal feed samples were contaminated by mycotoxigenic fungi (Table 8). The morphology of fungal colonies and molecular methods has been used to classify mycotoxigenic fungal isolates. All the growing fungal colonies were transferred from selective media plates (DRBC) to three identification Medias: malt extract agar (MEA), Cazpek yeast extract Agar (CYA) and glycerol-nitrate agar (G25N). Furthermore, the morphology of fungal colonies, including size, cells shape, color, spores' size and their microscopic characteristic were noted.

Table 8: Different fungal strains isolated from the mixed dairy cow feed

Isolate ID	**Color**						**Morphological**
	CYA		**MEA**		**G25N**		**Diagnosis**
	Observe	**Reverse**	**Observe**	**Reverse**	**Observe**	**Reverse**	
SC2	Bluish green	Pale Yellow	Bluish green	Orange yellow	Bluish green	Pale Yellow	*P. verrucosum*
SB1	Olive green to white	Cream	Green with exudate	Cream	Yellow	Yellow	*A. flavus*

SB3	Brownish black	Creamy white to green	More dense brownish black	Cream	Brownish spores	Yellow	*A. niger*
SA4	Olive green	Cream	Green	Cream	Yellow	orange yellow	*A. flavus*
SB5	Olive green to white	Cream	Green	Cream	orange yellow	Yellow	*A. flavus*
SA6	Jet black	Greenish to creamy white	dark green	creamy white	Less sporulation	Greenish to creamy	*A. carbonarius*
SC7	Golden brown to white	Golden brown to white	Bluish green	Orange yellow	Whitish yellow	orange yellow	*P. verrucosum*
SC8	Olive green to white	Cream	Green with exudate	Cream	orange yellow	orange yellow	*A. flavus*
SB9	Olive green to white with black exudate	Cream	Green with exudate	Cream	Orange	Orange	*A. flavus*
SG10	Golden brown to white color	Yellow	Bluish green	Orange yellow	pale green to orange	Golden yellow	*P. verrucosum*
SD11	Jet black	White	Jet black	creamy white to greenish	pale brown to black	creamy white to green-ish	*A. carbonarius*
SC12	Whitish green	Cream	Green	Cream	orange yellow	orange yellow	*A. flavus*

SE13	Bluish green	Pale yellow	Bluish green	Pale yellow	Less sporulation brownish white	Brownish white	*P. verrucosum*

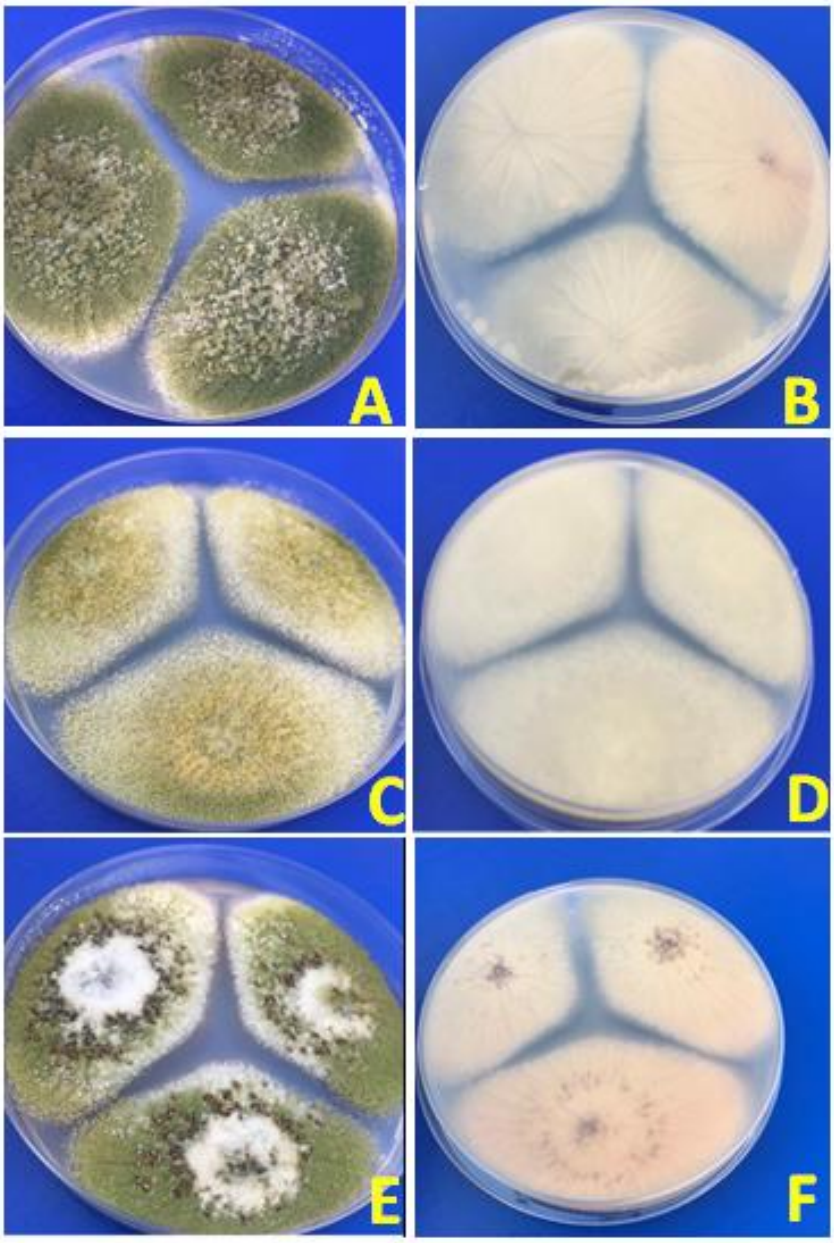

Figure 12. Identification of fungal colonies of *Aspergillus flavus* in the identification Medium, Malt Extract Agar, Glycerol nitrate agar and Czapek's agar.

[**A;** point inoculation of *A. flavus* on MEA media observe. **B;** point inoculation of *A.*

flavus on MEA media reverse. **C;** point inoculation of *A. flavus* on CYA media observe. **D;** point inoculation of *A. flavus* on CYA media reverse. **E**; point inoculation of *A. flavus* on G25N media observe. **F;** point inoculation of *A. flavus* on G25N media reverse].

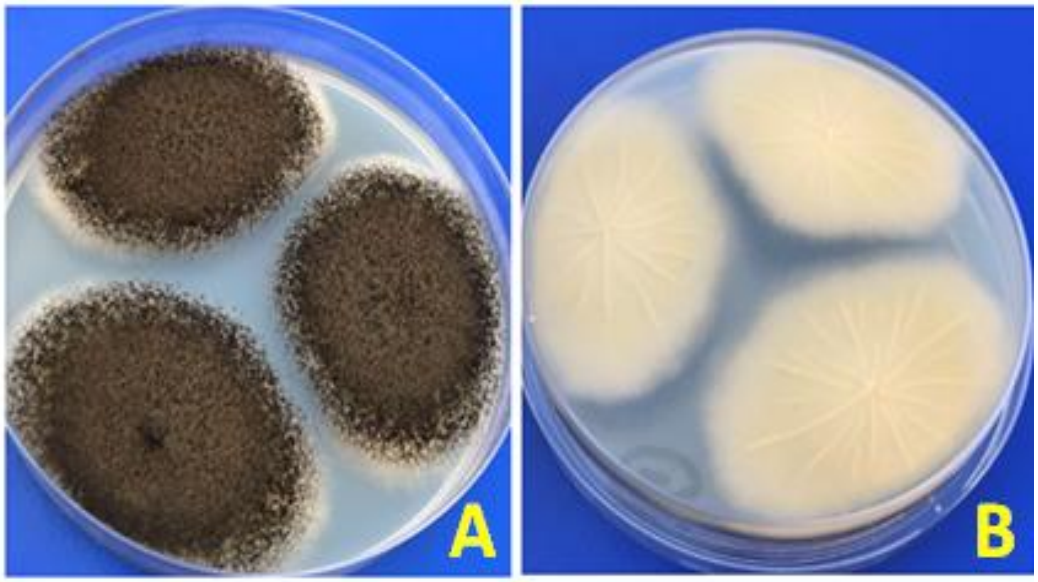

Figure 13. Identification of fungal colonies of *Aspergillus carbonarius* in the identification Medium including Glycerol nitrate agar
[**A;** point inoculation of *A. carbonarius* on G25N media observe. **B;** point inoculation of *A. carbonarius* on G25N media reverse].

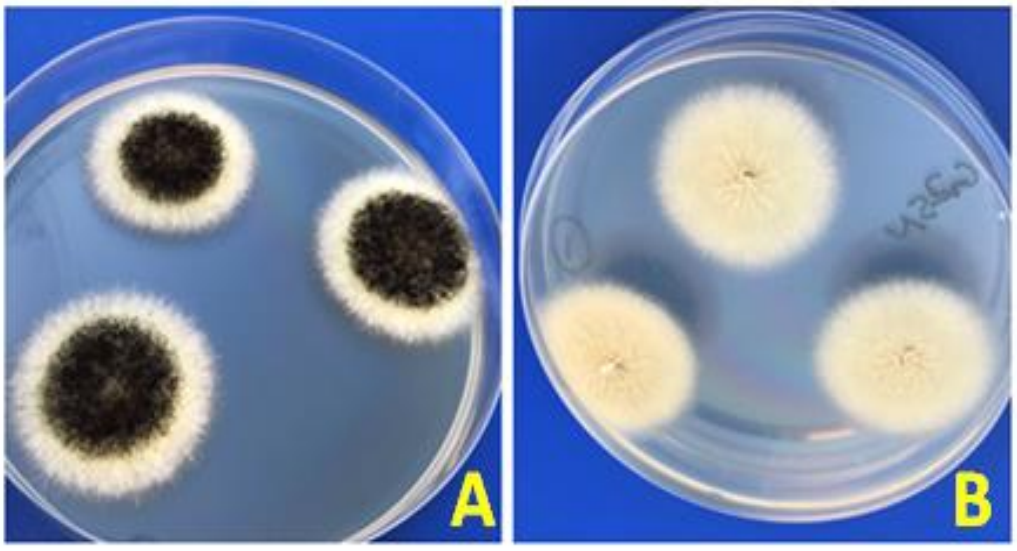

Figure 14. Identification of fungal colonies of *Aspergillus niger* in the identification Medium including Glycerol nitrate agar
[**A;** point inoculation of *A. niger* on G25 media observe. **B;** point inoculation of *A. niger* on G25N media reverse].

Based on their morphological features and their macroscopic characteristic (Figures 12, 13, 14) fungi were identified using the fungal identification key described in the literature. Two fungi, *A. flavus* and *A. parasiticus,* showed similar strains related to their morphological characteristics such as greenish yellow, olive green conidia on CYA. Another two species, *A. niger* and *A. carbonarius*, both produced black sporulation, therefore, they were differentiated based on the color of conidia. *A. niger* produced conidia that was brownish black in color, while *A. carbonarius* produced conidia that was jet black. Moreover, many other fungal species were isolated and identified based on morphology, such as genera *Penicillium* and *Aspergillus* (Thathana *et al.*, 2017).

4.3.2. Estimation of mycotoxins production by the toxigenic fungi:

The results show that, all fungal strains isolated from animal feed samples produce and synthesize mycotoxin compounds (Aflatoxins & Ochratoxins) more than detection limits. All the results were evaluated in context of European Union (EU) maximum limits and summarized on Table 9.

Table 9: Aflatoxin & OTA content synthesized by fungal strains isolated from cow feed samples

Source of samples	Sample type	Fungi strain	S. code	Concentration of AFs (µg/kg)
Qatar	Animal feed	*A. flavus*	SC	39 ± 1
Qatar	Animal feed	*A. flavus*	SA	2.7 ± 0.2
Qatar	Animal feed	*A. flavus*	SB	597 ± 4
Qatar	Animal feed	*A. flavus*	SE	330 ± 1
Qatar	Animal feed	*A. flavus*	SD	2270.2 ± 0.2

Sample source	Fungi type	Fungi strain	S. code	Concentration of OTA (µg/kg)
Qatar	Animal feed	*A. niger*	SD	28.91 ± 0.06
Qatar	Animal feed	*A. carbonarius*	1SD	1102 ± 2
Qatar	Animal feed	*A. carbonarius*	1SB	25.3 ± 0.4
Qatar	Animal feed	*A. niger*	SA	15 ± 2
Qatar	Animal feed	*A. niger*	SC	12.37 ± 0.09
Qatar	Animal feed	*P. verrucosum*	2SB	20 ± 1
Qatar	Animal feed	*A. niger*	3SB	14.4 ± 0.3
Qatar	Animal feed	*A. carbonarius*	1SC	7.7 ± 0.6

[1]Values in parenthesis indicate positive percentages; [2]EU maximum limits for AFs in animal feeds 20 ul/kg, and for OTA 250 ug/kg.

4.3.3 Molecular identification of toxigenic fungi:

Molecular identification of the fungal strains was done in order to identify the isolated fungal colonies. The species-specific PCR primers mentioned in Tables 5 were used to amplify specific fragments, and all the results of the amplification were compared with reference strain. Before applying species-specific PCR primers, DNA

from each isolated was tested for its suitability for PCR reactions by using universal fungal primers (ITS1/ITS4) (White *et al.*, 2006). All samples showed single and specific bands of 600 bp using ITS/ITS4 primers (Figure15).

