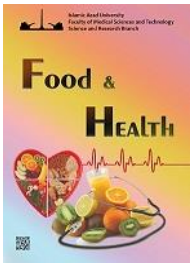

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Evaluating the relationship of fungal contamination and *ochratoxin* A content in nonalcoholic beers from different climatic regions

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by some species of fungi such as *Aspergillus* and *Penicillium*. It is found as a contaminant in a variety of animal and human foods. *Ochratoxin* A has teratogenic, hepatotoxic, nephrotoxic, nephrocarcinogenic and immunosuppressive effects on human and animals. This study was carried out to evaluate the content of *ochratoxin* A in non-alcoholic beers, which were randomly collected from different retail outlets. All samples were analyzed for *ochratoxin* A by ELISA. Identification of fungal isolates was based on both macroscopic characters (colony growth, colony diameter) and microscopic characters. The tease Mount technique was used in this study and the fungi were cultivated in YGC (yeast glucose chloramphenicol agar) medium. All of the samples were contaminated with *ochratoxin* A but the levels of the contamination were below the maximum permitted levels. However, the difference between local and imported beer samples was not statistically significant ($p>0.05$). The mycological survey showed that 100% of domestic and imported beer samples were contaminated with *Aspergillus*, while 31.4% of the domestic and 40% of the imported beer samples were contaminated with *Penicillium*. Among the *Aspergillus* species, the most representative specie was *Aspergillus niger*. Although the *ochratoxin* A concentrations of non-alcoholic beers were under the European maximum permitted levels, the long-term continual consumption may have considerable health problem despite the low levels of contamination.

1. Introduction

Ochratoxin A is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties (1), which has received growing interest from the scientific community and food committees in recent years. Sufficient evidence has been gathered to suggest the potential kidney carcinogenicity of OTA on humans (2, 3). The tolerable intakes have also been estimated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Canadian and European Commission at 100 ng/kg bw/week, 1.5~5.7ng/kg bw/day and not more than 5 ng/kg bw/day, respectively. However, many countries have not determined any regulatory limits for any commodities for OTA contamination (4). OTA can induce DNA damage, DNA repair and chromosomal aberrations in mammalian in vitro and DNA damage and chromosomal aberrations in mice treated in vivo (5, 6). This mycotoxin can cross the placenta and is embryotoxic and teratogenic in rats and mice (7). It also can inhibit the proliferation of B and T lymphocytes and affected the late stages of T-lymphocyte activation in vitro (8).

OTA has been suspected as a possible cause of a chronic kidney disease in South Eastern Europe known as "Balkan endemic nephropathy (BEN)", and urinary tract tumors (UTT) (9). OTA is nephrotoxic to all animal species studied so far and most likely to humans, who show the largest half-life time for elimination of this toxin amongst all species examined. OTA has been found in human blood serum (3), in human milk

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(10) and in a wide range of commodities, including cereal, coffee, pork and poultry, meat, pulses, beer, wine, and grape juice (11). Some studies had shown that OTA contamination was mainly associated with storage; therefore, suitable postharvest conditions such as temperature and moisture are important in preventing the growth of fungi and the production of the mycotoxin (12).

The only reported species capable of producing OTA belong to the genera *Aspergillus* and *Penicillium*. Some species of black aspergilla (*Aspergillus niger* group, *Aspergillus* section nigri, (13) have been described as capable of producing OTA (14, 15). Since *Aspergillus* and *Penicillium* species grow well on a variety of substrates and under different conditions of moisture, pH, and temperature, the natural occurrence of OTA in human food and animal feed is widespread. OTA production by *Aspergillus niger* was demonstrated for the first time in 1994 (14) and reviewed in 2001 (16), after several studies on these fungi.

The worldwide occurrence of *ochratoxin* A (OTA) contamination in raw and processed agricultural products has been amply documented (11, 17-21). It has been frequently found in beverages such as beer, wine, and grape juice (22), and predominantly found in cereals and derived products, which readily transferred into the beer from contaminated grain. The occurrence of *ochratoxin* A (OTA) in beer has been highly studied. Researchers conducted in various countries have shown that OTA is a common contaminant of beer (13, 23-25). The results of some earlier studies on OTA contamination in beer samples from various countries are listed in Table 1 (1). It is generally assumed that the mycotoxin problem is more serious in developing countries where the climatic conditions and compare it with the level in those nonalcoholic beers imported from European countries.

