

## MYCOFLORA STUDY IN A WHEAT FLOUR MILL OF ARGENTINA

Aringoli, E.E.\*; Cambiagno, D.E.; Chiericatti, C.A.; Basilico, J.C.; M.L.Z. Basilico

Cátedra de Microbiología, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829 (3000)  
Santa Fe, Argentina.

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### ABSTRACT

The mycoflora of the environment: wheat conditioning, milling and screening, and filling zone, as well as, raw material -wheat-, intermediate product -grits- and end product -flour- on day 1, and after cleaning improvements -days 45 and 90- were studied in an Argentine wheat mill. Samples were incubated at 28°C for 5-7 days on Malt Extract Agar with chloramphenicol (100 mg L<sup>-1</sup>) and the results were expressed in colony forming units per cubic meter of air (CFU m<sup>-3</sup>) or per gram of sample (CFU g<sup>-1</sup>), respectively. Fungal genera and species were isolated and identified and the potential toxicogenic capacity of the *Aspergillus flavus* and *Fusarium graminearum* isolated was studied. Time-Place and Time-Product multifactorial ANOVA were carried out. After cleaning improvements, CFU m<sup>-3</sup> of air decreased as a function of time. *Cladosporium* and *Alternaria* were abundant in every zone, *Aspergillus* predominated in the wheat conditioning zone and *Penicillium* and *Eurotium* decreased with time. Wheat was more contaminated than grits and flour; *Aspergillus*, *Eurotium* and *Mucoraceae* family were the most abundant. Deoxynivalenol was above the levels allowed in wheat, being acceptable in grits and flour. Aflatoxin and Zearalenone showed acceptable levels. When studied in vitro, 53% of *Aspergillus flavus* and 100% of *Fusarium graminearum* isolates, produced Total Aflatoxins, and Deoxynivalenol and Zearalenone, respectively.

**Key words:** mycoflora, environment, wheat mill.

### INTRODUCTION

In 2009, wheat production in Argentina was 13 million tonnes, 5 million tonnes of flour being produced in the 146 mills found in the country. (28).

During the process in the flour mills, bioaerosols are formed from particles of biological origin or with biological activity which can affect human beings because of processes of

infectivity, allergenicity, toxicity or others (22). Bioaerosols include molds and their toxic metabolites, air being an excellent vehicle for their dispersion (2, 12).

The Food and Agriculture Organization (18) reported that approximately 25% of the cereal-based foods produced in the world are contaminated with mycotoxins, which are secondary metabolites produced by filamentous molds causing diseases in vertebrates when ingested, inhaled or skin absorbed (19).

\*Corresponding Author. Mailing address: Cátedra de Microbiología, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829 (3000) Santa Fe, Argentina.; Tel.: 54 342 4571164 int. 2541 FAX:54 342 571162.; E-mail: [elaringo@fiq.unl.edu.ar](mailto:elaringo@fiq.unl.edu.ar)

Molds, on the other hand, are associated with human allergic diseases resulting from the exposition to spores, vegetative cells or metabolites characteristic of these microorganisms (17, 23, 27).

The basic and unquestionable aspects that an agro-food industry producing innocuous foods should achieve are those of the hygiene-sanitation of the production environment and the raw material. Therefore, the evaluation of the environmental fungal contamination is an important starting point in those cereal industrialization plants, in order to ensure healthy working conditions and the quality of raw material (3, 14, 30, 37, 39). Although several wheat studies have been carried out during harvests in Argentina (6, 13, 15, 21, 33, 34), scarce studies have been reported concerning the milling process (31).

The aim of this work was to determine fungal contamination in the environment air and in the raw material, as well as that resulting from the flour production in a wheat mill, as a function of the cleaning improvements carried out and wheat quality; the focus being on molds, since they represent the highest toxicological risk.

## MATERIALS AND METHODS

### Sampling plan

Sampling was carried out in summer, in an Argentine wheat flour mill located at the heart of the central plain -pampa húmeda-, the wheat-producing region of the country. The mill, with the latest technology equipment which produces milling products with appropriate moisture contents, has a storage capacity of 37000 tonnes and a flour production capacity of 150 tonnes day<sup>-1</sup>.

On day 1, air sampling was carried out in cuadruplicate in three of the zones considered more contaminated: Wheat Conditioning -WC-, Milling and Screening -MS- and flour Filling Zone -FZ-. Based on the results obtained in this case, cleaning system improvements were implemented, repeating the sampling at 45 and 90 days. Solid samples were also taken

at the entrance of the Wheat Conditioning zone -wheat-, the Milling and Screening zone -grits- and the Filling Zone -flour-. During sampling, the environmental conditions of temperature -°C- and relative humidity -RH %- were recorded with a psychrometer, using the wet bulb method.