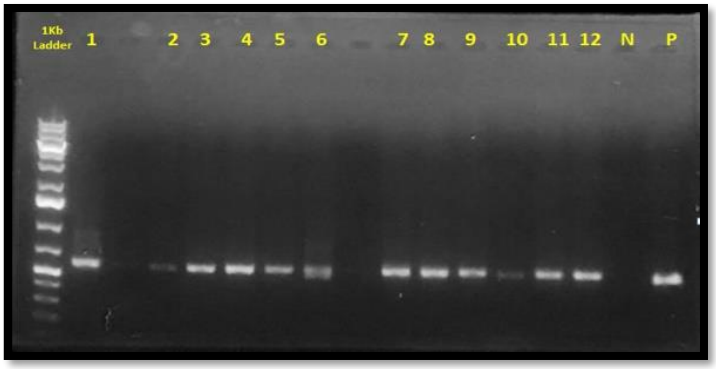

Figure 15: PCR amplification using universal primers for fungal isolates identification from cow feed samples. Lanes; 1- 12, primer ITS1/ITS4 the template DNA from *A. flavus, A. niger and A.carbonarius* (amplicon size 600 bp). Lanes; 13, represented the non-template (N) negative control of their previous lanes. Lanes 14; represented positive (P) control template from reference strain. 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp.

In addition, two of the isolates coded as (SCx & SC2x) showed a single amplification band against ITS1/NIG primer (product size 420 bp), which confirmed them as *A. niger* (Table 10, Figure16). Also, four of the *Aspergillus* confirmed by specific-species primers CAR1/CAR2 as *A. carbonarius* strains (Figure 16).

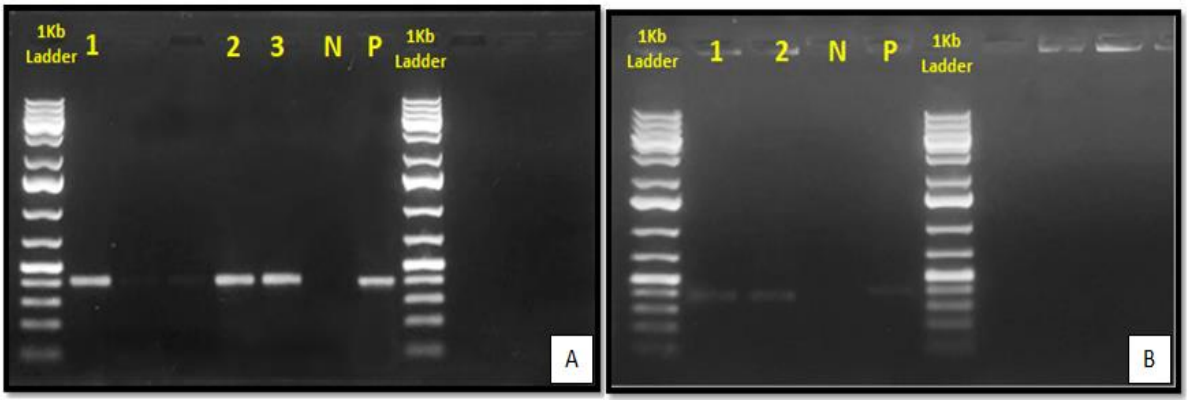

Figure 16: PCR amplification using species-specific primers for Black fungal isolates of cow feed samples.

[**(A)** Lanes; 1- 3, primer CAR1/CAR2 the template DNA from *A.carbonarius*,(amplicon size 420 bp). Lanes; N, represented the non-template negative control of their previous lanes. Lanes P; represent positive control template from *A.carbonarius* reference strain. **(B)** Lanes; 1- 2, primer ITS1/NIG the template DNA from *A. niger* (amplicon size 420 bp). Lanes; N, represented the non-template negative control of their previous lanes. Lanes P; represent positive control template from *A. niger* reference strain. 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp**].**

However, Six isolates coded by (SA, SC, SB1, SBY, SB2 and SD) produced single amplification band of 500 bp, using FLA1/FLA2 primers pair, which confirmed them as *A. flavus.* None of isolates showed *A. parasiticus* species (Figure17).

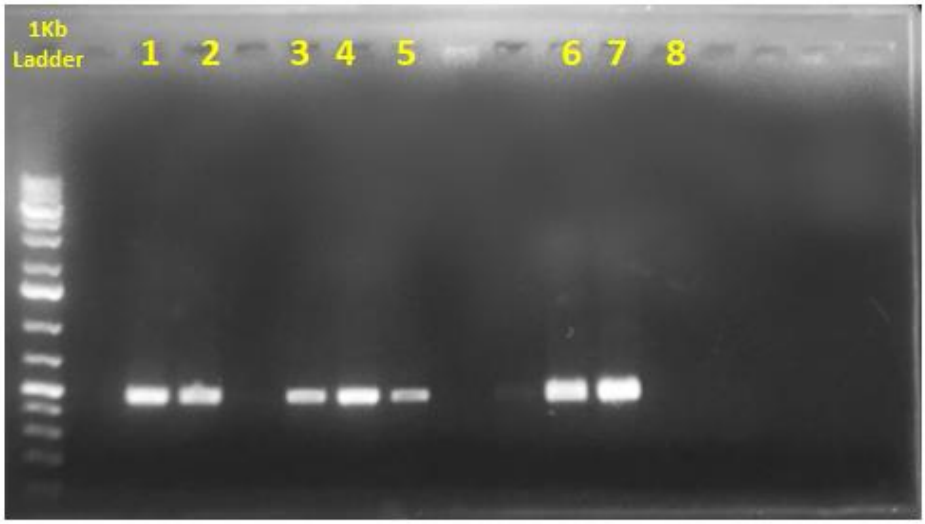

Figure 17: PCR amplification using species-specific primers for fungal isolates from cow feed samples. [Lanes; 1- 7, primer FLA1/FLA2 the template DNA from *A. flavus*, (amplicon size 500 bp). Lanes; 8, represented the non-template negative control of their previous lanes. 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp].

4.3.3.1. Evidence of aflatoxigenic genes:

Using specific primer pairs, PCR was used to predict the presence of specific mycotoxin genes. Table10 shows the identified aflatoxigenic genes presence in all the green *Aspergilli* species.

Table 10: Molecular identification using species specific primers for Green *Aspergillus*

Isolate ID	ITS1/ITS4	FLA1/FLA4	PAR1/PAR2	OMT-2	VER-1	APA
SA	+	+	-	+	+	+
SC	+	+	-	+	+	+
SB1	+	+	-	+	+	+
SBY	+	+	-	+	+	+
SB2	+	+	-	+	+	+
SD	+	+	-	+	+	+

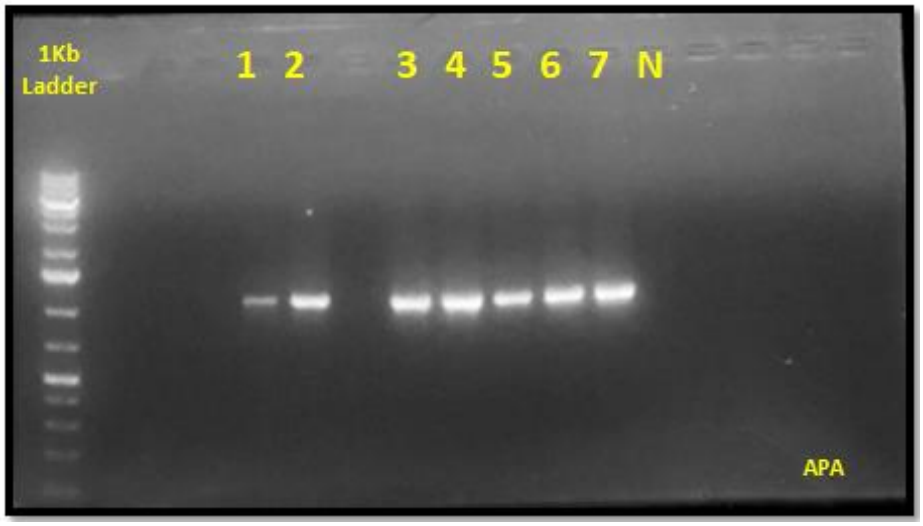

Figure 18: Gel electrophoretic analysis of PCR products using APA-2 primers and DNA obtained from fungi isolates of cow feed samples. All samples have (amplicon size 1032 bp). 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp.

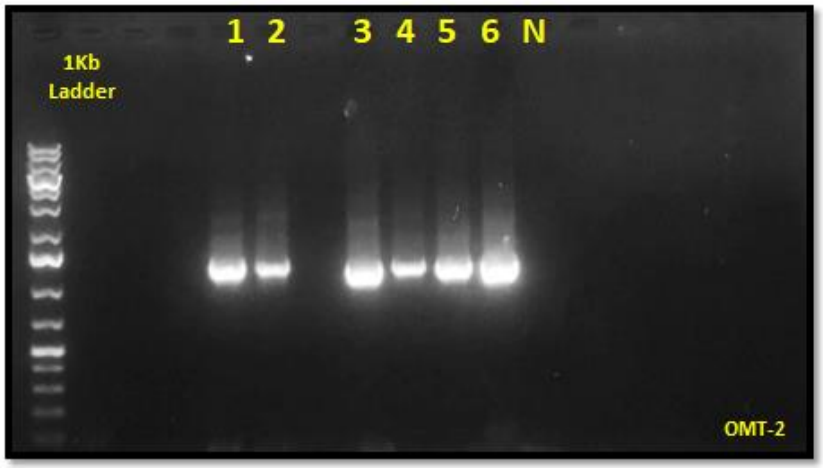

Figure 19: Gel electrophoretic analysis of PCR products using **OMT-1** primers and DNA obtained from fungi isolates of cow feed samples. All samples have (amplicon size 1024 bp). 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200,100bp.

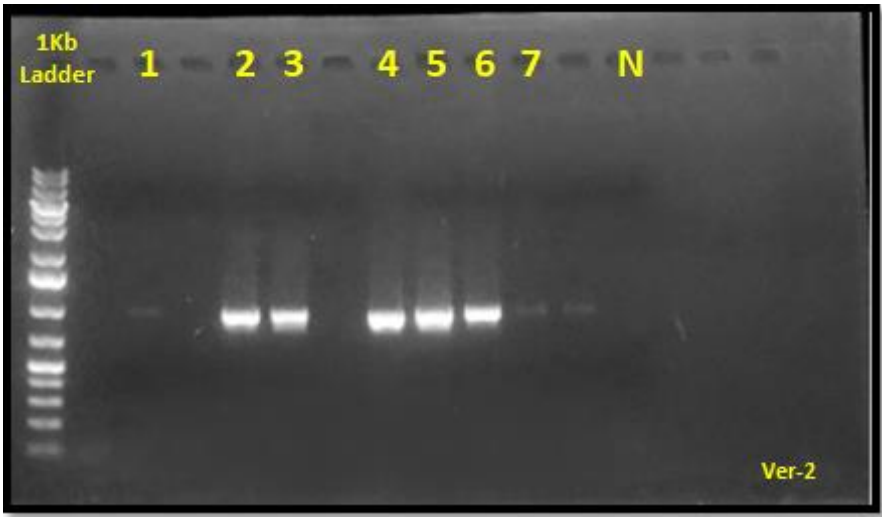

Figure 20: Gel electrophoretic analysis of PCR products using **Ver-2** primer and DNA obtained from fungi isolates of cow feed samples. All samples have (amplicon size 895 bp). 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp.

For green *Aspergilli*, three aflatoxigenic genes primers *ver-1*, *omt-1*, and *apa-2*, were used to check the synthesis of aflatoxin in green fungi isolates.

4.3.3.2. Evidence of ochratoxin genes:

On the other hand, for the black *Aspergilli*, there are three genes coding for ochratoxin synthesis in the pathway arranged by ***OTA-1, OTA-2, and OTA-3*** genes. All black *Aspergilli* isolates were positive for *OTA-2 gene*, but were negative for *OTA-1 and OTA-2* genes (Table 11).

Table 11: PCR results using species - specific primers (Black *Aspergillus)* .

Isolate ID	Primer ITS1/ITS4	ITS1/NIG	Carb1/Carb2	OTA-1	OTA-2	OTA-3
SB3X	+		+	-	+	-
SCX	+	+		-	+	-
SAX	+		+	-	+	-
SA2	+		+	-	+	-
SC2X	+	+		-	+	-
SB2	+		+	-	+	-

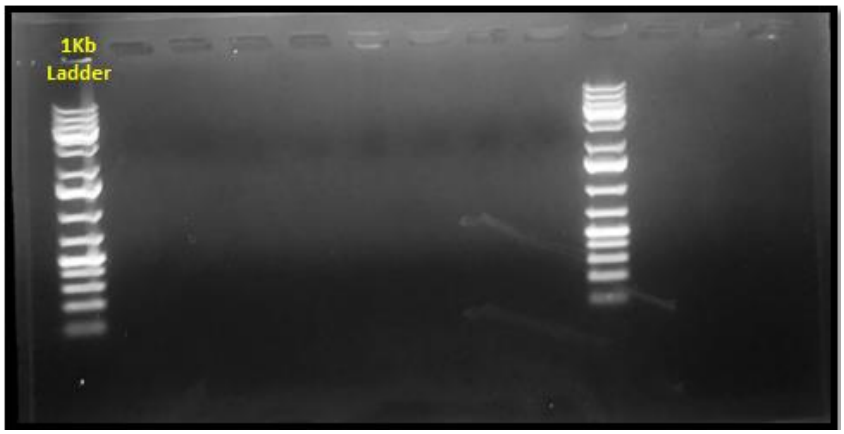

Figure 21. Gel electrophoretic analysis of PCR products using Ochratoxin genes OTA-1 primers and DNA obtained from fungi isolates of cow feed samples. None of the samples have (amplicon size 1000 bp). 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp.