2. Materials and methods

2.1. Sample collection

The number of collected samples for the study is based on the following statistical equation depending on the largest average variation in previous studies' value and standard deviation. 70 commercial beer samples (35 domestic and 35 imported samples) were randomly gathered from different retail outlets. The domestic beer samples were the products of 4 factories while the imported samples from 3 different factories, in various production periods.

2.2. Preparation of samples

Each bottled beer sample was gently shaken and approximately 100 ml was degassed by ultrasonication. Analytical grade of sodium hydrogen carbonate ($NaHCO_3$), hydrochloric acid (HCL) and dichloromethane were obtained from Merck. *Ochratoxin* A levels were determined by a commercially available *ochratoxin* A ELISA kit (RIDASCREEN), R-Biopharm AG, Darmstadt, Germany). In order to determine *ochratoxin* A by ELISA, 2.5 mL of 1 N HCl solution was added to 2 ml of each sample and shaken. The sample mixture was then extracted with dichloromethane. Following centrifugation at 3500g, for 15min, dichloromethane phase was collected and mixed with an equal volume of 0.13 M $NaHCO_3$ buffer pH 8.1. Then the removed aqueous phase was diluted with $NaHCO_3$ solution (pH=8.1) and used for the ochratoxin A determination.

2.3. ELISA determination of ochratoxin A

All samples were analyzed for OTA using 96-well RIDASCREEN OTA ELISA test kits (R-Biopharm from Germany) measured at 450 nm by a microplate reader (Sunrise, GmbH, Tecan, Austria). Ochratoxin A contamination in each sample was expressed as ng/ L. The detection limit of *ochratoxin* A for the technique was 25 ng/ L and the recovery rate was more than 85%.

2.4. Identification of fungal isolates

Tease Mount technique (26) was used for the identification of the fungal isolates which is based on both macroscopic (colony growth, colony diameter) and microscopic characters. This procedure is the most common and quickest technique used to mount fungi for microscopic examination. Briefly, a drop of lactophenol was placed on a clean microscope slide. With a long-handled inoculating needle, a small portion of growth midway between the colony canter and the edge was gently removed and placed in the lactophenol. With two dissecting needles, the fungus was gently teased apart so that it is thinly spread out in the lactophenol. A coverslip at the edge of the lactophenol is slowly placed without trapping any air bubbles under the coverslip. Excess lactophenol was removed from the edges of the coverslip by blotting with a paper towel. The edges of the coverslip were sealed with fingernail polish to preserve the mount. The cultivation medium YGC (yeast glucose chloramphenicol agar) was used in order to study the amount of fungal contamination.

2.5. Statistical analysis

Descriptive analyses were performed to describe the proportion of *ochratoxin* A contamination in local and imported samples. The independent t-test was used to compare the mean contamination of *ochratoxin* A between the local and imported samples. One-sample t-test was used for comparing the mean contamination of *ochratoxin* A with permitted values, and analysis of variance (ANOVA), was performed to compare the mean of *ochratoxin* A contamination between different brands. Test results are statistically significant at $p<0.05$.

Table 1. OTA contamination in beer samples from various countries (adapted from Cramer et al. 2010)

Origin of beer samples	No. of samples	Positive (%)	Mean (ng/l)	Range (ng/l)	Method	Researchers
Germany	161	63	31	20-330	LC+IAC	Meyer & Neugebauer (2000)
Belgium	15	40	49	1-135	LC+IAC	Visconti *et al.* (2000)
South African traditional beers	29	45	-	3-2340	LC	Odhav and Naicker (2002)
Belgium	62	97	33	10-185	LC+IAC	Tangni *et al.* (2002)
Beers from Turkish market	150	28	-	100-8100	ELISA	Gumus *et al.* (2004)
Japan	20	65	11	-	LC +IAC	Osamu (2005)
Korea	46	4	250	<100-300	LC+IAC	Park *et al.* (2005)
Spain	31	77	44	12-205	LC+IAC	Araguas *et al.* (2005)
Spain	31	84	36	<5-147	LC	Medina *et al.* (2005)
Beers from the Spanish market	69	100	70	8-498	LC+SPE	Medina *et al.* (2006)
Czech Republic	115	40	-	1-243.8	UPLC	Beˇlakova *et al.* (2011)
Italy	30	17	-	-	Clean-up	Prelle *et al.* (2013)