### Air sampling

An equipment -Standard RCS sampler -Biotest Diagnostic Corp., Denville, N.J., USA- based on the sampling of a known volume of air impacting on multiwell plates was used in this case. Malt Extract Agar -MEA- with the addition of chloramphenicol (0.1 g L<sup>-1</sup>) was used to prevent the bacterial growth, and dichloran 0.2 % (w/v) to inhibit rapid-growth molds (5, 35, 41, 43). Then, the multiwell plates were carried to the laboratory and incubated for 3-5 days at 27 °C (32). The equipment was cleaned with isopropyl alcohol before each sampling (5).

### Solid sampling

Samples of about 1 kg of wheat -W-, grits -G- and flour -F- ready to be filled were taken from zones WC, MS and FZ, respectively, and put in sterile bags. Once in the laboratory, they were subsampled under sterile conditions, the final 100 g of samples being ground in a cutting mill. An aliquot -10 g- taken from each sample was homogenized with 90 mL of 0.1 % (w/v) peptone water. Serial dilutions were made up to the 10<sup>-5</sup> dilution, in tubes containing 9 mL of the same diluent. After that, 1 g of each sample and 1 mL of each dilution was plated onto Petri dishes containing MEA, with the addition of chloramphenicol and dichloran (6, 10). This step was carried out in duplicate. At last the plates were incubated at 27 °C for 5 days for their subsequent analysis (32).

### Count, isolation and identification of the fungal flora

The count of the colonies grown on the multiwell plates was performed and the results were expressed as colony forming units per cubic metre of air (CFU m<sup>-3</sup>), taking into account factors such as sampling time and equipment factor.

Solid samples counts were expressed as colony forming units per gram (CFU g<sup>-1</sup>).

Colonies grown were subjected to a previous macroscopic and microscopic observation so as to determine the distinctive characteristics of each genus. They were then recultivated in MEA until pure colonies were obtained, which were then cultivated in different media and culture conditions for the identification of species (10, 20, 29, 32, 36). Non-sporulated colonies were streaked to plates with Spezieller Nährstoffarmer Agar -SNA- medium and incubated under the same conditions to allow for their sporulation (5).

#### **Mycotoxin determination in solid samples**

Total Aflatoxins, B and G groups -AF-, Deoxynivalenol -DON- and Zearalenone -ZEA- were determined in solid samples using Enzyme-Linked ImmunoSorbent Assay -ELISA-, recommended for analysis of cereals and derivatives -RIDASCREEN<sup>®</sup>FAST - R-Biopharm AG, Darmstadt, Germany-. Concentrations were measured photometrically at 450 nm with a STAT Fax 321/PLUS reader -Awareness Tech., Inc<sup>®</sup>, Palm city, USA.-. Quantification limits were 1.7 µg kg<sup>-1</sup> for AF, 200 µg kg<sup>-1</sup> for DON and 50 µg kg<sup>-1</sup> for ZEA, (38).

#### ***Aspergillus flavus* and *Fusarium graminearum* toxicogenic capacity**

Isolates of *A. flavus* and *F. graminearum* were recultivated on Oat Agar -OA- and Potato Dextrose Agar -PDA-, respectively (36) at 27 °C for 10-15 days to determine their toxicogenic capacity according to the slightly modified (16) method. It consists in growing each strain under the above-mentioned culture conditions, punching out a 1 cm centre of the colony and placing it into a hemodialysis tube with 500 µl of extractant solvent according to ELISA technique -RIDASCREEN<sup>®</sup>FAST - R-Biopharm AG, Darmstadt, Germany-. The production capacity of AF, DON and ZEA were then determined.

#### **Treatment of experimental data**

Fungal contamination expressed as CFU m<sup>-3</sup> and CFU g<sup>-1</sup>

were transformed to decimal log to perform the Multifactorial ANOVA tests: Time -1, 45 and 90 days-, Zone -WC, MS and FZ- for the air samples; and Time -1, 45 and 90 days-, Samples -W, G and F- for the solid samples (4). Abundance -n° CFU Genus / n° Total CFU x 100) was determined in air and solid samples. Excel and Statgraphics Version 08 3.0 were used as informatic tools.

