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Ochratoxin A and microbiological contamination of craft beers made in Cuenca, Ecuador

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ABSTRACT

Craft beers are usually produced on a small scale following atypical recipes and processes to achieve flavor innovation. Beers' hop composition offers a natural protection against biological contamination. Nevertheless, mycotoxins, such as ochratoxin A, can persist through the brewing process into the final product. In this study, the microbiological quality (aerobic mesophilic bacteria, mold, and yeast) via plate counts and ochratoxin A occurrence by HPLC-FLD were assessed in 42 samples of craft beer brewed in Cuenca, Ecuador. Aerobic mesophilic bacteria were present in 92.8% of analyzed samples and ranged from 6.6 to 8.4 log CFU mL^{-1}. Mold and yeast were present in 95.2% of craft beers, ranging between 6.6 and 8.2 log CFU mL^{-1}. Ochratoxin A was detected in 24% of craft beer samples, from which Ale type beers showed the highest contamination (0.03-0.51 ng mL^{-1}). None of the craft beer samples presented levels of ochratoxin A contamination above the permitted limit; while in most of the samples, microbiological contamination exceeded the reference limits applied for industrial beer. Although craft beer production does not follow a standardized process, to ensure the safety of this popular alcoholic beverage, minimum requirements for microbiological and chemical contaminants must be considered as well as further research into control and mitigation strategies throughout brewing.

Keywords: Ochratoxin A; mycotoxins; microbiological control; craft beer; food safety.

1. Introduction

Food contamination from biological and chemical sources is a serious hazard for consumers' health and an important economic detriment for producers (Singh et al., 2019). Cereals are the major energetic nutrient for humans and animals (Biel et al., 2020) and are utilized as raw material in diverse industries, for instance, barley in brewing. Unfortunately, cereals are highly susceptible to be contaminated, especially with mycotoxins (Khodaei et al., 2020; Mousavi-Khaneghah et al., 2019). Several studies have reported that ochratoxin A (OTA) is the most frequently occurred mycotoxin in barley (Palumbo et al., 2020; Pernica et al., 2019). OTA is considered a storage mycotoxin and is produced by filamentous fungi belonging to *Aspergillus* and *Penicillium* species, under favorable environmental factors such as water activity (0.99 - 0.90) and temperature (15 - 25 °C) (Cervini et al., 2020). OTA contamination could persist along brewing process and be detected in beer (Villacreces et al., 2022). Nevertheless, some studies have referred that mycotoxins can be reduced or metabolized along the process of industrial brewing (Wan et al., 2020). For instance, OTA partially decreases

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during malt kilning and wort boiling, that might be related to its slight solubility in water (Agriopoulou et al., 2020; Awuchi et al., 2021).

Craft brewing is a world-trend in alcoholic beverages production (Capitello & Todirica, 2021). Craft beer is generally produced on a small scale than the industrial process, also differing due to the employment of atypical recipes and procedures towards flavor innovation (Belmartino & Liseras, 2020). Several types of craft beer are available and their sensory properties are determined by the ingredients, in particular the yeast used and its treatment (Capece et al., 2018). According to the fermentation, Ale beer type results from a top-fermenting process at warm temperatures (18 - 25 °C); while Lager type is produced by a bottom-fermenting process at cold temperatures (5 - 15 °C) (Iorizzo et al., 2021). Beer color depends on the roasting grade of barley, applying low temperatures for blond and red Ale, and high temperatures for stout type (Ale variety) (Villacreces et al., 2022).

Craft beer might be vulnerable to spoilage bacteria, such as acetic acid bacteria, and wild yeast colonization. This has been attributed to the lack of pasteurization, the possible deficient manufacturing practices, the absence of filtration and the artificial carbonation along the craft brewing process (ASOCERV, 2016; Garofalo et al., 2015; Suiker & Wösten, 2022). Similar to the industrial process, a moderate removal of OTA from grain to wort has been reported in craft brewing (Volkova, 2013); while limited information is available on the removal of other mycotoxins for this type of processing (Schabo et al., 2021).

In the last decade, craft brewing production has become very popular in Latin America (Belmartino & Liseras, 2020; Toro-Gonzalez, 2017). Although Ecuador is not a country that produces barley, malt or hops, more than 200 microbreweries have been inventoried since 2012 (ASOCERV, 2016). In Ecuador, quality requirements are set for industrial beer but not for craft brewing. Consequently, craft beer is commercialized without any safety-related monitoring. In this study, OTA occurrence and microbiological indicators (aerobic bacteria, mold, and yeast) in craft beer produced and sold in Cuenca, Ecuador were assessed.