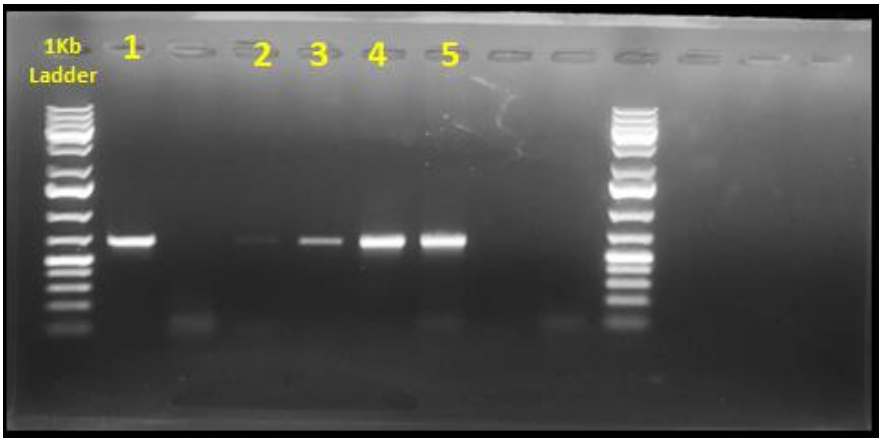

Figure 22. Gel electrophoretic analysis of PCR products using Ochratoxin genes

OTA-2 primers and DNA obtained from fungi isolates of cow feed samples. All samples have (amplicon size 650 bp). 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp.

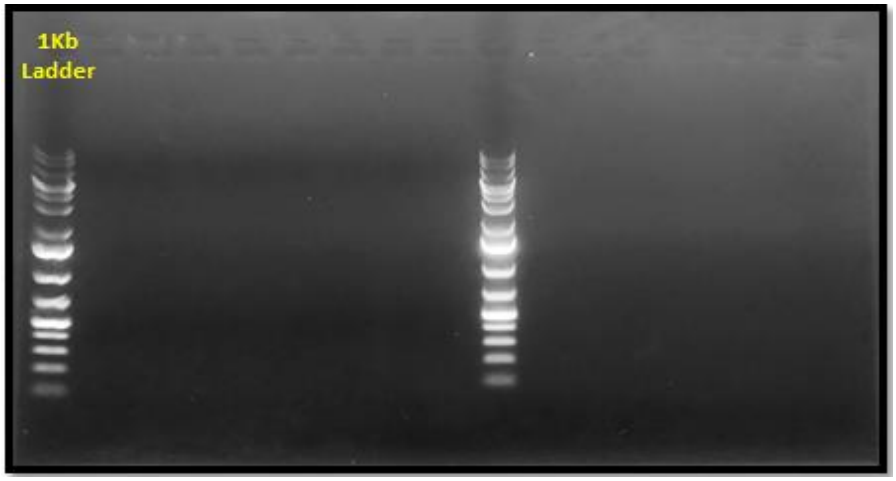

Figure 23. Gel electrophoretic analysis of PCR products using Ochratoxin genes OTA-3 primer and DNA obtained from fungi isolates of cow feed samples. None samples have (amplicon size 1000 bp). 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp.

Discussion:

All animal feed samples were tested for the presence of the aflatoxin and ochratoxin (A). The results of OTA and AFs, contamination in all animal feed samples was showed in Table 8. The samples showed the presence of OTA and AFs below the EU maximum permissible limits. This indicates the good quality of mixed dairy cow feed marketed in the Qatari market when compared with the contamination by mycotoxins in feed for animal consumption reported in other countries (Freitas *et al.*, 2015).

On other hands, each animal feed samples were tested for the presence of fungi.

The summarized results show that animal feed samples (Mixed dairy cow feed) present aflatoxigenic and ochratoxigenic fungi contamination. *A, flavus*, *A. parasiticus, A. carbonarius* and *P. verrucosum* were commonly detected in mixed dairy cow feed samples. The mixed dairy cow feed contained different ingredients like, yellow maize, barley, wheat bean, molasses, cotton seed, die calcium phosphate, soybean meal, salt, vitamins, trace minerals and sunflower seed. However, the origin of the samples is an important factor, such as in the present study, all samples mixed dairy cow feed were originated from Qatar, where the occurrence of aflatoxigenic is expected to be more frequentl because favorable weather conditions and relatively bad storage of cereals. The contamination of cereal grains with mycotoxigenic fungi has been published by many researchers (Hassan *et al.*, 2018; Al Jabir *et al.*, 2019). Also, studies conducted in the marketed feed cereals in Qatar, Hassan *et al.*, (2018) found a higher percentage of animal cerals and grains contaminated with many of mycotoxingenic fungi. Relatively lesser ecology of fungi contaminated noted in the present study, which shows again the good quality of dairy cow mixed feeds used for the dairy cow feeds.

All the isolated fungal colonies were transferred from DRBC selective medium to three identification media, such as, malt extract agar (MEA), Cazpek yeast extract Agar (CYA) and glycerol-nitrate agar (G25N). All the morphological characteristics like, colony shapes from both sides, were noted in Table.8. All fungal colonies were identified using fungal identification reference as described by Jedidi *(*2018) and Pitt (2009). Two fungi, *A. flavus* and *A. parasiticus* showed closely related morphological characteristics, like they appear on greenish yellow, olive green or deep conidia on CYA media.

Furthermore, the mycotoxin synthesis was determined by ELISA for all toxigenic fungi isolated from animal feed. Even through all the isolates in this research

were subjected to the same environmental conditions during incubation, four *A. flavus* isolates produced different concentrations of aflatoxins , with a highest total concentration level of 2270.2 ± 0.2 (µg/kg), detected in animal feed products. This concentration exceeded the maximum level of detection. On the other hand, two other papers found that *A. flavus* originally isolated from grains, cereals and wheat products, produced aflatoxins when re-inoculated onto wheat and liquid media respectively (Atalla *et al.*, 2003). Moreover, only one fungal strain was detected in animal feed products producing OTA at a concentration above maximum level of detection 1102 ± 2 (µg/kg).

In a second stage, we performed the molecular identification of the fungal strains using species-specific primers for both FLA1/FLA2 and PAR1/PAR2. The results showed that all fungal isolates indicate one species, *A. flavus*. However, six isolates coded by (SA, SC, SB1, SBY, SB2 and SD) produced single amplification band of 500 bp, using FLA1/FLA2 primers pair, corresponding to *A. flavus*. None of the isolates showed *A. parasiticus* species. By using a different approach, González-Salgado *et al.* (2005) identified *A. flavus* from wheat flour by using FLA1/FLA2 specific primer pairs, giving a PCR amplification product of 500 bp.

On other hand, two other species, *A. niger* and *A. carbonarius* were morphological identified also, both producing black sporulation and differentiated based on conidial color. In *A. niger*, the color is more brownish black, while in *A. carbonarius* it was jet black. All results of morphological characteristic are summarized in Table 8. Same as green *Aspergilla*, all black *Aspergilla* were identified at the molecular level by used species-specific primers ITS1/NIG and CAR1/CAR2 and the results show that, two of the isolates coded as (SCx & SC2x) showed a single amplification band against ITS1/NIG primer (product size 420 bp), corresponding to

A. niger (Table 10, Figure16). Also, three *Aspergilla* confirmed by specific-species primers CAR1/CAR2 as *A.carbonarius* strains.

However, González-Salgado *et al.* (2005) used ITS universal primers for the selective identification of black *Aspergilli.* Using ITS1/NIG species-specific primers for *A. niger* and CAR1/CAR2 primer for *A.carbonarius*, they were able to successfully differentiate between two *A. niger* species and results confirmed the three *A. carbonarius* species. In line with other studies, Hassan *et al*. (2018) identified species like *A. parasiticus, A. flavus and A.carbonarius,* which were isolated from feed samples marketed in Qatar. After identifying green and black *Aspergilli* species, mycotoxigenic genes were identified. For green *Aspergilli,* three aflatoxigenic genes primers ***ver-1, omt-1, and apa-2,*** were used to check the synthesis of aflatoxin in green fungi isolates. The results summarized in Table 10, show the identified aflatoxigenic genes found in all the green *Aspergilli* species. According to the RONI (1996), the use of *ver-1, omt-1* and *apa-2* as probes can discriminate between sterigmatocystin-producing fungi like some aflatoxigenic strains and both *A. parasiticus, A. flavus* known to produce aflatoxins, *ver-1, omt-1* and *apa-2.* These results are in a line with our present study. The fungi strains isolated from animal feed samples were checked for the presence of three genes encoding the aflatoxin synthesis.

On the other hand, for the black *Aspergilli,* there are three genes coding for ochratoxin synthesis in the pathway arranged by *OTA-1, OTA-2,* and *OTA-3* genes. All black *Aspergilli* isolates were positive for OTA-2 gene, but were negative for OTA-1 and OTA-3 genes (Figures 21-23).

Conclusion:

10 samples of mixed dairy cow feed were collected from different supermarkets located in Doha-Qatar. These animal feed products were tested for the presence of aflatoxin and ochratoxin (A) using ELISA assay. None of the samples showed any

presence of the detectable levels of mycotoxins. These results demonstrate the good quality of mixed dairy cow products in the local market. Despite of this, it is crucial to regularly monitor the level of mycotoxins in the feed for dairy cow to prevent the dairy products contamination, once the food is consumed by human, especially kids. However, many mycotoxigenic fungi have been isolated from the animal feeds. The morphological and molecular identification of these toxigenic fungi allowed the evidence of *A. flavus*, *A. niger*, *A. carbonarius* and *P. verrucosum* that synthesize during their growth mycotoxins compounds (Aflatoxins & Ochratoxins) at levels higher than detection limits and corresponding genes.

CHAPTER 5: ISOLATION AND APPLICATION OF LOCAL YEASTS STRAIN VOLATILE ORGANIC COMPOUNDS FOR THE BIOLOGICAL CONTROL OF TOXIGENIC FUNGI IN QATAR

5.1 Introduction:

Mycotoxins are a wide and diversified group of toxic secondary metabolites of low molecular weight synthesized by well-known group of filamentous fungi including the genera *Aspergillus, Penicillium, Alternaria* and *Fusarium*.

In 2019, more than 399 mycotoxins have been investigated, identified and published so far (Carolina Santos Pereira 2019). The widely discovered mycotoxins are AFA B1, DON, OTA, fumonisins and zearalenone, all considered problematic due to the health issues they cause. Biological control compounds using biological agents against mycotoxigenic fungi is considered as a safest options and now a day getting

popularity in food industry (Pfliegler, *et., al* 2015).

In new greenhouse studies, it was found that yeast can antagonize the mycotoxigenic fungi by different mechanisms, such as synthesis of antifungal compounds like gases or soluble molecules or formation of biofilm on the nutrients and space (Pfliegler, Pusztahelyi and Pócsi, 2015). Moreover, more than 30 yeast strain can produce compounds which prevent the growth of mycotoxigenic fungi (Farbo *et al.*, 2018).

In order to find suitable yeast strains useful in the biocontrol of toxigenic fungi and their toxins, we isolated in this project, 11 yeast strains locally from different food products, such as fermented food, dairy products and meat, to be used as biological control agents against the following mycotoxigenic fungal strains *A. parasiticus AF82, F. graminearum FGr14 36* and *P. verrucosum TF11 , A. Carbonarius* and other references strains.

In a first screening, *F. graminearum, A. parasiticus, A. carbonarius* and *P. verrucosum* and their respective mycotoxins were exposed to the yeast VOCs.

The synthesis of different mycotoxin, such as AFB1, DON and OTA were measured from the fungal colonies that were exposed to the yeasts VOCs. Several yeasts strains have fungal biocontrol activites against the fungi, inhibiting their growth and mycotoxins synthesis.

Moreover, volatile organic compounds molecules synthesized by the yeast strains were investigated by SPAM/GC analysis.

5.2 Isolation of yeast strains from fermented food, dairy products and meat products:

Directly from fermented food and dry meat products 14 yeast strains

were isolated on YPDA and YGCA media. Isolation, purification and molecular identification by conventional taxonomic methods and Sanger sequencing of the rDNA intergenic sequence flanking the 5.8s rDNA for yeast strains were used in this study.

5.2.1 Molecular identification of the isolated yeast strains:

5.2.1.1. Amplification of the yeast DNA using universal pair of primer ITS1/ITS4:

The molecular identification of the yeast strains isolated in this work was carried out by PCR/ sequencing using ITS-1 primer (foreword primer) and ITS-4 primer (reverse primer) (Figure.24).

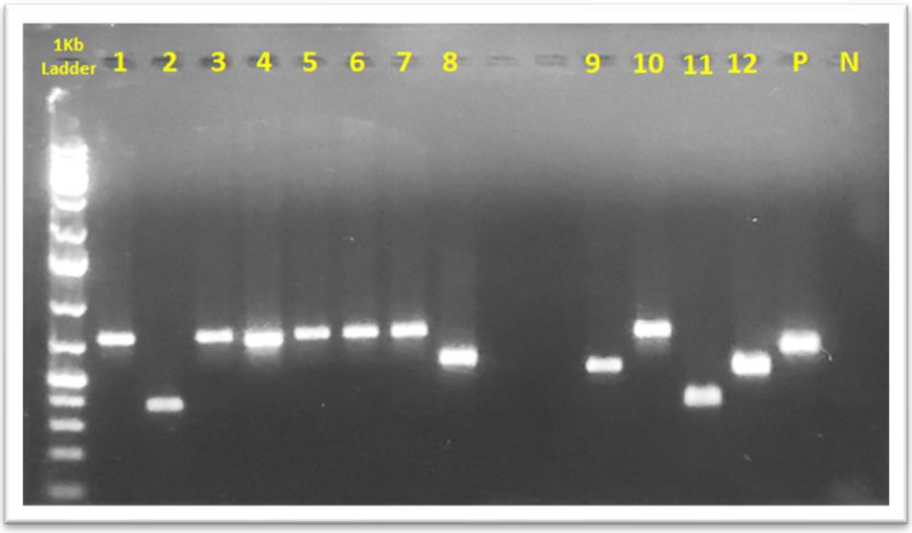

Figure 24**.** PCR amplification using universal primers for the identification of yeasts isolates from fermented food, dairy products, meat and animal feed samples
[Lanes; 1-12, primer ITS1/ITS4 the template DNA from yeast (amplicon size 600 bp). Lanes; N, represented the negative control of their previous lanes. Lanes P; represented positive control template from reference strain. 1kb plus DNA marker with fragment sizes 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 850, 650, 500, 400, 300, 200, 100bp].