## **RESULTS AND DISCUSSION**

Table 1 shows the values of environmental fungal count in the different zones -WC, MS and FZ- as a function of time -1, 45 and 90 days- and Table 2 the (Time-Zone) Multifactorial ANOVA values, which showed significant differences (p-Value < 0.05) in Time and Zone for the Total Count.

The significant differences found as a function of time show the improvements in the cleaning conditions adopted; being necessary to point out that solid residue on floor and machines could be observed during the first sampling. As regards the Zone, a higher production of fungal bioaerosols appeared on FZ for the days under study, originated in the flour screening process and the hopper discharge for subsequent fractionation and filling. Although no data on environmental pollution on wheat mills have so far been reported, the levels of counts here encountered would be in agreement with those found by Adhikari *et al.* (2) for environments of agricultural product storage. As for the genera identified, *Cladosporium* and *Alternaria* were the most abundant ones -Table 1-, no significant differences being shown for Time and Zone in the ANOVA study, which agrees with studies made in house environments in the region (4, 5). On the other hand, genus *Aspergillus*, was more abundant in WC, whereas *Penicillium* and *Eurotium* decrease in function of cleaning, like other authors state (2, 7). *Cladosporium* and *Alternaria* genus are considered allergenic, and some *Alternaria* species are potential mycotoxins producers. *Epicoccum* can also cause allergenic and antigenic cross-reactivity, thus enhancing those processes (8), and this genus was isolated mainly from the MS zone. The main species with those characteristics

were *Cladosporium cladosporioides*, *Alternaria alternata*, *fumigatus* and 3% to *A. clavatus* respectively; and isolates of *Epicoccum nigrum*; isolates of *Aspergillus*, of which 80% corresponded to *A. flavus*, 12% to *A. terreus*, 5% to *A. fumigatus*, of which 58% corresponded to *F. graminearum*, 30% to *F. equiseti* and 12% to *F. oxysporum*.

**Table 1.** Environmental fungal counts in CFU m<sup>-3</sup> of air for Time (day 1, 45, 90) and Zone (WC: wheat conditioning, MS: milling and screening, FZ: filling zone).

Genus (species)*	Fungal Counts (CFU m <sup>-3</sup> )								
	day 1			day 45			day 90		
	WC	MS	FZ	WC	MS	FZ	WC	MS	FZ
Totals	9400	16400	24000	11000	8800	12200	5200	8400	10200
<i>Cladosporium</i>	2200	5400	5800	3400	2000	2200	2600	1800	1800
<i>Alternaria</i>	1400	3200	1600	3800	3800	2800	1000	3000	2600
<i>Aspergillus</i> *	3000	200	2000	200	ND	3600	ND	800	3000
( <i>A. candidus</i> )	(ND) <sup>†</sup>	(36)	(ND)	(128)	(ND)	(120)	(ND)	(12)	(240)
( <i>A. clavatus</i> )	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
( <i>A. flavus</i> )	(3000)	(164)	(2000)	(ND)	(ND)	(3240)	(ND)	(788)	(2760)
( <i>A. fumigatus</i> )	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
( <i>A. terreus</i> )	(ND)	(ND)	(ND)	(ND)	(ND)	(180)	(ND)	(ND)	(ND)
<i>Penicillium</i>	1000	1800	8200	400	ND	1200	200	ND	ND
<i>Eurotium</i>	1600	2400	400	2000	600	1600	400	ND	ND
<i>Epicoccum</i>	ND	2000	ND	200	1400	400	400	2000	800
<i>Fusarium</i>	ND	ND	800	600	600	200	ND	ND	ND
( <i>F. equiseti</i> )	(ND)	(ND)	(80)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
( <i>F. graminearum</i> )	(ND)	(ND)	(720)	(576)	(600)	(200)	(ND)	(ND)	(ND)
( <i>F. oxysporum</i> )	(ND)	(ND)	(ND)	(24)	(ND)	(ND)	(ND)	(ND)	(ND)
<i>Absidia</i>	ND	ND	ND	ND	ND	ND	ND	ND	48
<i>Mucor</i>	ND	ND	ND	ND	ND	ND	ND	ND	12
<i>Rhizopus</i>	200	400	400	400	ND	ND	ND	ND	1740
Yeasts**	ND	200	3800	ND	ND	ND	600	ND	ND
Others****	ND	800	1000	ND	400	200	ND	800	200

\*Potential toxicogenic species

\*\* not genus were determined

\*\*\*Genus: *Stemphylium*, *Aureobasidium*, *Moniliella*, *Trichoderma* and sterile mycelia

†ND: Non detected. Detection Limit: 12 CFU m<sup>-3</sup>

day 1: pre-cleaning system improvements, day 45 and day 90: controls of cleaning system improvements

