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Mycobiota associated with yerba mate sold in Paraguay

Micobiota asociada con yerba mate comercializada en Paraguay

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Resumen: La yerba mate (*Ilex paraguariensis*) es una hierba ampliamente consumida que se originó en Paraguay, pero que crece en la región entre el sur de Brasil, el norte de Argentina, Paraguay y Uruguay. Se informa que la planta posee múltiples propiedades medicinales que hacen que la yerba mate sea popular para el consumo en esta región. Actualmente también se considera un producto nutracéutico y un alimento funcional que es consumido en diferentes países del mundo. En medicina popular, se ha reportado científicamente que posee múltiples propiedades. Treinta y nueve marcas de muestras de yerba mate se analizaron mediante placas de dilución en serie y se incubaron a 25°C durante 5 días se determinó la incidencia fúngica. De las 39 muestras analizadas, el 15,4% estuvieron libres de la presencia de hongos y levaduras. El 84,6% restante presentó contaminación con incidencias variables de *Aspergillus*. Se observó la presencia de *Aspergillus* secciones *nigri, flavi, circumdati, fumigati, wentii, usti, versicolor* y *Emericella nidulans*, siendo la prevalente *Aspergillus* sección *nigri,* representando el 65,3%. Teniendo en cuenta los riesgos a la salud asociados con estos hongos, es importante considerar la formulación de regulaciones con respecto al tamaño de las partículas y la presencia de ocratoxinas y aflatoxinas en los productos comerciales de yerba mate.

Palabras claves: *hierbas, hongos, inocuidad, riesgo, seguridad alimentaria.*

Abstract: Yerba mate (*Ilex paraguariensis*) is a widely consumed herb which originated in Paraguay but grows widely in the region between Southern Brazil, Northern Argentina, Paraguay, and Uruguay. The plant is reported to possess multiple medicinal properties which makes yerba mate popular for consumption as tea in this region. It is also being considered as a nutraceutical product and a functional food consumed in different countries around the world. In popular medicine, and it has been scientifically reported to possess multiple properties. Thirty-nine brands of yerba mate samples were analyzed by serial dilution plates. and incubated at 25 ° C for 5 days. Based on their morphological characteristics, the fungal incidence and the number of colonies forming units were determined. Of the 39 samples analyzed, 15.4% were free from the presence of fungi and yeasts. The remaining 84.6% of the samples were contaminated with variable contents of the fungi *Aspergillus.* The presence of *Aspergillus* sections *nigri, flavi, circumdati, fumigati, wentii, usti, versicolor* and *Emericella nidulans* was observed the most prevalent among them was *Aspergillus* section *nigri,* representing 65.3%. Considering the health risks associated with these fungi, it is important to consider formulating regulations regarding the particle size and the presence of ochratoxins and aflatoxins in commercial yerba mate products.

Keywords: *innocuity, food safety, fungi, herbal, risk.*

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Introduction

Yerba mate (*Ilex paraguariensis*) is a plant native to South America, specifically from the region between Southern Brazil, Northern Argentina, Paraguay, and Uruguay (Marcowicz *et al*., 2007), Is one of the products which is deeply rooted in Paraguayan culture. Its aqueous extract is consumed in a massive way in different forms such as: mate (hot water), tereré (with cold water) or cocido (hot infusion of toasted yerba) (Jerke *et al*., 2009; Pérez Paiva *et al*., 2013).

In popular medicine, it is recommend to trait arthritis, headache, rheumatism, hemorrhoids, obesity, fatigue, slow digestion, and liver disorders among other health problems (Bastos *et al*., 2005). Yerba mate has been reported to possess multiple properties, such as vasodilatory effects, reducing cholesterol and triglyceride (Stein *et al*., 2005), preventing DNA damage (Mohadjerani & Roodgar, 2016), antimutagenic and radioprotective effects (Bracesco *et al*., 2018), antidiabetic effects (Heck & De Mejia, 2007; Rocha *et al*., 2018), prevention of the senescence of retinal cells and mobilization of the cellular pathways (Tate *et al*., 2020), antioxidant and antimicrobial effects (Kungel *et al*., 2018), preservation of the cardiovascular health (Cardozo Junior & Morand, 2016), anti-obesity effects (Gambero & Ribeiro, 2015; Kim *et al*., 2015; Uecker *et al*., 2019), prevention of liver redox imbalance, high triglycerides and microsteatosis (de Oliveira *et al*., 2018), among others. It is also an excellent source of polyphenols such as caffeic and chlorogenic acids (Tate *et al*., 2020).