Seven samples showed single and specific bands of 600 bp, and one samples (coded as samples no.2) showed specific bands of 400bp, one

samples (coded as samples no.11) showed single and specific bands of 500bp using ITS/ITS4 primers.

Yeast strain	Similarity %	Code
Kluyveromyces marxianus	*100%*	***QKM#4***
Clavispora lusitaniae	*98%*	***QCL#2***
Kazachstania servazzii	99%	***QKS#3***
Kazachstania servazzii	100%	***QKS#6***
Kazachstania servazzii	96.48%	***QKS#6***
Kazachstania servazzii	89%	***QKS#7***
Aureobasidium pullulans	99.09%	***QAP#8***
Cryptococcus	83.02%	9
Kazachstania sp.	79.47%	***QKS#13***
Clavispora lusitaniae	98.82%	***QCL#14***
Cutaneotrichosporon curvatus	99.16%	***QCC#15***

5.2.1.2. Sequencing of the 26s rDNA and identification of the yeast strains:

All PCR fragments were sequenced. The sequences are displayed in (Appendix A).

The Blast alignment of these sequences using the link (ncbi.nih.nlm.gov) of ncbi Genbank database at the National Center for biotechnology information, allowed the identification 11 yeast strains Table 12. The yeast strains were belonging to the following genera *Kluyveromyces marxianus, Clavispora lusitaniae, Kazachstania servazzii, Aureobasidium pullulans, Cutaneotrichosporon curvatus.*

Table 12: Yeast strains identified in this study

5.3 Spectrum of antifungal activities of yeast strains VOCs

5.3.1 Growth inhibitory effect of different yeast's VOCs on *A. parasiticus AF82*:

The diameter size of *A. parasiticus* inoculated as spores on PDA plates tightly sealed to YPDA plates containing growing yeast colonies , was in comparison to that of the control system with YPDA plates without yeast cells (negative control plates). It was clearly observed that the yeast VOCs (Figure25-26) four yeast strains reduced the growth of the *A. parasiticus AF82* in comparison to the control colonies after 4^{th} and 7^{th} days. However, the decrement in the diameter on the 7^{th} day was shows highly different than colony diameter in the negative control (control: 30.6 mm, treated Y4: 17.4 mm & treated Y2: 18.7) in yeast *Kluyveromyces marxianus* QKM#4 and yeast *Clavispora lusitaniae* QKM#2, while not much difference between control and remaining treated yeast strains. The VOCs affected the pigmentation and/or sporulation of the fungal cells as well. The color of the fungal colonies was totally different from that of the nonexposed to the VOCs. The fungal colonies exposed to different yeasts VOCs, had white cottony shape, different from those of the negative control colonies which retained the yellow color on the edges and greenish in the centre of the colony.

5.3.2 Effect of different yeast's VOCs on *F. graminearum* FGr14 growth:

From the diameter measurement for the control and tested colonies of *F. graminearum* with yeast VOCs, there was a very significant decrease in the fungal growth according to diameters measurement of the colonies and in comparison to the negative controls colony on the 4th and 7th days (Figure25-26). The yeast VOCs were able to inhibit the growth of the point inoculated *F. graminearum* colonies when sealed to the different isolates of yeast cells, and in comparison to the control plates. The results of the inhibition effects of different yeast strains VOCs, on the 4th and 7th days, on the inoculum of *F. graminearum* are summarized on Figure 25 and 26**.** Same as *A. parasiticus* strains, the pigmentation losses were observed also for the treated colonies and it is completely white from the centre of the colonies, in comparison to the negative control colonies, which was completely pinkish from the centre of the colonies.

5.3.3 Effect of different yeast's VOCs on *P. verrucosum* TF11 growth:

The 11 yeast strains VOCs reduced significantly the growth of *P. verrucosum* TF11, making *P. verrucosum* strain as the most sensitive fungus among the three studied fungi, *A. parasiticus, F. graminearum FGr14 and A. corbaniares.*, to yeasts VOCs. The measured diameters of the tested *P. verrucosum* colonies reached to 5.62 mm and 8.52 for the yeast strains QKM#4 and QKM#2 respectively , compared the negative control colony size of 15.17mm after 7th day, (Figure 26). The VOCs affected the pigmentation and sporulation of the fungal colonies exposed to the yeast's VOCs, where the tested colonies showed white cotton pigmentation in comparison to the colonies of the negative control that appeared as dark green bluish in centre of the colonies.

5.3.4 Effect of different yeast's VOCs on *A. carbonarius* growth:

A. carbonarius strain is considered as the most sensitive fungal strain to the

VOCs of the eleven yeast strains. In fact, the point inculated colonies sealed to yeast strains showed a great inhibtion in their dimeter mesurments. The diameters of ***A. carbonarius*** treated colonies with respectively QKM#4 and QKM#2 were 4.8mm and 6.53mm in comparison to the negative control colony dimeter ws 25.2mm after 4th days, (Figure 25). Four of the colonies exposed to the 8 other yeast's VOCs were almost same as that of the negative control colony of the 7th days. These results confirm that QKM#4 and QKM#2 VOCs have the highest antifungal activities .

In addition the loss of pigmentation for the colonies exposed to yeast's VOCs is still remaining until the 7th days, while the fungus in the negative control, is very dark greenish and black colour.

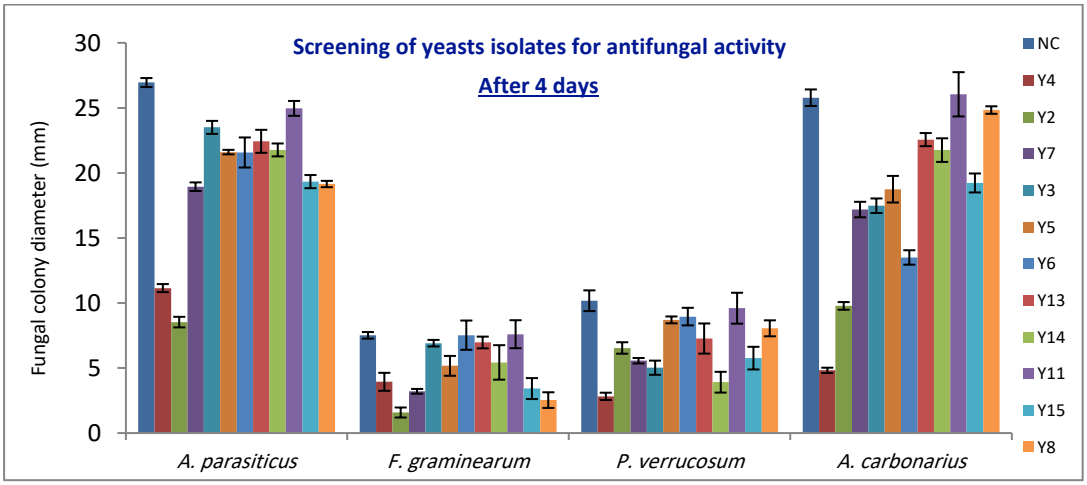

Figure 25. Comparison of yeast strains VOCs antifungal activities against *A. parasiticus, F. graminearum, P. verrucosum and A. carbonarius* after 4th days post point inoculation [**NC**; negative control plate without yeast VOC,**Y4**; yeast strain coded by 4,**Y2**; yeast strain coded by 2,**Y7**; yeast strain coded by7,**Y3**; yeast strain coded by3,**Y5**; yeast strain coded by5,**Y6**; yeast strain coded by6,**Y13**; yeast strain coded by 13,**Y14**; yeast strain coded by 14, **Y11**; yeast strain coded by11, **Y15**; yeast strain coded by15,**Y8**; yeast strain coded by 8.

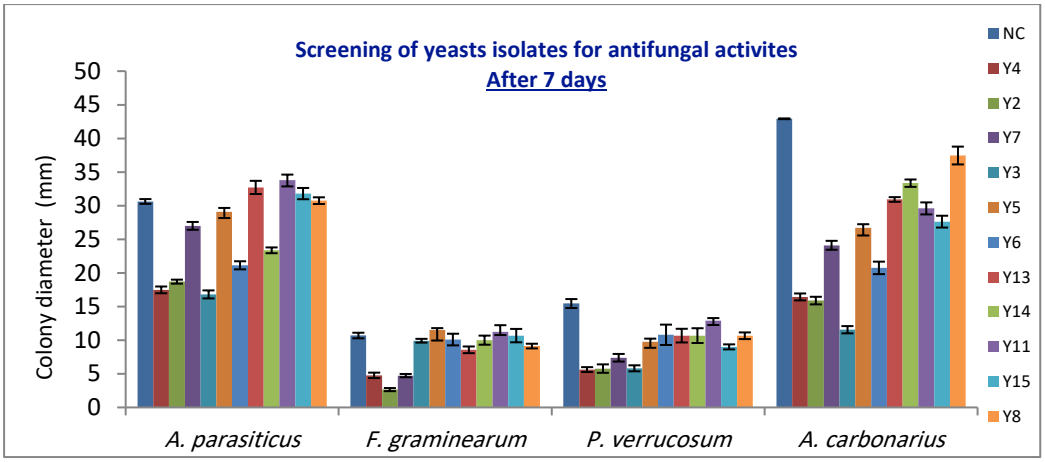

Figure 26. Comparison of yeast strains VOCs antifungal activities against point inoculated *A. parasiticus, F. graminearum, P. verrucosum and A. carbonarius* after (7^{th}day) [**NC**; negative control plates,**Y4**; yeast strain coded by 4,**Y2**; yeast strain coded by 2,**Y7**; yeast strain coded by7,**Y3**; yeast strain coded by3,**Y5**; yeast strain coded by5,**Y6**; yeast strain coded by6,**Y13**; yeast strain coded by 13,**Y14**; yeast strain coded by 14, **Y11**; yeast strain coded by11, **Y15**; yeast strain coded by15,**Y8**; yeast strain coded by 8.

5.4. Spectrum of the QKM#4 yeast VOCs antifungal activities against toxigenic fungi

The effects of the QKM#4 yeast VOCs antifungal activities were explored against different toxigenic fungi *A.* paraticus, *F. graminearum*, P. verrucosum *and A. carbonarius*. The diameters of the exposed *A. carbonarius* colonies (Figure 27, A-B) were smaller than that of the control. QKM#4 yeast VOCs reduced significantly the growth of the *A. carbonarius* on the 4th and 7th days, respectively (Figure28).

The pigmentation and/or sporulation of the fungi were also affected too. The color of the fungal colonies, exposed to VOCs, was totally different than that in the negative control not exposed to the VOCs. The fungal colonies exposed to the yeast VOCs, had a white cottony shape, different from the negative control colonies which retained the yellow on the edges and greenish in the centre of their colony. Figure (27) shows how exposure to yeast VOCs has resulted in undefined margins in the fungal colony.

From the diameter measurement for the control and tested colonies of *P. verrucosum* with yeast VOCs, there was a very major drop in the fungal growth on the 4th and 7th day. The yeast VOCs were able to prevent the growth of the point inoculated *P. verrucosum* colonies when sealed to yeast cells and in comparison to the control plates. The yeast VOCs had an inhibition effect of 72.01% and 63.53%, on the 4th and 7th days (Figure 28). Same as *A. carbonarius*, the QKM-4 VOCs affected the pigmentation and sporulation of the fungal colonies exposed to the yeast VOCs, where the tested colonies showed a white cotton pigmentation in comparison to the colonies of the negative control plate which appeared as dark green bluish in centre (Figure 27, C-D).

On other hand, after 4 days of sealing, the growth of *F. graminearum* (Third reference strain used in the current study) showed to a maximum growth inhibition with a diameter of 2.94 mm, significantly lower than 9.41mm of the negative control**,** giving an inhibition percentages of 47.50 % and 55.33 % respectively on the 4th and 7th days (Figure 28). After 7 days of sealing, the growth of *F. graminearum* was not increased compared with controls also (Figure 27). However, the VOCs affected the pigmentation and sporulation of the fungal colonies exposed to the yeast VOCs, where the tested colonies showed a white cotton pigmentation in comparison to the colonies of the negative control plate which appeared as dark pinkish in centre (Figure 27, E-F).

While for the fourth fungal strains, *A. parasiticus* point inculated colonies sealed to yeast showed a less inhibtion in their dimeter mesurments. The diameter of the treated colonies was 10.93 mm and 17.28 mm on the 4th and 7th days, which accounted to an inhibition percentatge 73.50% and 181.46 , respectively for the 4th and 7th days (Figure 27). Also, the loss of pigmentation for those colonies which exposed to yeast's

VOCs is still present until the 7th days, when compared with the negative control colonies very dark yellowish colour. The bio-control activity of the QKM-4 VOCs is attributed to the release of antifungal VOC compounds, which clearly inhibited the growth of fungi even at very low number of yeast colonies at dilution ($x10^3$). Figure (29) shows the broad range spectrum of *QKM-4* VOCs antifungal activities against 17 mycotoxigenic fungi.