**Table 2.** Multifactorial ANOVA of environmental fungal genus for Time (day 1, 45, 90) and Zone (wheat conditioning, milling and screening, and filling zone)

	Multifactorial ANOVA			
	Time		Zone	
	F-Ratio	p-Value*	F-Ratio	p-Value*
Total log	19.26	0.0000	13.31	0.0000
log <i>Cladosporium</i>	3.12	0.0580	0.39	0.6788
log <i>Alternaria</i>	2.53	0.0959	2.95	0.0670
log <i>Aspergillus</i>	0.43	0.6512	10.11	0.0004
log <i>Penicillium</i>	21.17	0.0000	2.84	0.0739
log <i>Eurotium</i>	8.60	0.0110	2.02	0.1494
log <i>Epicoccum</i>	1.88	0.1700	10.76	0.0003
log <i>Fusarium</i>	3.44	0.0446	0.19	0.8275
log <i>Rhizopus</i>	0.96	0.3939	2.12	0.1373
log Yeasts**	4.42	0.0204	2.06	0.1440
log Others***	0.28	0.7591	4.23	0.0237

\*p<0.05

\*\* not genus were determined.

\*\*\*Genus: *Stemphylium*, *Aureobasidium*, *Moniliella*, *Trichoderma*, *Absidia*, *Mucor* and sterile mycelia.

Table 3 shows total fungal counts for the products under study -W, IP and F- as a function of time -1, 45 and 90 days-. Fungal contamination can be seen to decrease with milling process progress, wheat showing the higher variability in contamination depending on the lots considered (6).

**Table 3.** Products (W: wheat, G: grits, F: flour) fungal counts in CFU g<sup>-1</sup> for Time (day 1, 45 and 90)

Genus (species)*	Fungal Counts (CFU g <sup>-1</sup> )								
	day 1			day 45			day 90		
	W	G	F	W	G	F	W	G	F
Totals	135	110	140	270	60	95	360	170	145
<i>Cladosporium</i>	ND <sup>†</sup>	ND	ND	75	ND	20	ND	5	ND
<i>Alternaria</i>	ND	ND	ND	40	ND	35	ND	ND	ND
<i>Aspergillus</i>	15	65	105	25	35	5	15	90	105
( <i>A. candidus</i> )	(15)	(65)	(105)	(25)	(30)	(ND)	(14)	(89)	(100)
( <i>A. clavatus</i> )	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
( <i>A. flavus</i> )	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
( <i>A. sydowii</i> )	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(2)	(4)
<i>Penicillium</i>	5	5	15	ND	20	20	ND	5	25
<i>Eurotium</i>	110	30	10	100	5	ND	20	55	15
<i>Epicoccum</i>	ND	5	ND	20	ND	ND	ND	ND	ND
<i>Fusarium</i>	ND	ND	ND	10	ND	ND	ND	ND	ND
( <i>F. graminearum</i> )	(ND)	(ND)	(ND)	(9)	(ND)	(ND)	(ND)	(ND)	(ND)
( <i>F. oxysporum</i> )	(ND)	(ND)	(ND)	(1)	(ND)	(ND)	(ND)	(ND)	(ND)
<i>Absidia</i>	1	2	ND	ND	ND	ND	20	ND	ND
<i>Mucor</i>	ND	ND	ND	ND	ND	ND	5	ND	ND
<i>Rhizopus</i>	4	2	ND	ND	ND	ND	300	15	ND
<i>Eupenicillium</i>	ND	ND	10	ND	ND	ND	ND	ND	ND
<i>Emericella</i>	ND	ND	ND	ND	ND	5	ND	ND	ND
SM**	ND	ND	ND	ND	ND	10	ND	ND	ND
Yeasts***	60	20	ND	15	5	ND	15	ND	ND

\*Potencial toxicogenic species

\*\* Sterile mycelia

\*\*\* not genus were determined

<sup>†</sup>ND: Non detected. Detection Limit: 1 CFU g<sup>-1</sup>

day 1: pre-cleaning system improvements, day 45 and day 90: controls of cleaning system improvements

Values of Time-Product Multifactorial ANOVA were significant only for the log<sub>10</sub> of total counts as a function of Products (F-Ratio, 4.04 and p-Value, 0.043); the highest counts being found for W, in agreement with Berghofer *et al.* (7). After leaving the silos, the grain undergoes a process of control and cleaning which includes sieving before entering the WC zone, the same authors (7) demonstrated that the microbial load in the flour depended directly on the wheat microbial quality and that, as grain processing advances, the fungal contamination decreases in the final product -grits and flour-.