Yerba mate is a plant rich in vitamins and produces a feeling of well-being, vigor, and intellectual lucidity, based on the presence of the alkaloid matein. It is diuretic and digestive product with laxative properties (Jerke *et al*., 2009; Riachi & De Maria, 2017).All these held benefits have made yerba mate a popular product, being considered as a nutraceutical and a functional (Alkhatib *et al*., 2017; Kujawska, 2018; Riachi & De Maria, 2017) currently consumed in different countries around the world (Alkhatib & Atcheson, 2017).

During its process, the herb undergoes changes in temperature changes and storage periods for aging to improve its flavor. This period can vary from 2 months to 1 year or more, which favors the development of filamentous fungi in the final product (Silva *et al*., 2019). The Paraguayan regulations establish a limit of $1{,}8\times10^2$ CFU/g of total fungi and yeasts for processed yerba mate (Instituto Nacional de Tecnología Normalización y Metrología, 2007). Several species of filamentous fungi have been reported in yerba mate, among them *Aspergillus* of the Sections *Nigri* and *Flavi* (Jerke *et al*., 2009; Pérez Paiva *et al*., 2013; Silva *et al*., 2019), which are potential producers of mycotoxins.

Considering its beneficial effects, yerba mate has become a popular product around the world (Alkhatib *et al*., 2017; Alkhatib & Atcheson, 2017), and an increase in its consumption has been seen. Therefore, it is necessary to have a quality control on the commercial products to ensure their safety. The aim of this work was to evaluate the content of fungi and yeasts in the commercial brands of yerba mate being sold in Paraguay.

Materials y Methods

Sample collection

39 brands (1 kg packages) of yerba mate elaborated (*Ilex paraguariensis*), all products with RSPA (sanitary food product registration) and RE (establishment registration) in force from the National Institute of Food and Nutrition, INAN. Each package, adquired in supermarkets of the country was considered as a unit of analysis.

Fungal analysis for colony forming units

Samples were analyzed by serial dilution plates. A 25 g subsample of each sample was diluted in 225 mL of sterile distilled water. An aliquot of 0.1 ml was spread on Potato Dextrose Agar (PDA) (90 × 15 mm plates) and incubated at 25 °C for 5 days. The number of colony forming units (CFU) and fungal incidence were determined based on morphological characteristics (Pitt & Hocking, 2009).

Table 1. Colony Forming Units for gram (CFU/g) in Yerba Mate samples, considering the crop management system (conventional and organic).

Management	n (samples)	Minimum CFU/g	Maximum CFU/g	Median CFU/g
Conventional	35	0	3.0×10^4	6.0×10^2
Organic	4	0	4.0×10^3	1.0×10^1

Fungal analysis for isolation of *Aspergillus*

Samples were analyzed by serial dilution plates. A 25 g subsample of each sample was diluted in 225 mL of sterile distilled water. An aliquot of 0.1 mL was spread 90 × 15 mm plates with MAAS, (selective medium for isolation of Aspergillus from soil) (5 g of peptone, 1 g of potassium hypophosphite, 30 g of sodium chloride, 10 g of glucose, 0.5 g of magnesium sulfate heptahydrate, 20 g of agar and 1000 mL of distilled water) (Dhingra, OD; Sinclair, 1995; Klich, 2002) and incubated at 25 °C for 5 days. The number of colony forming units (CFU) and fungal incidence was determined based on morphological characteristics (Barnett & Hunter, 1972; Pitt & Hocking, 2009).

Subsequently, each colony was purified, and monosporic cultures were performed using serial dilutions. The fungi were seeded on PDA (90 × 15 mm plates) and incubated at 25 °C for 5 days. Those who presented characteristics of belonging to the genus *Aspergillus* were identified using a taxonomic key(Raper *et al*., 1965).

For the analysis, aspects related to the geographical origin of the samples, brand, grain size, handling (conventional and organic) were considered. Maximum, minimum, and median values and were calculated.

Results

A total of 39 yerba mate brands marketed in Paraguay were examined for the presence of fungal contamination. Two thirds (26) of the samples declared conventional processing while the remaining one third (13) were promoted as organic. In terms of granulometry, 74.3% declared intermediate grinding, 12.8% coarse grinding, 10.3% of fine grinding and 2.5% with stick.

Of the total sample analyzed, 15.4% (n=6), were found to be free from the presence of fungi and yeasts. The remaining 84.6% (n=33) of the samples were found to be positive for fungal contamination and the fungal load per sample ranged from 2×10^1 CFU/g to 3.2×10^4 CFU/g.