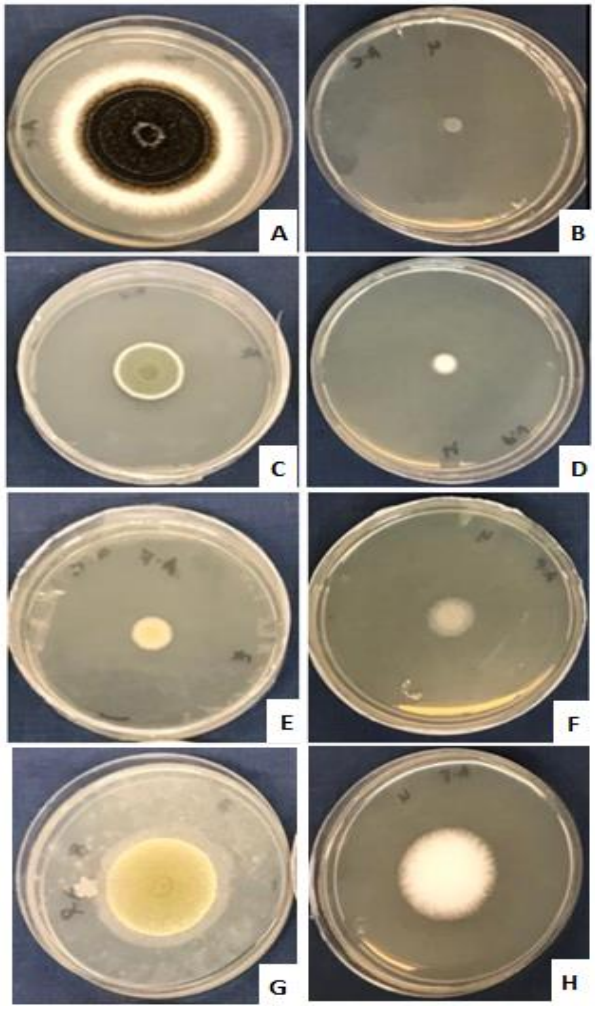

Figure 27. *Kluyveromyces marxianus QKM#4* VOCs spectrum of antifungal activities against *A. carbonarius* (A-B), *P. verrucosum* (C-D), *F. graminearum* (E-F), and *A. parasiticus* (G-H) Morphology of Colonies of *A. carbonarius;* **(A)** not with yeast VOCs; **(B)** with VOCs from (10^3 cells/mL), *QKM-4* cells. Morphology of Colonies of *P. verrucosum;* **(C)** not with yeast VOCs; **(D)** with VOCs from $x10^{-3}$ dilution of *QKM-*

4 cells; **(E),** Morphology of Colonies of *F. graminearum;* **(E)** not with *QKM-4* cellsVOCs; **(F)** with VOCs from (10^3 cells/mL), *QKM-4* cells. Morphology of colonies of *A. parasiticus;* **(G)** without yeast VOCs; **(H)** with VOCs (10^3 cells/mL) of *QKM-4* cells.

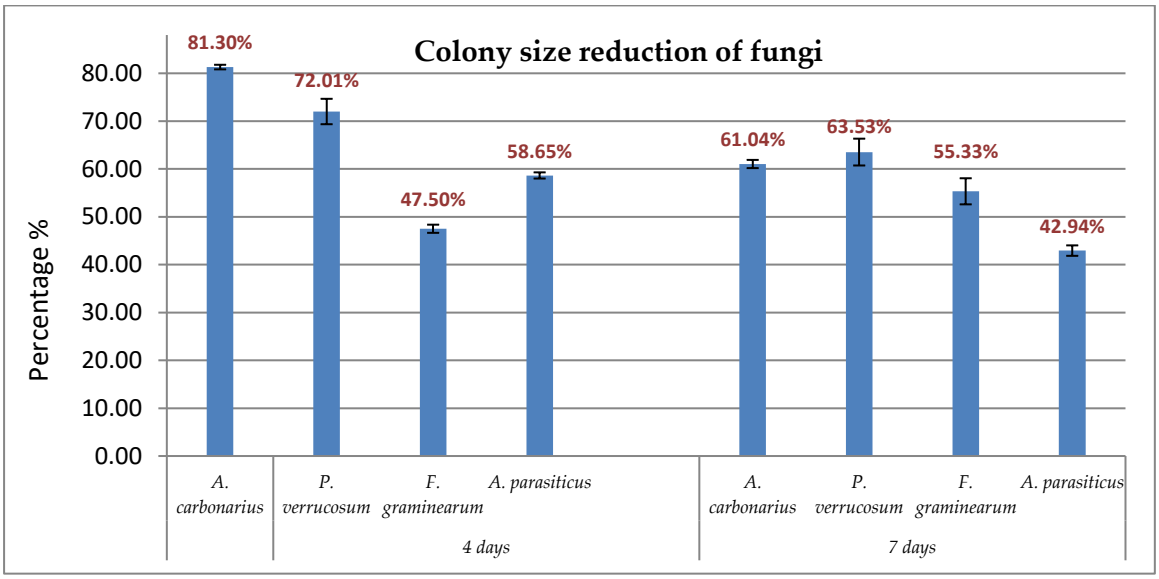

Figure 28. Inhibition of Fungal Growth measured as reduction (%) of colony size in comparison to the control at days 4 and 7 post exposure to *Kluyveromyces marxianus QKM#4* VOCs. Spores of selected *A. carbonarius, P. verrucosum, F. graminearum and A. parasiticus* were inoculated at the middle of PDA plates sealed for 48 hr yeast colonies plated on YPDA plate's media.

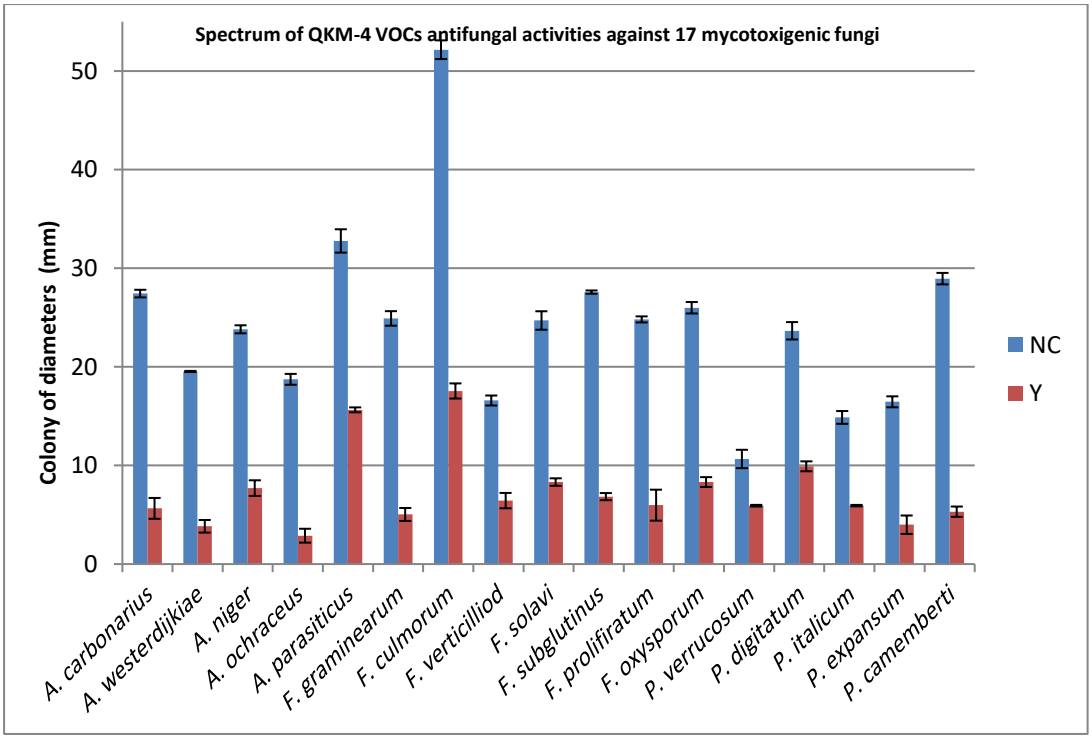

Figure 29. Spectrum of *Kluyveromyces marxianus QKM#4* VOCs antifungal activities against 17 mycotoxigenic fungi. **NC**, negative control, fungi not exposed to yeast strain VOCs; **Y**, fungi exposed to QKM-4 strain VOCs.

5.5. Investigation of the major VOCs of the local yeast strain QKM#4 of *Kluyveromyces marxianus* GC results:

The major volatiles from QKM-4 were identified by SPME-GC/MS analysis to be nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36) and tetracosane (C24) (Table 13). The absence of these compounds in the volatiles of negative control flasks (YPD media without yeast) confirmed that these compounds are synthesized by the yeast only. The synthesis and antifungal activities of alkanes by biocontrol agents such as yeasts and bacteria have already been reported in several studies (Zhang et al., 2013). In a paper (Zhang et al., 2013), VOC analysis of antifungal strain of *Bacillus atrophaeus* (strain CAB-1) showed several alkanes such as heptadecane, tetrapentacontane, eicosane, silane and others.

These researchers later on confirmed antifungal activities of synthetic hexadecane and eicosane against *B. cinerea*. Likewise, a broad-spectrum antimicrobial activity of *Pseudomonas putida* was associated with its potential to produce alkanes such as, heneicosane and tetratetracontane along with other volatile compounds (Sheoran et al., 2015). The presence of already published work on the antifungal activities of alkanes, refrained us to perform testing with synthetic alkanes. The chromatogram of the volatile there was a peak with the retention time of 37.63 min, while the lower retention time was tetracosane shows a peak with 44.94 min. In the control (medium without yeast cells), the detected volatiles showed negligible areas, and not detected in the control headspace.

Table 13: Volatiles organic compounds synthesized by the yeast strain QKM#4:

S. No	Name of compounds	Retention time (min)	Peak area %
1	Nonadecane	37.63	8.00
2	Eicosane	39.20	8.52
3	Docosane	40.77	10.91
4	Heptacosane	42.17	10.95
5	Hexatriacontane	43.58	10.59
6	Tetracosane	44.94	9.59

5.6 Effect of yeasts VOCs on the mycotoxins synthesis:

We explored the effect of the QKM#4 volatiles on the synthesis of mycotoxins by the tested mycotoxigenic strains. *A. carbonarius* and *P. verrucosum*. The fungi exposed to the *QKM-4 VOCs* for 10 days showed a significant reduction in ochratoxins

(OTA) synthesis by *A. carbonarius* and *P. verrucosum*. In the plugs isolated from colonized media with *A. carbonarius* fungi which were exposed to yeast VOCs, significant lower OTA contents (31.4 ± 0.89µg/Kg) were noticed, in comparison to those obtained from unexposed fungi to VOCs (2422.6 ± 4.04 µg/Kg). While, for the plugs of the isolates of *P. verrucosum* colonies exposed to yeast VOCs, significant lower OTA contents (0.12 ± 0.03µg/Kg) were observed, as in comparison to the control plates, of fungi not exposed to the yeast VOCs (31.1 ± 0.54 µg/Kg).

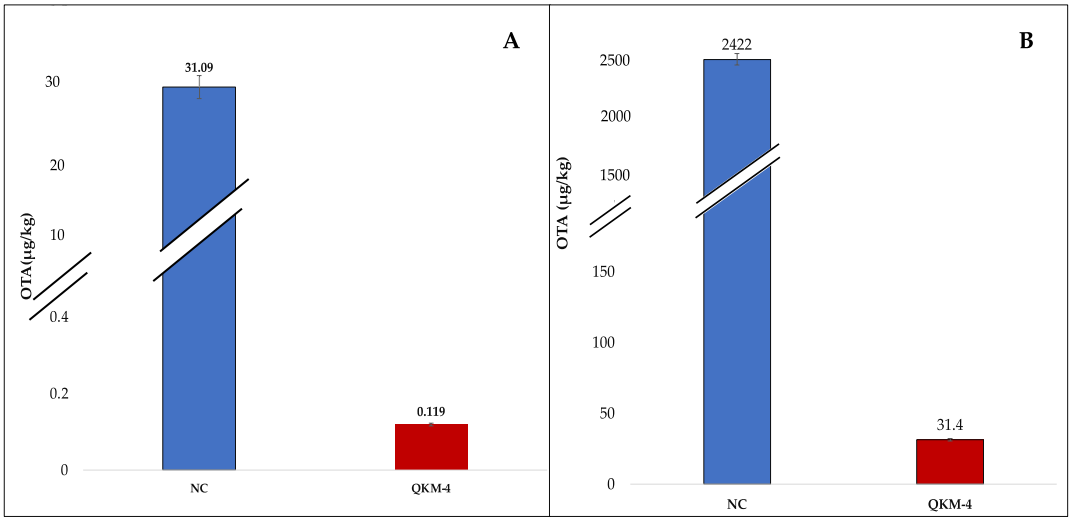

Figure 30. Effect of QKM-4 volatiles on the mycotoxin synthesis of toxigenic fungi **(A)** Comparison of ochratoxins synthesis between VOCs exposed and unexposed *A. carbonarace*. **(B)** Comparing of OTA synthesis between yeast VOCs exposed and unexposed *P. verrucosum*.

5.7. Exploration of the Removal of mycotoxins by *Kluyveromyces marxianus QKM4* living and heat inactivated cells:

5.7.1. Exploration of the Ochratoxins binding to yeast cells:

The choice of mycotoxins binder mainly depends on the spectrum of its activity against a range of target mycotoxins, high absorption potential, safety, irreversibility and finally the proven *in vivo* supporting experiments. In fact, the spectrum of a

mycotoxin binder depends on several factors such as the polarity of the target toxin, nature of binder, pH of the medium and level of mycotoxin contamination.