3. Results

3.1. Determination of ochratoxin A

All beer samples were contaminated by *ochratoxin* A. The mean and range of OTA contamination in local beers were higher than the imported ones. The mean contamination of domestic beer samples was 96.04±42.68 ng/l with a range between 0.50-524.50 ng/l, and the mean of OTA contamination in imported beer samples was 60.71±47.82 ng/l with a range between 0.90-228.60 ng/l. The difference between local and imported beer samples was not statistically significant ($p>0.05$). In addition, no significant difference in the OTA contamination between brands was observed. Higher OTA levels than the mean were observed in 25.7% of local and 40% of imported products. As illustrated in Fig.1, all of the imported beers contamination falls below 250 ng/l, whereas 88.6% of domestic product contaminations were below 250 ng/l while the contamination in the other 11.42% reached to 550 ng/l. In the range of 0-150 ng/l, the OTA content of domestic beer products was lower compared to the imported ones, however, imported beer products showed lower OTA concentration with less standard deviation as compared to domestic ones (Fig.2).

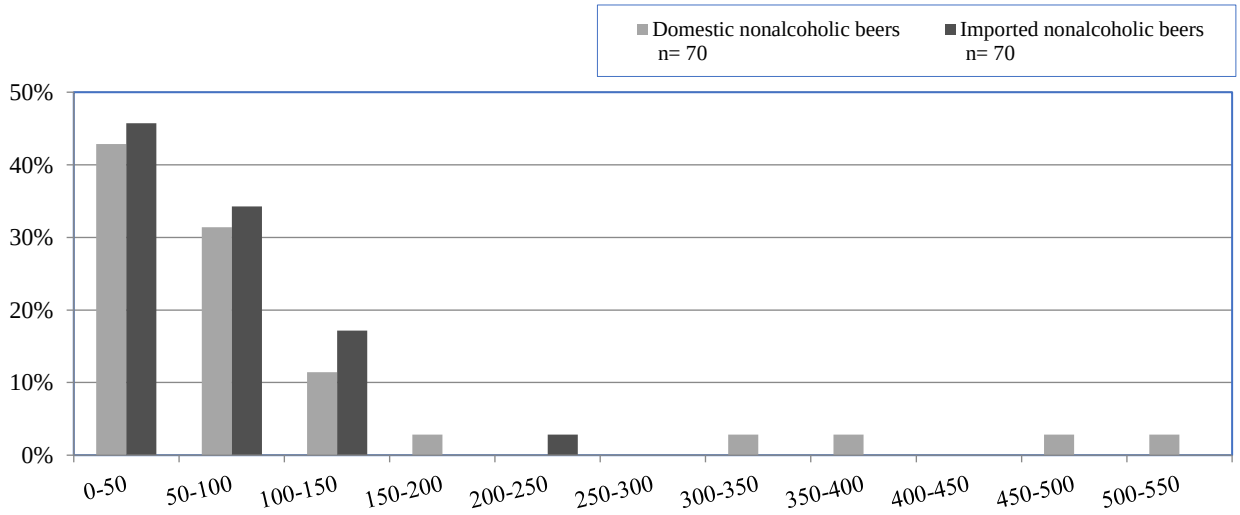

Fig. 1. The percentage of *ochratoxin* A contamination (ng/L) in domestic & imported products.

3.2. *Identification of fungal isolates*

The mycological survey showed that *Aspergillus niger* was responsible for the majority of contamination in both domestic and imported beer samples (Fig.3 and Fig.4). However, *Penicillium* contamination is seen in 31.4% of the domestic beers and 40% of the imported beer samples. Other fungi contaminations were also observed but at minimal levels.