Almost 40% of the positive samples (n=33) were found to have contamination values over 1.8×10^3 CFU/g, which are higher than the Paraguayan regulations. In Paraguay, in a study, 22 brands of elaborated yerba mate, it was determined that 10 brands exceeded the limits established by the national regulations 8×10^3 CFU/g (Pérez Paiva *et al*., 2013).

Our study revealed that 54.5% (n=18) of the positive samples exhibited contamination levels below 1.0×10^3 CFU/g. Of these samples, 93.4% were processed conventionally, while 6.6 % were organic. With regards to granulometry, 88.9% declared intermediate grinding, 11.1% of fine grinding, and 5.5% of coarse and stick grinding, respectively. Regarding of 27.3% (n=9) of the samples showed levels, between 1.0×10^3 and 9.0×10^3 CFU/g; 11.1% were organic and 88.9% conventional, in other hand granulometry, 22.2% suffered coarse grinding and 88.8% intermediate grinding (Table 1).

Six samples (18.2%),all from different manufacturers, exceeded the contamination limit (1.0×10^4 CFU/g) by the World Health Organization (WHO, 2007).

Considering the geographical origin, the highest levels of contamination were found in brands produced in the departments of Itapúa and Guairá, where the maximum values of CFU/g were 3×10^4 and 2×10^4 respectively (Table 2).

With respect to granulometry, fine grinding leads to the highest level of contamination, 3.2×10^4 CFU/g with a median of 6.8×10^2 CFU/g, followed

Table 2. Colony Forming Units per gram (CFU/g) in yerba mate samples considering the geographic origin (place of planting).

Geographic Origin	n (samples)	Minimum CFU/g	Maximum CFU/g	Median CFU/g
Alto Paraná	3	0	4.0×10^3	3.0×10^2
Guairá	12	0	2×10^4	5.5×10^2
Itapúa	21	0	3×10^4	9.0×10^2
Misiones	2	80	1.0×10^2	9.0×10^2
Central	1	0	0	0

by intermediate grinding with 2.2×10^4 CFU/g and a median of 6.1×10^2 CFU/g; Coarse grinding, led to a maximum of 7.1×10^3 CFU/g and a median of 1.6×10^2 CFU/g and the product with stick, with a maximum of 8.0×10^1 CFU/g (Table 3).

Presence of Aspergillus

The 89.7% of the samples presented variable levels of contamination with fungi of the genus *Aspergillus*. The presence of *Aspergillus* Section *Nigri*, *Flavi*, *Circumdati*, *Fumigati*, *Wentii*, *Usti*, *Versicolor* and *Emericella nidulans* was determined.

Aspergillus Section *Nigri* was prevalent, representing 65.3%, followed by Aspergillus Section *Flavi* with 26.9%. These two groups represented 92.2% of the isolated colonies. The other 7.8% were represented by *Aspergillus* Section *Fumigati* (2.9%), *Versicolor* (2.4%), *Circumdati* (1.4%), *Wentii* (0.4%), *Emericella nidulans* (0.5%), and *Usti* (0.1%).

Discussion

Yerba mate undergoes extensive processing before it is ready to be packaged including selection of the leaves, blanching and drying, the final product is extremely dehydrated, and for these, impairs the growth of pathogenic organisms but fungal species, are ubiquitous in the environment and may easily contaminate yerba mate leaves during storage or manipulation (Vieira *et al*., 2010).

A study done in Brazil in 2010 reported substantial growths of fungi in the range of 0.1×10^1 to 4.9×10^2CFU/g in 7 of the 8 brands analyzed. Also in Brazil, in 2019, 33.3% (n=15) of the samples studied exhibited contamination levels below 1.0×10^3 CFU/g; 60.1% showed intermediate contamination levels between 1.0×10^3 and 9.0×10^3 CFU/g, and two samples exceeded the contamination limit (1×10^4 CFU/g). In both studies, the levels of contamination detected are higher than those obtained in our study, where 6 brands did not present any type of microbiological contaminant. In Argentina, an analysis of 36 samples of different brands from establishments of the provinces of Misiones, Corrientes, and Buenos Aires determined that on average the fungal count was 6.1×10^3 CFU/g (Jerke *et al*., 2009), with the highest incidence of molds (89%) than yeasts, higher than that obtained in our study of 5×10^3

Table 3. Colony Forming Units per gram (CFU/g) in yerba mate samples considering the Granulometry.