In the present study, live and heat inactivated QKM-4 cell were used to test their adsorption potentials to remove OTA , DON and AFs toxins from a buffer solution at pH 5 (acidic buffer). Adding of living yeast cells caused the reduction of OTA at 24% & 11% in the supernatant of the buffer artificially spiked with 0.9 µg/L and 1.8 µg/L, respectively (Figure 31). The addition of inactivated QKM-4 cells to OTA contaminated buffer solution showed a higher binding of 58% and 30% at levels of contamination of 0.9 µg/L and 1.8 µg/L, respectively.

The efficacy of inactivated yeast was significantly higher than the living QKM-4 cells and in both cases, the binding potential showed almost 50% decline at higher tested level of OTA. The quantity of removed OTA from the supernatant has been mainly detected in tube pellets.

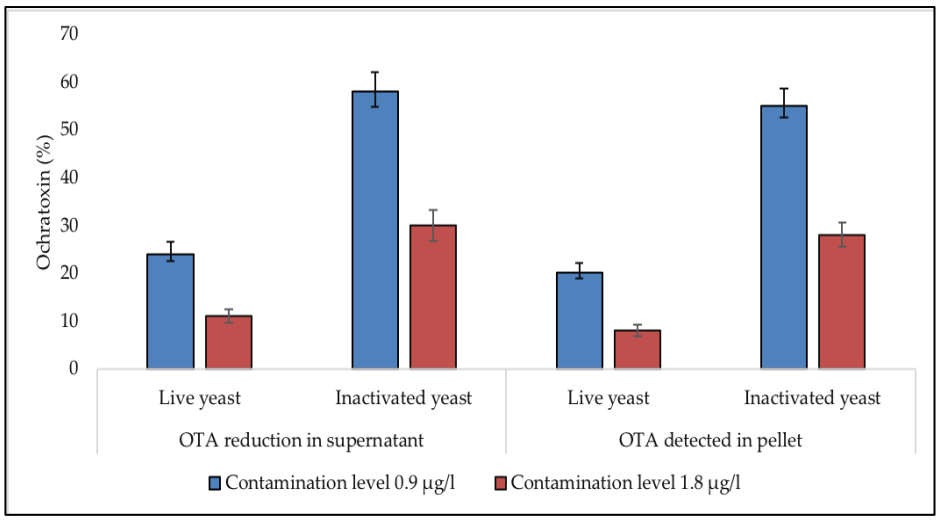

Figure 31. The absorption of OTA by living and heat-inactivated QKM-4 cells from a buffer solution at pH 5. Living (20 µL) or inactivated yeast (5 mg) was added to tubes having OTA at 0.9µg/L or 1.8 µg/L and incubated for 1 hr. Mycotoxins levels in the supernatant and pellet were tested by ELISA. Inactivated yeast showed promising activity against OTA as in comparison to live cells.

5.7.2. Exploration of the Deoxynivalenol binding to yeast cells:

In another experiment, the adsorption potential of QKM-4 was also tested against DON mycotoxin. At low DON contamination level (40 µg/L); living and inactivated yeast cells showed 12% and 6.5% reduction in the mycotoxin in the supernatant of the tubes. This reduction (or adsorption) reached to 49% and 41% by live and inactivated yeast, respectively, when the buffer solution was artificially contaminated with the higher (80 µg/L) level of the DON. In all cases the removed mycotoxin was detected in the pellet of the tube (Figure 32). In line with these results, Zeidan *et al.* (2018) found that, DON toxins showed the least adsorption potentials to the yeast cell wall among the OTA, AFs mycotoxins. But, the removal of DON by the live yeast cells was not more than 17%.

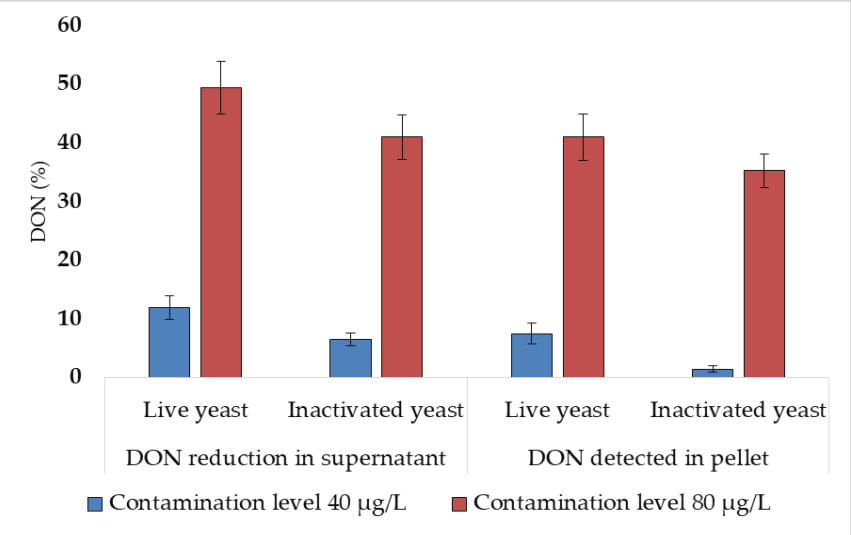

Figure 32. Adsorption of DON on live and inactivated yeast cells. QKM-4 at both physical states (live and inactivated) showed a significant adsorption of DON at its higher contamination level (80 µg/L) as in comparison to low contamination (40 µg/L).

5.7.3. Exploration of the Aflatoxins binding to yeast cells:

Regarding AFs, it was noticed that the toxins adsorption on yeast cell wall is higher in comparison to the OTA and DON at the pH 5, containing 0.2 µg/L and 0.4

µg/L , the adsorption of AFs on live yeast cells was 70% and 71%, respectively, and 55% and 95% were detected on the pellets. While in buffer solution containing with AFs at 0.2 µg/L and 0.4 µg/L , adding of non-living yeast cells caused 100% and 100% removal of the toxins at pH 5 and 88% and 98% were detected on the pellets.

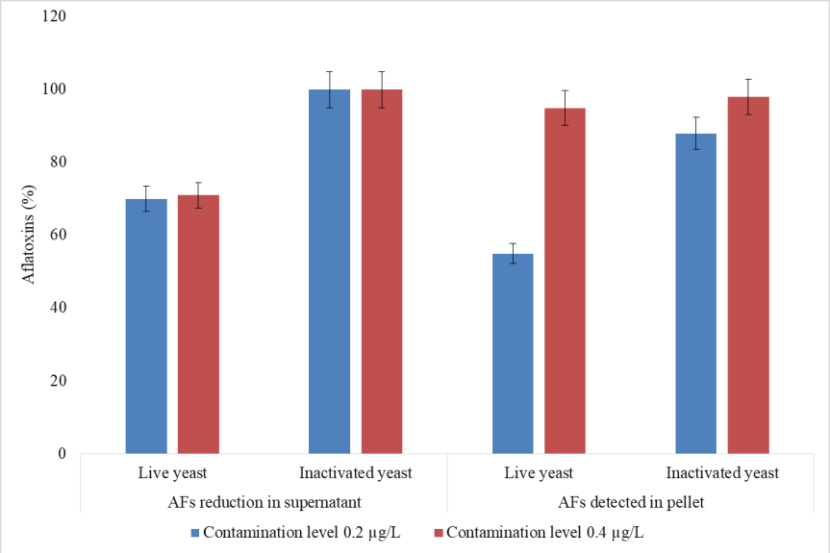

Figure 33. Adsorption of AFs on live and inactivated yeast cells. QKM-4 at both physical states (live and inactivated) showed a significant adsorption of AFs at its higher contamination level (0.4 µg/L) and low contamination (0.2 µg/L).

Discussion:

Now a day, the biological control of toxigenic fungi and their mycotoxin synthesis is considered as the safest approach as in comparison to the chemical and physical methods. Furthermore, uses of environmental-friendly biological strains such as non-pathogenic yeast strains or bacterial strains, not only having potential to control the fungal growth and mycotoxins synthesis of toxigenic fungi, but also adding a beneficial characteristic to the feed and food matrix.

In the present study, we isolated, from different food products, 11 yeast strains belonging to the following genera *Kluyveromyces marxianus, Clavispora lusitaniae, Kazachstania servazzii, Aureobasidium pullulans* and *Cutaneotrichosporon curvatus.* These strains were explored for the antifungal effects against four toxigenic fungal strains, *A. parasiticus, A. niger, P. verrucosum* and *A. carbonarace.*

However, strains showed very important antifungal activities due to the Volatile Organic Compounds that they produce. The VOCs released by the yeast strain QKM#4 of *Kluyveromyces marxianus*, showed a wide antifungal spectrum of antifungal activities against seventeen fungal toxigenic reference strains. The diameter of fungal colony treated with QKM-4 VOCs at day 4 and 7 was 4.8mm, 16.4mm as in comparison to the control fungi having diameters of 25.71mm and 42.91mm at day 4 and 7. Furthermore, these finding are in line with those of Fiori *et al*., 2014, reporting a similar decreases in the colony diameter of *A. carbonarius* when exposed to yeast VOCs. Also, Sui sheng (2014), reported a decrease in *A. parasiticus* growth by another yeast *Pichia anomala* VOCs.

The biocontrol activity of the yeast VOCs is attributed to the release of antifungal compound, identified as 2-phenylethanol which is very known know to inhibit the fungal growth even at very low concentration (Hua *et al.*, 2014). In the

present study, the major volatile compounds from QKM-4 yeast strain were identified by SPME-GC/MS analysis to be nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36) and tetracosane (C24) (Table13). GC/MS total ion chromatogram (TIC) showing the detection of six molecules corresponding fragmentation electron impact. The chromatogram of the volatile compounds shows a peak with the retention time of 37.63, 39.20, 40.77, 42.17, 43.58, 44.94 min. All volatile compounds were identified and summarized on Table 13. as organic compounds namely as alkanes. The synthesis and antifungal activities of alkanes by biocontrol agents such as yeasts and bacteria have already been reported in several studies (Zhang *et al.,* 2013; Sheoran *et al.,* 2015). In a study (Zhang *et al.,* 2013), VOC analysis of antifungal strain of *Bacillus atrophaeus* (strain CAB-1) showed several alkanes such as heptadecane, tetrapentacontane, eicosane, silane and others. These researchers later on confirmed antifungal activities of synthetic hexadecane and eicosane against *B. cinerea*. Likewise, a broad-spectrum antimicrobial activity of *Pseudomonas putida* was associated with its potential to produce alkanes such as, heneicosane and tetratetracontane along with other volatile compounds (Sheoran *et al.,* 2015). The presence of already published work on the antifungal activities of alkanes, refrained us to perform testing with synthetic alkanes.

The *in-vitro* investigation of the antifungal potential of the newly isolated best yeast strains coded as QKM#4, on the vegetative growth of four fungal strains in a first screening and seventeen strains in a second screening, it was shown that yeast VOCs successful inhibited the fungal cells growth and the sporulation and synthesis of OTA in both black *Aspergillus* and *Penicillium*. In line with the present study, Farbo *et al*., (2018) demonstrated that the major compound inhibiting the growth and synthesis of OTA in *Aspergilli* species is the 2-phenylethanol.

Post-harvest mycotoxins management of cereals and grains involves multiple strategies, among which decontamination of feed and food includes addition of mycotoxins adsorbing substances to the toxins contaminated substrates. In commercial agriculture and livestock settings, materials such as clays (bentonites, zeolite, hydrated sodium calcium aluminosilicates, montmorillonite, smectite), activated charcoals, yeast cell walls and their derivatives are being used as individually or in different combinations.

The potentials of mycotoxins, OTA, DON and AFs to adsorb to *QKM-4* were explored *in-vitro* by both living and inactive yeast cells' adsorption rates, from both living yeast cell culture and inactivated yeast cells were used to inoculate acidic buffers of pH 5 and later were contaminated with mycotoxins. Selected pH 5, to test viability of yeast strain to survive under gastro-intestinal (GI) conditions and to study the OTA, DON and AFs mycotoxins binding ability under acidic pH 5, comparable to those found along with ruminant GI tract. The tubes having mycotoxins were shake incubated for 60 min at 37 C° and were centrifuged, then the supernatant were separated from pellets. The adsorption potential were calculated to detected concentration of contamination on the pellets by dividing the determined concentration of toxins in the pellet over original concentration used in the acidic buffer solution and multiplied by 100% to get the adsorption potential percentage.

The OTA adsorption to living yeast cells resulted in the reduction of OTA by 24% and 11% in the supernatant of buffer solution contaminated at 0.9 µg/L and 1.8 µg/L, respectively (Figure 31). The addition of inactivated QKM-4 cells to OTA contaminated buffer solutions showed a higher binding of 58% and 30% in buffers containing 0.9 µg/L and 1.8 µg/L, respectively. The efficacy of inactivated yeast was significantly higher than the living QKM-4 cells and in both cases, the binding potential

showed almost 50% decline at higher tested level of OTA. The quantity of removed OTA from the supernatant was mainly detected in the pellet of the tube.

While for the DON adsorption potential, at low concentration level (40 µg/L); living and inactivated yeast cells showed 12% and 6.5% reduction in the mycotoxin in the supernatant of the tubes. This reduction (or adsorption) reached to 49% and 41% by living and inactivated yeast, respectively, when the buffer solution was artificially contaminated with the higher (80 µg/L) level of the DON. In all cases the removed mycotoxin was detected in the pellet of the tube (Figure 32). However, OTA showed the highest adsorption potentials to the inactivated yeast cells in comparison to the DON. In line with these results, Zeidan *et al.* (2018) found that, DON toxins showed the least adsorption potentials to the yeast cell wall among the OTA, AFs mycotoxins. But, the removal of DON by the live yeast cells was not more than 17%.