A low fungal contamination was found in all W samples studied, although Kozak *et al.* (25) showed that fungal counts below the detection limit, based on viable count methods, can

be even high enough to cause breathing problems. Genera *Aspergillus* and *Eurotium* were present during the three days of study, while *Mucoraceae* family, which includes principally isolates of the genera *Rhizopus*, were the most abundant in W at day 90.

Although wheat is a susceptible cereal to *Fusarium* species in Argentina among other countries (6, 13, 15, 21, 31,33, 34) low counts were observed in the present study, probably due to the good climatic conditions of the year, in storage and production quality of the environment -average RH 62 %- . Unlike the environmental counts, genera *Cladosporium* and *Alternaria* showed low figures, this would confirm that both genera would constitute an ordinary environmental flora.

Isolates of genera *Aspergillus*, 62% corresponded to *A. candidus*, 23% to *A. flavus*, 8% to *A. sydowi*, 5% to *A. fumigatus* and 2% to *A. clavatus*. Genus *Fusarium* was only isolated from W in low count at day 45, -*F. graminearum* 60% and *F. oxysporum* 30%-. Both genera and species identified are in agreement with data reported by other authors (6, 7, 32,36) as regards abundance in cereals. Likewise, the same authors' report that most genera and species identified can grow with a relatively low water activity.

*Fusarium* species can produce plenty of bioactive metabolites, group B trichotecenes among them (1, 24), being DON the main wheat contaminant in Argentina (6, 13, 15, 21, 33,). DON occurrence is related with the presence of *F. graminearum*, also a good producer of another type of toxins of ZEA family. When analyzing the toxicogenic capacity of the *A. flavus* isolates, 53 % were found capable of producing total aflatoxins, whereas 100 % of the *F. graminearum* isolates turned out to be DON and ZEA producers under the working

conditions.

Table 4 shows the values of mycotoxins considered in the solid samples. Despite *A. flavus* isolate, AF levels in wheat were lower than the quantification level, similar results being found for ZEA. Neither toxin was detected in the other products studied, which is in agreement with Berghofer *et al.* (7), probably because during milling the outer layer of wheat is removed, thus dragging the fungal load and decreasing the possible levels of mycotoxins to be detected in the end product (26). Aflatoxins contamination was shown to be of higher importance in other cereals and oilseeds than in wheat (40).

DON levels, conversely, overcame the maximum limits recommended for wheat -1250  $\mu\text{g kg}^{-1}$ - and, although the levels decreased in G and F, figures close to those allowed were detected in flour -750  $\mu\text{g kg}^{-1}$ - (11). Although the presence of these mycotoxins has been studied in different foods, little is known regarding the occupational exposure to them and the effects on the workers' health (14, 42).

**Table 4.** Aflatoxins (AF), Deoxynivalenol (DON) and Zearalenone (ZEA) by immunoenzimatic methods (ELISA) in products (W: wheat, G: grits, F: flour)

Time	Mycotoxin Determination ( $\mu\text{g kg}^{-1}$ ) <sup>(a)</sup>								
	W			G			F		
	AF	DON	ZEA	AF	DON	ZEA	AF	DON	ZEA
day 1	ND <sup>(1)</sup>	ND	ND	ND	100	ND	ND	ND	ND
day 45	ND	3600	ND	ND	800	ND	ND	700	ND
day 90	1.7	1800	ND	ND	700	ND	ND	700	ND

<sup>(1)</sup> ND: Non Detected

<sup>(a)</sup> Quantification limits for total aflatoxins, deoxynivalenol and zearalenone were 1.7  $\mu\text{g kg}^{-1}$ , 200 1.7  $\mu\text{g kg}^{-1}$  and 50 1.7  $\mu\text{g kg}^{-1}$  respectively.

A decrease in the fungal load was observed after cleaning conditions in the mill were improved as suggested, both in the environment and in the machinery used, as was shown in bakeries (9). Grain quality is important not only for the workers at their worksite but for the end product to be obtained. There exists an environmental flora, intrinsic to the mill itself, as well as another depending on the fungal load, which comes from the raw material being processed. Many allergenic and toxicogenic molds were detected, both in the environment and in the solid products. DON values in wheat

decreased throughout the processing line, attaining allowed levels in the ready-to-use flour. However, cleaning conditions should be kept and controls in the wheat entering the plant and the mill environment should be done in order to adopt measures that ensure healthy conditions for the plant workers and high quality in the end product.

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