Granulometry	n (samples)	Minimum CFU/g	Maximum CFU/g	Median CFU/g
Fine grinding	4	1.1×10^2	3.2×10^4	6.8×10^2
Intermediate grinding	29	0	2.2×10^4	6.1×10^2
Coarse grinding	5	0	7.1×10^3	1.6×10^2
With stick	1	8.0×10^1	8.0×10^1	8.0×10^1

CFU/g. Besides evaluating different brands, this study also considered other important aspects such as: handling, geographical origin of the products, and the granulometry of the grinding.

Considering product management, the highest levels of contamination were found in brands produced under conventional process, with the maximum CFU value being 3.0×10^4 CFU/g, and a median of 6×10^2 CFU/g. On other hand, organic management demonstrated much lower contamination, the higher level being 4.0×10^3 CFU/g and the median 1.0×10^1 CFU/g (Table 1).

In soils with organic management, there is a greater heterogeneity of the microbiome when compared to soils with conventional management, this could help control pathogens during cultivation and decrease the initial inoculum present in yerba mate (Lupatini *et al*., 2017).

In Paraguay, to be able to label a product as organic, it must be demonstrated to have some type of certification that indicates its organic management, although there is no specific certification that is mandatory. The definition of "organic yerba mate" is not standardized or regulated in current system (Instituto Nacional de Tecnología Normalización y Metrología, 2007: Norma Paraguaya NP 35 001 93 [C.D.U. 633]: Yerba Mate Elaborada, Requisitos Generales).

In sense of geographic results, Itapúa and Guairá are the Departments with the highest yerba mate production in the country in terms of cultivated area and productivity (Ministerio de Agricultura y Ganadería, 2018). The higher level of contamination can probably be related to the high volumes of product handled in these departments 73,000 and 20,000 tons respectively, compared to Alto Paraná with 6,500 tons and Misiones with 52 tons.

With respect to granulometry, fine grinding leads, the existence of a correlation between the content of molds and yeasts and the size of the Yerba mate particles could not be determined due to the small size of the sample. However, the present data allows us to believe that a fine grinding of the product increases the chances of contamination compared to a coarse grinding.(Silva *et al*., 2019; Vieira *et al*., 2010).

In the sense of Aspergillus presence, a study from 2013 including 22 commercial brands of elaborated yerba mate commercialized in Paraguay determined the presence of *Aspergillus niger, Aspergillus flavus, Pullularia sp. Penicillium sp.* and *Cladosporium* sp. (Perez Paiva *et al.*, 2013). In Argentina, where 36 samples of processed yerba mate were analyzed, it was determined that the incidence of the *Aspergillus* genus was 86%. In Brazil, 8 brands of yerba, it was determined that *Aspergillus niger* and *Aspergillus fumigatus* were the prevalent fungi (Vieira *et al*., 2010). At 2019 also in Brazil was reported as prevalent with 76.5% to *Aspergillus* Section *Nigri* followed by *Aspergillus* Section *Flavi* with 1.6%(Silva *et al*., 2019). In Argentina,36 samples of yerba mate processed, 1215 strains corresponded to *Aspergillus* Section *Nigri*, these being the prevalent fungi (Castrillo *et al*., 2013). All these results are like those obtained in our study, thus corroborating the importance of this fungal group as a contaminant of yerba mate. The occurrence of fungi belonging to *Aspergillus* Section *Nigri* and Section *Flavi* in yerba mate demands more attention due to the risk of mycotoxin production (Silva *et al*., 2019).

A concerning issue that arise during the development of this work was the unevenness of the labeling of the commercial product. In the various labels, terms such as selected, premium, organic, natural herb medium grinding or fine grinding were found, however none of these definitions are found in current regulations.

This situation is worrying from the point of view of the quality and safety of the products; since there is no legal definition about the different parameters, the final product is therefore highly heterogeneous.

It is important to analyze and consider in the future regulations regarding yerba mate the particle size of the herb and the contents of mycotoxins present in these products.

In yerba mate, high fungal counts may indicate a failure during processing or a recontamination

at some stage of storage, transportation or marketing, due to an increase in moisture content (Silva *et al*., 2019).

Conclusion

Based on the results obtained, we conclude that yerba mate is a favorable substrate for the growth of fungi and yeasts; therefore, periodic microbiological control is essential. Regarding the presence of fungi of the genus *Aspergillus*, their importance should be considered due to their potential for mycotoxin production. In our work of 39 samples tested, 84.6% (n=33) were positive for fungal contamination and 39.4% presented values higher than 1.8×10^3. CFU/g, above the limits established by Paraguayan regulations. *Aspergillus* Section *Nigri* was the prevalent group 65.3%, followed by *Aspergillus* Section *Flavi* with 26. 8%.

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