2. Methodology

2.1. Sampling frame

This study was conducted in Cuenca, Ecuador during October 2017. Local pubs, liquor stores and local supermarkets that commercialized different types of craft beers were identified and registered. In total, 42 samples were collected, three samples from twelve pubs (in bulk) and two stores, one per craft beer type (blond Ale, red Ale, and stout).

2.2. Samples pre-treatment

Collected samples were stored in sterilized amber bottles in the refrigerator at 2 - 8 ºC. Before analysis, samples were degassed by horizontal shaker (VWR, 3500, New Jersey, USA) for 60 min at 200 rpm, followed by ultrasonication (3510R-DTH, Branson, USA) for 30 min at room temperature (Lasram et al., 2013; Nguyen & Ryu, 2014).

2.3. Chemicals and reagents

Peptone water was purchased from Fluka Analytical (Madrid, Spain) and used to dilute craft beers samples (1:10 v/v). Plate count agar (PCA) was supplied by Difco™ (New Jersey, USA). PCA was prepared by dissolving 23.5 g in 1.0 L of distilled water, pH was adjusted to 7.0 (Mettler Toledo, 8603, Switzerland) and then it was sterilized (Tuttnauer, 2340MK, Israel) for 15 min at 121 ºC. Davis's yeast salt agar was prepared with ammonium sulphate 1.0 g by Merck (Darmstadt, Germany), ammonium nitrate 1.0 g by Merck (Darmstadt, Germany), disodium hydrogen phosphate 4.0 g by Merck (Darmstadt, Germany), potassium dihydrogen phosphate 2.0 g by Sigma Aldrich (Japan), sodium chloride 1.0 g by Fisher Scientific (USA), D-glucose 10.0 g by Sigma (México), commercial dried yeast 1.0 g and base agar by Merck (Darmstadt, Germany) using 15.0 g per liter. All salts were dissolved in distilled water and sterilized as indicated for PCA (INEN, 2012, 2013a). At 50 ºC, this growth media was acidified with citric acid solution (10 % p/v) up to pH 3.5 (57 mL per liter approx.). Ultrapure water was obtained from Type I water purification system (Barnstead; Iowa, USA). Glacial acetic acid (HAc) was supplied by Merck KGaA (Darmstadt, Germany). Phosphate-buffered saline pH 7.4 solution; solid standard of ochratoxin A from *Aspergillus ochraceous* (CAS Nº 303-47-9), acetonitrile (ACN) and methanol (MeOH) HPLC grade were supplied by Sigma Aldrich (St Louis, USA). Immunoaffinity cartridges (Ochraprep) were purchased from R-Biopharm Rhône (Glasgow, Scotland).

OTA standard was reconstituted with ACN (1 mg mL^{-1}), then divided into aliquots and dried under nitrogen stream. A stock solution of 1000 ng mL^{-1} was reconstituted and further diluted with

ACN/H2O (50:50, v/v). A calibration curve was constructed with ten concentration points within a range of 0.01 to 40 ng mL^{-1}.

2.4. Ochratoxin A extraction and HPLC analysis

Analytical procedure scheme is outlined in Figure 1. Thirty mL of degassed beer were diluted with 15 mL of phosphate buffer solution (PBS, pH 7.4). The mixture was centrifuged at 820 g for 10 minutes at 10 °C (Hettich, Mikro 220R, Germany) (Cao et al., 2013). Thirty mL of the supernatant (equivalent to 20 mL of beer) were passed at 1 mL min^{-1} through the Ochraprep immunoaffinity cartridge (pre-conditioned with 3 mL of PBS).

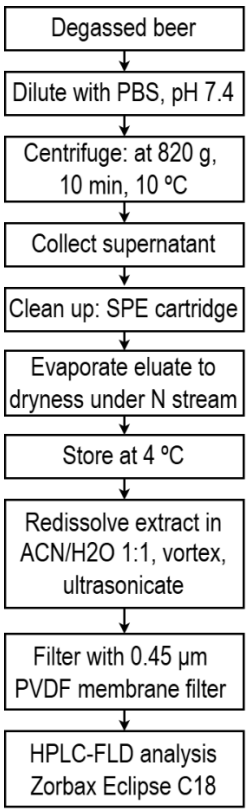

Figure 1. Procedure scheme for extraction of Ochratoxin A and HPLC analysis.