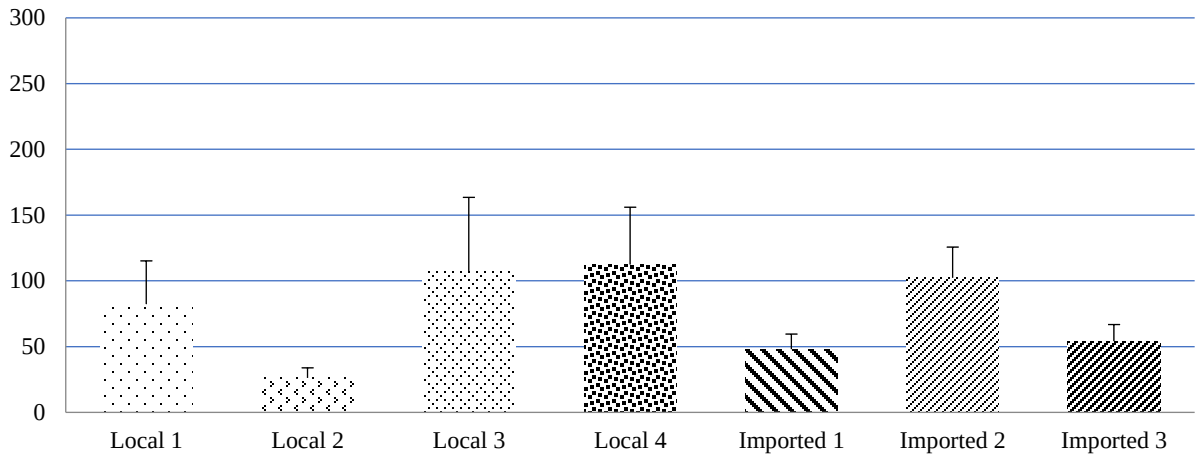

Fig. 2. The mean of *ochratoxin* A concentration (ng/L) in local & imported samples.

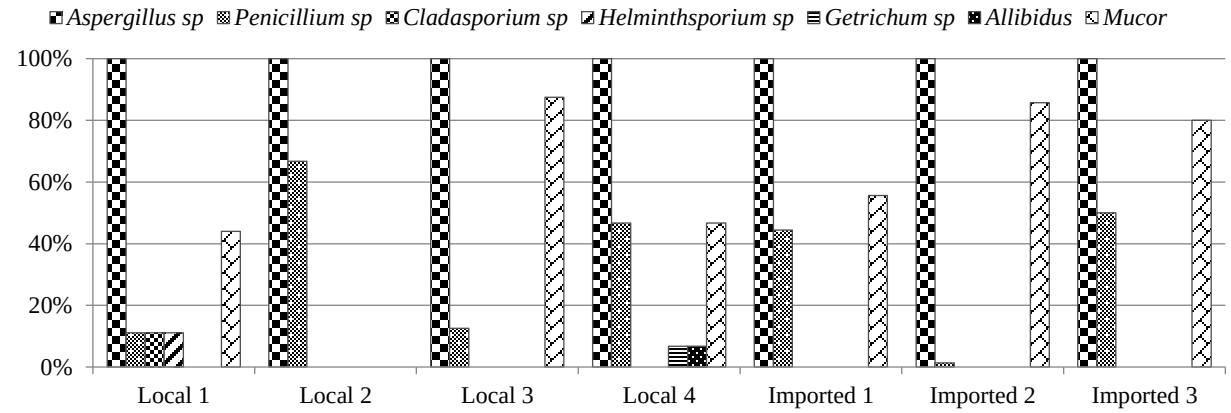

Fig. 3. Percentage frequency distribution of fungal contamination.