According to the results on Figure 33, the adsorption efficacy of AFs to the living and non-living yeast cell wall was higher than OTA and DON. Under acidic condition, and at levels of contamination of 0.2 µg/L and 0.4 µg/L , the AFs adsorption on living yeast cells was 70% and 71%, respectively, and 55% and 95% were detected on the pellets. While in the solutions containing AFs at 0.2 µg/L and 0.4 µg/L, the addition of non-living yeast cells gave in both 100% removal of the toxins at pH 5 and 88% and 98% were detected respectively in the pellets. The heating for yeast cell (inactivate yeast cells) could play a role on increasing the permeability of the external layer of the yeast cell wall due to the suspension of some of the mannans from cell wall surface, leading to increased hidden binding sites. On line with this finding, Rahaie *et al.*, 2010 found that, *S. cerevisiae* cells were able to remove large amounts of AFs B1, even at high contamination levels (20ppb) under acidic conditions, also, heating , could increase yeast binding ability to 55% and 75% with primary AFB1 concentration at 10

and 20 ppb. However, AFs showed the highest adsorption potentials to the activate and inactivated yeast cells in comparison to OTA and DON.

However, there are many factors playing an important role on the adsorption of mycotoxins to yeast cell wall such as, characteristics of the yeast cell wall. Some of the features of yeast cell wall very important for the mycotoxins adsorption, like porosity, surface area or charge and its distribution around yeast cell wall.(Dogi *et al.*, 2011). Moreover, pH 5 buffer is also considering an important factor which can have a highly effects on the adsorption potentials. On other hands, yeast cell wall is derived from glucans, which can find this compound in the inner layer of the cell wall attached to the chitin compounds. The outer yeast cell wall is composed of mannoproteins which have function for cell wall communication and transfer compounds into cell. So, the adsorption potential process of different mycotoxins can easily explained by the fact that functional groups founds on the yeast cell wall get attached to some other functional groups found on the mycotoxins compounds. The glucan compound attached to the yeast cell wall has a main function for the binding which occurs between the mycotoxins and yeast cell wall. Different chemical binding forces help on the adsorption and cell wall binding like, van ser Wal forces and weak hydrophobic interaction(Jouany, Yiannikouris and Bertin, no date).

Conclusion:

In the present study, we isolated, from different food products, 11 yeast strains belonging to the following genera *Kluyveromyces marxianus, Clavispora lusitaniae, Kazachstania servazzii, Aureobasidium pullulans* and *Cutaneotrichosporon curvatus.* These strains showed very important antifungal activities due to the Volatile Organic Compounds that they produce. The VOCs released by the yeast strain QKM#4 of *Kluyveromyces marxianus*, showed a wide antifungal spectrum of antifungal activities

against seventeen fungal toxigenic reference strains. VOCs molecules were identified by GC analysis as organic compounds namely as alkane. The interesting part of present study, the strain yeast strain QKM#4, that has a great potential not only to decrease vegetative growth of toxigenic fungi, but also to inhibit fungi sporulation and synthesis of OTA toxins. This yeast strain is safe. Its VOCs can be considered as biological control agents active against mycotoxigenic fungi. This might be considered as one of the best and the safest approach as in comparison to the chemical and physical methods. On the other hand, this project also focused on the adsorption efficacy of two mycotoxins OTA and DON to an isolated yeast QKM-4. Our results confirmed that, QKM-4 yeast strain has a great adsorption potential to AFs, OTA and DON mycotoxins and this strain proved the great biocontrol agent in mycotoxin decontamination even when the contamination level of the mycotoxin increased. Same as GI tract pH 5 buffer, both living and inactive yeast cell walls were still able to decontaminate the mycotoxins in low and high concentration levels. We conclude that QKM-4 yeast strain shoes a great potential to adsorb mycotoxins *in-vitro* experiments, but more studies need to be conducted on the efficiency of the yeast cells adsorption potentials to mycotoxins *in-vivo* experiments.

CHAPTER 6: *IN-VITRO* APPLICATION OF THE YEAST *KLUYVEROMYCES MAXIANNS* QKM-4 VOCS FOR THE PROTECTION OF TOMATO AND GRAPE FRUITS FROM *F. OXYSPORUM* AND *A. CARBONARIUS* CONTAMINATION

Introduction:

Over the past few decades, laboratory and field trial experiments have achieved a significant amount of data regarding the use of beneficial microorganisms as biological control agents to control the mycotoxigenic fungi because of their direct destructive effect on agriculture and the potential of mycotoxins, which is dangerous to animal and human health (F. Abdallah *et al.*, 2019). Mycotoxigenic fungi which contaminate the food and feed post and pre-harvest pose a great concern due to their ability to synthesize mycotoxins which contaminate the harvest products.

Yeasts are considered as beneficial biocontrol agents that were already used against OTA contamination in grape and AFs contamination in tomato. Yeasts are interesting because of their capability to live in various environmental conditions, in addition to a quick development rate and no formation of lethal substances (Mwakinyali, *et al*., 2019).

In this part of the research, we proposed to explore, *in vitro,* the biocontrol potentialities of the VOCs synthesized by *Kluyveromyces marxianus QKM-4* strains, to protect tomatoes and grapes inoculated *in vitro* with these fungi.

6.2. Study of *Kluyveromyces marxianus* QKM-4 VOCs' protection potentialities of tomatoes from the infection with *Fusarium oxysporum:*

Kluyveromyces marxianus QKM-4 VOCs potentials to affect the growth and spores' germination of *Fusarium oxysporum in vitro* contaminating tomato fruits surface, was evaluated by transferring *F. oxysporum* spores suspension to the surface of five tomatoes fruits and incubating them in a closed glass box (tubberware) containing a YPDA plate containing 48 h streaked *Kluyveromyces marxianus* colonies, and incubating them at 27.5 C° for 10 days. As controls, in another glass box five tomatoes were also infected under the same conditions with *F. oxysporum* and YPDA plate but without yeast cells. After 10 days of incubation period, the boxes were opened to evaluate the shape and degree of infection of the tomatoes by the fungus in both boxes. It was noticed that the tomatoes which were exposed to *Kluyveromyces marxianus* VOCs, were 100% safe without any fungal growth and any fungal spores germination (Figure 34). In addition, the surface of the tomatoes was intact, rigid and remained fresh. However, in the control box, where five tomatoes were inoculated with the fungal spores, but without yeast, the spores germinated and the fungal mycelia covered the tomatoes surface. Figure 34 shows the difference between the VOCs exposed and non-exposed tomatoes.

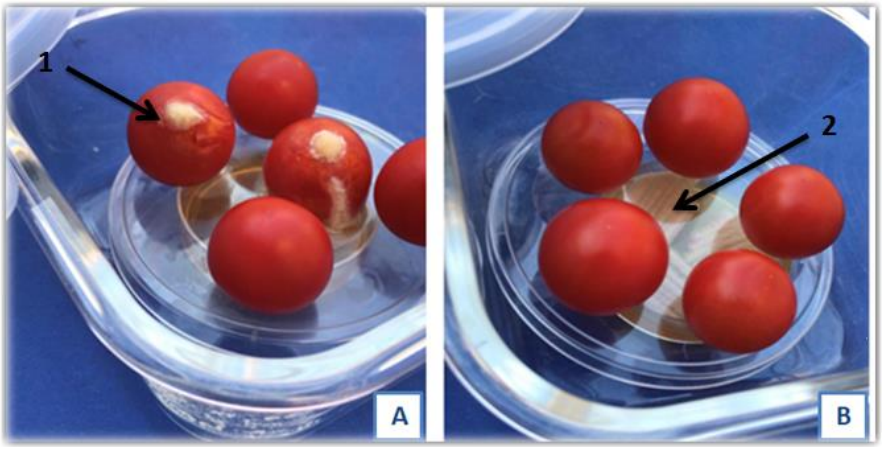

Figure 34: *In-vitro* biocontrol of *F. oxysporum* added on the surface of tomatoes by the application of *Kluyveromyces marxianus QKM#4* VOCs

[**A:** Tomatoes inoculated with *F. oxysporum* which were not exposed to yeast VOCs. **Arrow# 1** shows the surface of tomatoes fruits infected by *F. oxysporum* and covered by a mycelium], [**B:** Tomatoes infected with *F. oxysporum* and exposed to yeast's VOCs. **Arrow #2** shows the plate of YPDA streaked with yeast.

6.3. Study of *Kluyveromyces marxianus* QKM-4 VOCs' protection potentialities of grape fruits from the infection with *Aspergillus carbonarius:*

Kluyveromyces marxianus QKM-4 VOCs potentials to affect the growth and spores' germination of *Aspergillus carbonarius in vitro* contaminating tomato fruits surface, was evaluated by transferring *A. carbonarius* spores suspension to the surface of five grape fruits and incubating them in a closed glass box (tubberware) containing a YPDA plate containing 48 h streaked *Kluyveromyces marxianus* colonies, and incubating them at 27.5 C° for 10 days. As controls, in another glass box five tomatoes were also infected under the same conditions with *A. carbonarius* and YPDA plate but without yeast cells. After 10 days of incubation period, the boxes were opened to evaluate the shape and degree of infection of the tomatoes by the fungus in both boxes. It was noticed that the grape fruits which were exposed to *Kluyveromyces marxianus* VOCs, were 100% safe without any fungal growth and any fungal spores germination (Figure 35). In addition, the surface of the grape fruits was intact, rigid and grapes

remained fresh. However, in the control box, where five grape fruits were inoculated with the fungal spores, but without yeast, the spores germinated and the fungal mycelia covered the grape surface. Figure 35 shows the difference between the VOCs exposed and non-exposed tomatoes.

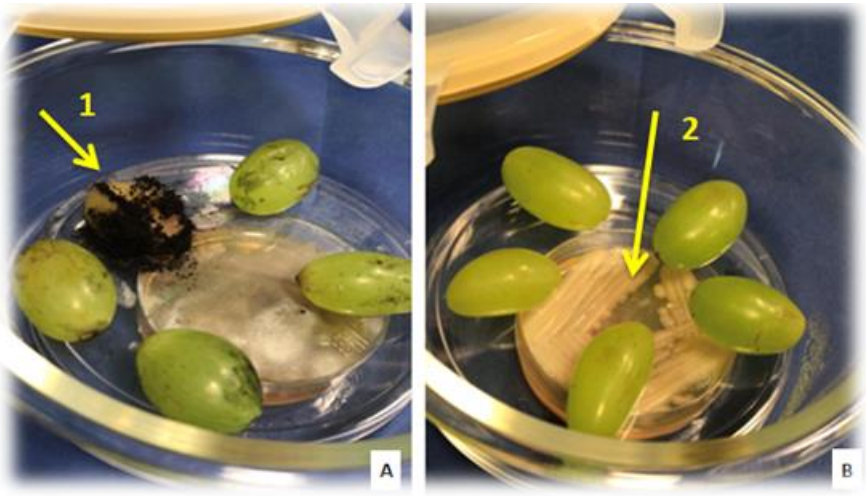

Figure 35: *In-vitro* biocontrol of *A. carbonarius* contaminating the surface of tomatoes by the application of *Kluyveromyces marxianus QKM#4* VOCs
A: Grape contaminated with *A. carbonarius* but not exposed to yeast's VOCs. **Arrow #1** shows the surface of grape fruits infected by *A. carbonarius* spores and covered by mycelium], **B:** Grapes infected by *A. carbonarius* and exposed to yeast's VOCs. **Arrow #2** shows the plate of YPDA streaked with yeast.

Discussion:

The great potentials of the VOCs synthesized by QKM-4 *Kluyveromyces marxianus* yeast strain were *in-vitro* explored against fungal contamination of *F. oxysporum* of tomato and *Aspergillus carbonarius* of grape fruits. After both fruits infection and incubation with and without yeast cells, for 10 days at 27.5C°, it was noticed that the QKM#4 VOCs inhibited the growth and germination of spores on both fruits. Both tomatoes and grapes remained solid and looking as fresh ones while in the controls, the fruits were colonized with the respective fungi and the grapes and tomatoes were damaged, ruptured and their walls were breaking and were watery from inside.

In line with present study, Zeidan *et al*., 2018, reported similar results, when they infected tomatoes with similar fungal strain and exposed to yeast VOCs, and gave a complete absence of infection .

Other yeast strains were reported as biological control agents to antagonize mycotoxigenic fungi contaminating fruits, with high impact on spores germination and fungal growth. (Luo *et al.*, 2020). On the other hands, some bacterial strains produce VOCs inhibiting *F. oxysporum* growth (Naing *et al.*, 2015).

Our results corroborate with those of Farbo *et al.*, (2018) about yeast VOCs application in the protection of grapes from *A. carbonarius* infection and those of Chen *et al.*, (2018) about yeast's VOCs application for the protection of strawberry fruits surface from *B. cinerea, .*

Conclusion:

In this part, we reported the application of the biological control potentialities of a local yeast strain QKM-4 of *Kluyveromyces marxianus* that could protect efficiently tomatoes and grapes from mycotoxigenic fungi infection.