The cartridge was post-washed with 20 mL of water at 5 mL min^{-1}. Elution was conducted with 1.5 mL of MeOH/HAc (98:2, v/v) followed by 1 mL of water. The eluate was evaporated up to dryness under a stream of nitrogen gas (Dominick hunter, G4510W, England) at room temperature. The dried extract was stored at 4 °C until analysis (Cao et al., 2013; Lasram et al., 2013). The extract was redissolved in 1 mL of ACN/H_2O (50:50, v/v), followed by vortexing for 3 min and ultrasonication for 5 min at room temperature (Branson, 3510R-DTH, USA). The final extract was filtered (0.45 µm, PVDF membrane filter) and subjected to HPLC analysis.

OTA analysis was carried out by High Performance Liquid Chromatography (HPLC) with fluorescence detection (FLD) (Agilent Technologies, 1200, USA), following a standardized method (Ortiz et al., 2013). Briefly, after injection of 100 µL of filtered extract, separation was achieved using a Zorbax Eclipse C18 column (250 x 4.6 mm, 5 µm) kept at room temperature (25 ºC). An isocratic elution was applied using a mixture of ACN/H2O/HAc (50:49:1, v/v/v) at a flow rate of 1 mL min^{-1} for 15 minutes. The fluorescence detector was set to 247 nm and 480 nm of excitation and emission wavelengths, respectively.

Retention time window was between 10.45 and 10.65 min. Limits of detection and quantification were established at 0.0063 and 0.0126 ng mL^{-1} of beer, respectively. Linear range was from 0.1 to 40 ng mL^{-1} (R^2 = 0.9999).

2.5. Microbiological analysis

Microbiological analyses were performed according to the requirements set by the Ecuadorian Service of Normalization (INEN), that described the quality control for industrial beers (INEN, 2003). All microbiological analyses were done in duplicate.

Aerobic mesophilic bacteria. One mL of sample was thoroughly mixed with 20 mL of liquid PCA at 45 ºC. After solidification, inverted plates were incubated at 30 ºC for 48 to 72 hours (Memmert, INB200, Germany) (INEN, 2013b). Two consecutive dilutions were necessary for colony counting (within 15 to 300 colonies). Results were expressed as Colony-Forming Units per mL of sample (CFU mL^{-1}) after considering the dilution factor (10^n) (INEN, 2012).

Molds and yeasts. One mL of sample was plated on Davis's yeast salt agar. Plates were incubated at 25 ºC for 5 days. Plates with less than 150 colonies from two consecutive dilutions were considered for colony counting. Results were expressed as CFU mL^{-1} of molds and yeast multiplied by 10^n (INEN, 2013a).

2.6. Statistical analysis

OTA concentration was calculated by interpolation of resulting peak areas of the samples within the calibration curve. Differences among beer types were evaluated by ANOVA considering a significance level of 5%. Microbiological mean counts were compared with Ecuadorian and international quality control requirements. Statis-tical analyses were performed in the software STATA 10.0.

3. Results and discussion

3.1. OTA occurrence in craft beer

In total, 42 craft beer samples were analyzed (14 blond Ale, 14 red Ale and 14 stout type). Results are summarized in Table 1 OTA was detected in 24% of craft beer samples, corresponding to 9.5% blond Ale type, 9.5% red Ale type and 4.8% stout type. Ale type beers showed the highest contamination level in a range of 0.03 - 0.51 ng mL^{-1}. Similar concentration were previously reported in Ale beers in Czech Republic (41.6%, 0.001 - 0.24 ng mL^{-1}) (Běláková et al., 2011) and in Tunisia (48%, 0.04 - 0.35 ng mL^{-1}) (Lasram et al., 2013). Higher concentrations, but at lower rates, have been reported in Brazilian beers (4.8%, 1 - 18 ng mL^{-1}) (Kawashima et al., 2007) and European beers (10%, 3.2 - 5.1 ng mL^{-1}) (Rubert et al., 2013). Specific data on red Ale and stout beer is scarce. An European report found that 1 out 10 red Ale beers were contaminated with OTA at 5.1 ng mL^{-1} (Rubert et al., 2013); whereas 44.4% of Czech stout beers were contaminated within 0.002 to 0.05 ng mL^{-1} (Běláková et al., 2011).