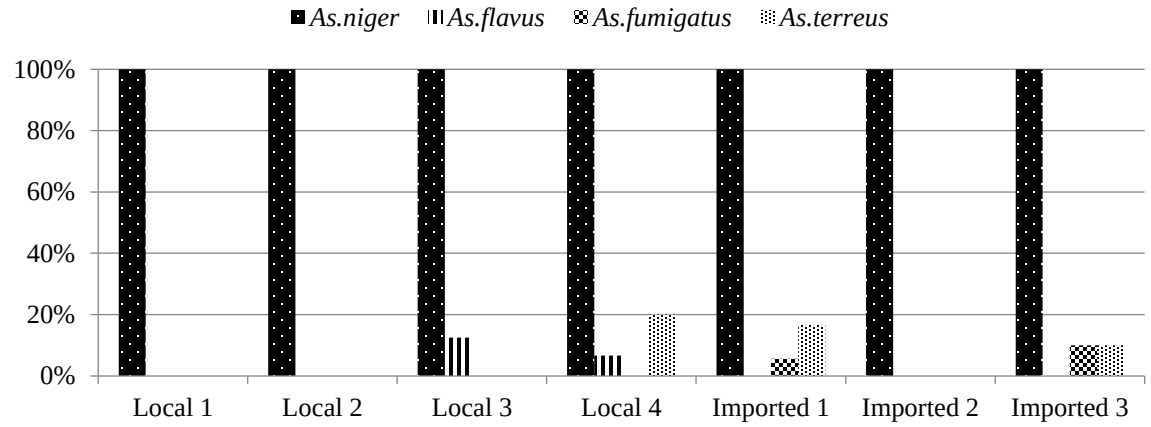

Fig. 4. Percentage frequency distribution of *Aspergillus* species.

4. Discussion

OTA in beer comes from barley and malt extract, where the ochratoxigenic fungi are present from the earliest stages of barley growth (13, 27). Barley may be contaminated as early as the ripening period. Their subsequent development and OTA production are influenced by different factors, particularly those related to climatic conditions during

ripening (27). Temperature is an influential factor; for example, OTA production peaks at 15-20˚C in *Aspergillus carbonarius* and at higher temperatures (20-25˚C) in *Aspergillus niger*. Also, the growth of toxin-producing fungi is influenced by the humidity of the barley. Additional factors potentially affecting unwanted fungal development include fungicide rate and application time, as well as storage conditions of the harvested barley and drying time (28).

Despite the fact that different climatic conditions affect the distribution of OTA-producing fungi, the origin of the beer can be a determining factor of its final OTA content. A relationship between OTA levels and production location have been reported in Mediterranean countries like Italy and Greece, where barley grown in southern Europe is especially prone to develop fungus and produce greater amounts of the toxin. In general, beers from the Mediterranean Basin are suspected to be more contaminated than beers produced in other European areas (29). OTA has been reported to be produced by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus niger* as the main producers commonly found in warm climates such as southern Europe and by *Penicillium verrucosum* and *Penicillium nordicum* in more temperate climates (30). In this study, *Aspergillus niger* and *Penicillium verrucosum* were found in both domestic and imported samples. The wider range of OTA concentrations found in the domestic products was probably due to both the widespread and higher occurrence of this toxin in barley and/or malt produced domestically as compared to the imported European products. However, comparing the incidence of ochratoxigenic fungi in beer. Although chromatographic techniques isolate a greater number of different chemical structures (31). Overall, the results from this survey are reassuring, and do not raise concerns for moderate consumer health as regards exposure to mycotoxins from nonalcoholic beers, while the long-term continues consumption may have considerable health problem despite the low levels of contamination.

5. Conclusion

Though many studies have been carried out to control OTA formation in different foodstuffs and products, there is no clear model to arrest the formation of this metabolite. It has been shown that reduced temperature and/ or humidity controlled OTA formation. Although the present study unable to find significantly differentiate the occurrence of OTA in the domestic and imported non-alcoholic beers, the controlled OTA formation observed is suggested to some extent determined by the numbers of competing fungi survived which is highly corresponded with regional and seasonal climatic conditions during beer production and storage. Prevention of mycotoxin contamination of food raw materials is now considered more important than subsequent cure and hazard analysis and critical control point (HACCP) approaches are being developed to examine the critical control points at which mycotoxicogenic molds and mycotoxins may enter to a range of food chains.

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