The great potentials of the VOCs synthesized by QKM-4 *Kluyveromyces marxianus* yeast strain were *in-vitro* explored against fungal contamination of *F.*

oxysporum of tomato and *Aspergillus carbonarius* of grape fruits. After both fruits infection and incubation with and without yeast cells, for 10 days at 27.5C°, it was noticed that the QKM#4 VOCs inhibited the growth and germination of spores on both fruits. Both tomatoes and grapes remained solid and looking as fresh ones while in the controls, the fruits were colonized with the respective fungi and the grapes and tomatoes were damaged ruptured and their walls were breaking and were watery from inside.

CHAPTER 7: CONCLUSION AND FUTURE PERSPECTIVES

In this M. Sc. Thesis, we isolated and characterized toxigenic fungi from mixed dairy cow feed products, to estimate the contamination levels in animal feed products by mycotoxins and mycotoxigenic fungi. On other hands we explored the biological potentialities of 14 yeast strains locally isolated from fermented food, dairy products and meat. We evidenced the presence of OTA and AFs below the EU maximum permissible limits. Mycotoxigenic fungal strains have been isolated from the animal feeds. The morphological and molecular identification of these toxigenic fungi allowed the evidence of *A. flavus*, *A. niger*, *A. carbonarius* and *P. verrucosum* that synthesize during their growth mycotoxins compounds (Aflatoxins & Ochratoxins) at levels higher than the detection limits and the corresponding genes were evidenced.

In order to set up safe and efficient biological control strategy of these mycotoxigenic fungi, we decided to use yeasts known to be very safe for such purpose. Thus, in the second part of this Thesis, 14 yeast strains were isolated from different fermented food, dairy and meat products. These strains showed a great antifungal and spectrum of activities through the synthesis of Volatile Organic Compounds (VOCs). Among these yeast strains, a particular strain of *Kluyveromyces marxianus* QKM-4, had the strongest antifungal VOCs that we were able to identify. GC/MS based analysis of yeast VOCs showed long chain alkanes including nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36) and tetracosane (C24) that are able to control the mycotoxigenic fungal strains and their mycotoxins synthesis. Testing the mycotoxin binding potential of the live and heat-inactivated QKM-4 cells, showed the reduction of OTA and DON up to 58% and 49%. The findings of the present study clearly demonstrate a strong antifungal potential of *Kluyveromyces marxianus* QKM-4 for its possible application in the agriculture and food industry**.** In an applied part of the

present work, the strain QKM-4 of *Kluyveromyces marxianus* showed a great inhibition potential of the mycotoxigenic fungal growth, spore's germination and 100% protection of tomato and grape fruits from the *in vitro* infection of the latter by mycotoxigenic fungi fungi for more than 10 days

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APPENDIX

Appendix (A): Sequences of ITS1-IT4 of yeast strains and Blast results

QCL#2# - strain: Clavispora lusitaniae - percentage: 98%
GGCTTATTTCGTTACCTTTGCATTTGCGACAAAAGACATTACACTTCTAA TATATTTTTATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGA TGAAGAACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTGAA TCATCGAATCTTTGAACGCACATTGCGCCTCGAGGCATTCCTCGAGGCA TGCCTGTTTGAGCGTCGCATCCCCTCTAACCCCCGGTTAGGCGTTGCTCC GAAATATCAACCGCGCTGTCAAACACGTTTACAGCACGACATTTCGCCC TCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAAAAAGCG GAGGAA
QKS#3# - Strain: Kazachstania servazzii - percentage: 65%
GCCCTGGAAGTAATTTTTCGTTGCTTTGCGAGGAGACACTATACTGCTG GACCAGCGCTTAATTGCGCGGTTTGGTGGGTCTCTGTAGCTCAGTAGCA CTATTACACACAGTGGAGATTTTTATAATTCTTTGCATGCTTCTTTGGGC TGCTTCGGCGGCCCAGGAGTGACAAACACAAACAATTTTGTAATTTTAT GAACTAGTCAAAACCAGAATTCCAGGAAGATTTATCTTTTTGTAATATT AAAACAAATATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCG ATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCC GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCAGG GGGCATGCCTGTTTGAGCGTCATTTCCTTCTCAAACAGCAATGTTTGGTT GTGAGTGATACTCTTTCGGACTTAGCTTGAAAGTGCTGGACAATGGCTG ATGAGGCGGAGGGGGTTTGTGTCTTTCACACCCCGGGCCCCCAAAAGG GCCCAAGAGACCTATCTAAAA
ATTTAAAAAAACTCCCCTGTGTGTAAAAAGGGAATCGGGAGCTCCAGA CCCCCCCAAACCCGGCAAATTAACGCTGGGGCCGCACAGATATAGGGT CCCCTCGCAAAGGAACCAAAAAATTTATATTTTCTATAAGGGATCCTTC CCGAAGTTACCCTTAGGAAAGGGAGAATTC
QKM#4# - strain: Kluyveromyces marxianus- percentage: 100%
GGCCCTTGATAGATGATAGATTACTGGGGGATCGTCTGACAAGGCCTGC GCTTAATTGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCT CATCCTAAACACAATGGAGTTTTTTCTCTATGAACTACTTCCCTGGAGAG CTCGTCTCTCCAGTGGACATAAACACAAACAATATTTTGTATTATGAAA AACTATTATACTATAAAATTTAATATTCAAAACTTTCAACAACGGATCT CTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCAATATGTATTGT GAATTGCAAATTTTAGGGAATCATGAGATCTTTAAAATCAGATGGTTCC CTCAGATCTTTCAAGTGACATTGCTAGCGGAAATTCTCTGTGTTCTTTCA TCGATGCGAGAATCAAGAGATCCGTGTTGAAAAGTTTTGAAATATTAAA TTTTTATAGTATAATAGTTTTTCATAATACAAAAATATTTGTTTGTGTTTT ATGTCCCACTGGGAGAGACGAGCTCTCCCACGGGAAGTAGTTTCATAAG AGAAAAAACTTCCATTTGTGTTTAGGATGAGAAATAAAAAACTGATAGC AGAGAATCCAGGAACTGGCCCCGCCAATTAACCGGAGGGCTTGGTCC

QKS#5# -strain: Kazachstania servazzii - percentage: 28%
GCGGGACCTAGCTTATTTTCGTTGCTTTGCGAGGAGACACTATACTGCT GGACCAGCGCTTAATTGCGCGGTTGGAGAGGAAGCAATAGCCTCGTGC CTTAACGCCTCGTAAAAAGGAGGCAAAACGAGAGGATGTGTCTGGGGA CGTCCCTCACCCATAATTCTTCTCCCAAAGGGGCGTTTTCCCATATACAA AAGGAGAGGGCA
QKS#6# -strain: Kazachstania servazzii - percentage: 66%
GACCTGGAATTATTTTTCGTTGCTTTGCGAGGAGACACTATACTGCTGG ACCAGCGCTTAATTGCGCGGTTTGGTGGGTCTCTGTAGCTCAGTAGCAC TATTACACACAGTGGAGATTTTTATAATTCTTTGCATGCTTCTTTGGGCT GCTTCGGCGGCCCAGGAGTGACAAACACAAACAATTTTGTAATTTTACC CCACTAGTCAAAACCAGAATTCCAGGAAGATTTATCTTTTTGTAATATT AAAACAAATATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCG ATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCC GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCAGG GGGCATGCCTGTTTGAGCGTCATTTCCTTCTCAAACAGCAATGTTTGGTT GTGAGTGAAAATCTATCGGACTTCGCGGGAATCTGCTGGACAATAGCTG ATAAGGGCAGAATTGTTTTTGTTTGTGACACCCGGGGCGCCGAAAAGGG CCCAAAAGAAGTGCGTATAGATATAAAAATCTCCCCCTCTGTGTGAAAT AGCGACATGAGATTCACAGAACCCCCCCCAACCGCCCAAATTTAACGCT GGGGCAGGGCGATATAGGGTCTCCTCGCAAAGGAACCAAAAAATATAT ATTTCTTATAGGGATCCTTCCCAAGGGTTCCCCTACGGAGGGT
QKS#7# strain: Kazachstania servazzii - percentage: 83%
AGAACCTGAAATTATTTTTTCGTTGCTTTGCGAGGAGACACTATACTGCT GGACCAGCGCTTAATTGCGCGGTTTGGTGGGAAACTGAAGCTCAGTAGC ACTATTACACACAGTGGAGATTTTTATAAGGGGATTGCATGCTTCTTTG GGCTGCTTCGGCGGCCCAGGAGTGACAAACACAAACAATTTTGTAATTC CCTTACCAAAAGAAAACCAGAATTCCAGGAAGATTTATCTTTTTGTAAT ATGGAAGCAAATATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCA TCGCTGGAGAACGCACAGAAGGGCGATCCGTAATGTGAAATTGCCGAA GACCGGGGAATCTTCTACTCTTAGAACGCCTATGGGTTCTTCGGGGTAT TTCAGCGGGGAGGGAAGAAAAAGGATCCCATTGGCTTTAAGAAC
QAP#8# strain: Aureobasidium pullulans - percentage: 98%
GGCCCTGGGTAGGTGCTCAGCGCGACCTCCACCCTTTGTTGTTAAACTA CCTTGTTGCTTTGGCGGGACCGCTCGGTCTCGAGCCGCTGGGGATTCGT CCCAGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTAAACCG GTCGTCTGAGTTAAAATTTTGAATAAATCAAAACTTTCAACAACGGATC TCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC CCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATTACACCACTC AAGCTATGCTTGGTATTGGGTGCCGTCCTTAGTTGGGCGCGCCTTAAAG ACCTCGGCGAGGCCTCACCGGCTTTAGGCGTAGTAGAATTTATTCGAAC GTCTGTCAAAGGAGAGGACTTCTGCCGACTGAAACCTTTTATTTTTCTAG

GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAT AAAGCGGAGAA
QC#11# -Strain: Cryptococcus - percentage: 30%
AGGCCTCGCAGCCTCGTAGCCTTTACCGGCTGGTAGGGTGTTGTGCCGG TCTCTTTTGGGAGCCGGCCCAAATCTAAATAAACATAGAAACTGTGGCC TCGGGCATTTACACAAACTGCTCCTAATGACAGTAACGTCATAACTCCA CCTAAAACTTTGGGCAACGGATCCCGGGAGTCTGGAGTCTATTGATTAC TCACCCCAAGAAGCGCTTCGAAAGCCAATGGGGGAAAGGGGGGATTCA AGGATCCATGAACCCCTGCTTTCGCCATTTGGAATTCCTTAAGGGCATTC CGGTGCGTTTTTTCATCAAACCCGAAAACCAAAAGATCCTTTTGTTAAA AGTTTTATGTTTGTTTTGAAGGTTACATTCTTACAACAGTTTTGGGTGAA AAGGCCCCAAGGCCCACTTTACGGGGGGGGAGAAATTTAGGGCGGGTC CCCAAAGAAACCCGGACAACCCTAACCCCCCCGTAAAAGCGCCAGGGGG GAACATAATGGATTGTGGGGGGGGGTTCACCCTCCGGGAC
QK#13# Strain: Kazachstania sp - percentage: 41%
GGACCCCCGTCAGTAGTTTATCCGTTGCTTTGCGAGAGCACTATACTTGC TGGAACAGCGCTTAATTGCGCGGTTTGCGGGAAAAGAGAAGGAGTAGA TAAACCAACGGACTTTATTTAAGAGGAAAAAGTCCGCAGAGCCCCTTAC CCGCAGCCCTCTCCCACAGAGAATCATCATTTACGGGGGGCAGATTCAG AATTCCATAGATACTCGGCACCAAATTCCTCTCCAAAACAGTCCTCTCC ACCCGTCCCCGGGAACCCAGGCGCCTGTGTGTTTCGATCCATGAGTCCG CAATTCTCCAATCTCCTTTCTATCGCTTTCTCGCGTCTTCATGATGCGAG ACCAAGAGATCCCTGTTGAAAGTTTAATATTGTTTTATATTACAAAAGA TAATCCTCCTTGATTCCTGGGTTTGCCATTCCTAAGATAAGGAGGTTGGT TGGGTTCGCCATTCCGG
QCL#14# Strain: Clavispora lusitaniae - percentage: 96%
GGCCCTAGTCGTTCCTTTGCATTTGCGACAAAAGACATTACACTTCTAAT ATATTTTTATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGAT GAAGAACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTGAAT CATCGAATCTTTGAACGCACATTGCGCCTCGAGGCATTCCTCGAGGCAT GCCTGTTTGAGCGTCGCATCCCCTCTAACCCCCGGTTAGGCGTTGCTCCG AAATATCAACCGCGCTGTCAAACACGTTTACAGCACGACATTTCGCCCT CAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGA GGAA
QCC#15# Strain: *Cutaneotrichosporon curvatus* - *percentage:* 78%
GGTAGGGATGCTTCGGGGCTACTATATCCATAACACCTGTGACTGTTGA TTGACTTCGGTCAATATTTTTACAAACATTGTGTAATGAACGTCATGTTA TAATAACAAATATAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGA TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG TGAATCATCGAATCTTTGAACGCAACTTGCGCTCTCTGGTATTCCGGAG AGCATGCCTGTTTGAGTGTCATGAAATCTCAACCATTAGGGTTTCTTAAT GGCTTGGATTTGGACGTTTGCCAGTCAAATGGCTCGTCTTAAAAGAGTT

AGTGAATTTAACATTTGTCTTCTGGCGTAATAAGTTTCGCTGGGCTGATA
GTGTGAAGTTTGCTTCTAATCGTCCGCAAGGACAATTCTTGAACTCTGG
CCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCATAAACCC
GGAGAGGAAAAGGGTACCCACTTCCCTCCGGCTACTGGTGGGGGGGGG
GGGGGGGGGTGTTTTATTACTTCGGGCAGTATTTTTTCAAAAATTGTTTT
AATGACTTATGTTACCAT