Table 1
Occurrence of ochratoxin A in craft beer commercialized in Cuenca, Ecuador (n = 42). All expressed in ng mL^{-1} of beer

Type of beer	Positive	Mean ± SD	Min - Max
Blonde Ale	4/14	0.15 ± 0.24	0.03 – 0.51
Red Ale	4/14	0.12 ± 0.17	0.03 – 0.38
Stout	2/14	0.20 ± 0.18	0.07 – 0.33

In general, without distinction between industrial or craft process, OTA contamination in European beer occurs at rather low concentrations (< 0.2 ng mL^{-1}) (Bellver-Soto et al., 2014; Coronel et al., 2012). Overall, no limits have been established by the European Commission (EC), since it is considered that the safety of this alcoholic beverage should be controlled at the earlier stage of the production chain (EC, 2010). When considering beer as a barley derivative product, the maximum permitted limit for OTA is 3 µg kg^{-1} (EC, 2010). Maximum limits of OTA in beer in different European countries range from 0.2 to 0.5 ng mL^{-1} (Medina et al., 2005). Our findings did not exceed the reference permitted limits.

Various foods represent potential OTA dietary sources (Coronel et al., 2012; Skarkova et al., 2013), including alcoholic beverages like wine and beer, mainly in adults' diet (Bui-Klimke & Wu, 2015). Exceeding safety intake levels depends on the intensity, frequency, and duration of exposure. For instance, it has been reported that beer consumption contributes with 5% of OTA intake in Europe (EC, 2002). In countries such Ecuador, 79.9% of adults have shown high preference for beer consumption (WHO, 2018), but there is no data available on the daily or total consumption of OTA.

3.2. Microbiological control

Microbial occurrence was higher in red Ale type beer, followed by blond Ale. Results of microbiological analysis are summarized in Table 2. Aerobic mesophilic bacteria were present in 92.8% ranging from 6.6 to 8.4 log CFU mL^{-1}. Similarly, mold and yeast were present in 95.2% ranging between 6.6 and 8.2 log CFU mL^{-1}.

Results were compared with the previous Ecuadorian regulation set in 2002 for non-pasteurized beer (INEN, 2003), whose limit for aerobic mesophilic bacteria and for mold and yeasts is 1.9 log CFU mL^{-1}. Only three samples for aerobic mesophilic bacteria and two for mold and yeast complied with this regulation, while counting in other samples were substantially higher. Since 2013, the Ecuadorian regulation was replaced by another that focuses solely on pasteurized beer (INEN, 2013b). No regulation for craft beer is set in Latin American countries, except for the Argentinian Food Code that encompasses quality requirements but no microbiological indicators (Bigeon et al., 2017; CAA, 2017).

Table 2
Microbiological control assessment in craft beer commercialized in Cuenca, Ecuador (n = 42). All expressed as log Colony-Forming Units (log CFU mL^{-1})

Type of beer	Mesophilic aerobic bacteria			Mold and yeast		
	Positive	Mean ± SD	Min – Max	Positive	Mean ± SD	Min – Max
Blonde Ale	13/14	7.1 ± 7.6	1.3 – 8.2	14/14	7.0 ± 7.5	2.6 – 8.1
Red Ale	13/14	5.6 ± 6.1	1.3 – 6.6	13/14	5.7 ± 6.0	1.5 – 6.6
Stout	13/14	7.2 ± 7.8	1.2 – 8.4	13/14	7.1 ± 7.6	1.2 – 8.2

4. Conclusions

This is the first report of OTA contamination and microbiological indicators in craft beer produced in Ecuador. OTA contamination was similar to levels reported in other countries. Those levels are quite low, but they do contribute to the overall dietary exposure to this toxin. The presence of microbial indicators can be considered high, but there is not any regulation on craft beer to compare with. Although our findings may not represent any risk to consumers, they suggest a possible coexistence of common beer microorganisms, spoilage agents and even pathogens in the final product. This work could serve as a baseline study towards contaminant mitigation strategies, as well as to increase awareness and control measures in craft beer production. Future studies should focus on strategies to control and mitigate undesirable microorganisms and the carry-over of mycotoxins from raw ingredients for craft beer production.

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