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La granja:
Revista de
Ciencias de la Vida

Editorial

ABYA YALA | UNIVERSIDAD POLITÉCNICA SALESIANA

Dear reader:

We are pleased to present our Special Issue on Natural Products in this 36th issue of La Granja. A Natural Product is understood as any biomolecule that has a benefit for human beings, in areas such as pharmaceuticals, cosmetics, food and industrial products in general. The abundance of Natural Products is in accordance with the biodiversity that a region or country may have; therefore, they are valuable goods for nations having these resources in their territory.

In this issue of La Granja we present research on this subject. First, there is a paper from Panama written by Karol Gutiérrez-Pineda and Misay Herrera, who describe the qualitative phytochemistry of the species *Terminalia Catappa* L. (Panama Almond), in several extracts, giving a first look at possible applications of the plant. It is a study conducted by Fundación Pro-Conservación de los Primates Panameños (FCPP), and Sociedad Mesoamericana para la Biología y la Conservación de la Naturaleza (SMBC).

From Ecuador, Anderson Pazmiño and his team from the Technical University of Manabí, the Agrarian University of Ecuador and Escuela Superior Politécnica del Litoral work with essential oils of cinnamon and oregano incorporated in biodegradable films to measure their potential for inhibiting bacteria, and propose them as preservation additives in the food industry. In the miscellaneous section, in the area of conservation, researchers from Universidad Antonio Nariño and Fundación Universitaria Agraria de Colombia, in a paper led by Olga Lozano Camelo, present strategies for the modified preservation and description of *Batrachochytrium Dendrobatidis* Fungalysin. Regarding sustainable development, Miguel García-Parra and leading researchers from prestigious universities: Universidad del Cauca, Fundación Universitaria Juan de Castellanos, Universidad Nacional Abierta y a Distancia and Universidad Nacional de Colombia, along with Universidad de Concepción de Chile, present a Literature review on one of the most current topics: the Sustainable Development Goals in the American region.

Likewise, Adán Ramírez García and his team from Universidad Autónoma Chapingo, along with Instituto Tecnológico de Sonora of Mexico and Escuela Superior Politécnica del Litoral del Ecuador, present the adaptive capacities of coffee and cocoa crops in possible climate change scenarios, one of the main concerns in food sovereignty. While in Hydrology, Mario Guallpa, Rolando Céleri and Patricio Crespo, from the University of Cuenca and ETAPA del Ecuador, demonstrate the effect of the theoretical discharge coefficient of landfills on the measurement of flows in our Andean rivers.

There are two contributions in the field of environmental pollution. The first refers to the effectiveness on the use of diatoms in wastewater filtration, a study led by Cristian Joao Vázquez, from the Ministry of Public Health in collaboration with the National University of Chimborazo in Ecuador. The second shows the contrasting effects of environmental pollution in the Metropolitan District of Quito, research led by Daniel Cornejo-Vásconez, from the University of the Armed Forces and the Pontifical Catholic University of Ecuador. Both studies show the areas in which pollution affects water and air.

Finally, in veterinary sciences, Teodosio Huanca, from the National Institute of Agrarian Innovation of Peru, shows the effect of embryotrophic factors at different oxygen tensions *in vitro* on the embryonic development of alpacas in the Andean region. Meanwhile, from Food Science, Sixto Reyna and José Arteaga from the Technical University of Manabí, present an extensive study on the risks of contamination of milk and its derivatives.

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Without doubt, this material will be useful in the research carried out in the region and its communities in order to have benefit and use of natural products and environmental resources.

Sincerely,

Sheila Serrano Vincenti MsC.
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Special Issue/ Número Especial

Natural Products

Preliminary phytochemical characterization of the ethanolic extracts of leaf, green and ripe fruit of *Terminalia catappa L.* (Almendro) in Panama

Caracterización fitoquímica preliminar de los extractos etanólicos de hoja, fruto verde y maduro de *Terminalia catappa L.* (Almendro) en Panamá

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Abstract

The almond tree (*Terminalia catappa L.*) in Panama is for timber, nutritional, and medicinal use, among others. Little is known about the secondary compounds present in the different parts of this plant species. This work aimed to carry out the phytochemical characterization of the green leaf (GL), green fruit (GF) and ripe fruit (RF) of the almond tree. The sampling was carried out between May to June 2017. The GL, GF and RF of the almond tree were placed separately, in direct extraction treatment with 95% ethanol for one day. The ethanolic extract was obtained by gravity filtration and concentrated in a vacuum rotary evaporator (BUCHI Rotavapor^{TM} R-210). Phytochemical screening was performed to determine the three main groups of secondary compounds (phenolics, nitrogenous and terpenes). An Infrared Spectroscopy (FTIR) and Ultraviolet-Visible (UV) Spectroscopy analysis were performed to find out which functional groups were present. Past 4.02 program was used to generate a graph of relative abundance of the secondary compounds present, and Whittaker index was applied to evaluate the percentage of difference in the phytochemical composition of the plant's parts. With these data, it was able to identify if the semi-quantitative phytochemical characterization is variable for each part, with nitrogen compounds (alkaloids) and phenolic compounds (tannins, flavonoids) presenting greater abundance. No presence of cardiotonic glycosides was found, or gums and mucilage. This information indicates that *T. catappa L.* is a potential resource for health, being of great ethnobotanical, pharmacological value and for the food industry in Panama.

Keywords: Secondary compounds, ethnobotany, bioactive functions, medicinal plant, natural products, *Terminalia catappa*.

Resumen

El almendro (*Terminalia catappa L.*) en Panamá es de uso maderable, nutricional, medicinal, entre otros. Se sabe poco sobre los compuestos secundarios presentes en las diferentes partes de esta especie vegetal. Este trabajo tuvo como objetivo realizar la caracterización fitoquímica de la hoja verde (HN), fruto verde (FV) y maduro (FM) del almendro. El muestreo se llevó a cabo entre los meses de mayo a junio del 2017. Se colocaron las HN, FV y FM del almendro por separado en tratamiento de extracción directa con etanol al 95% durante un día. El extracto etanólico fue obtenido por filtrado de gravedad y concentrado en un rotavapor al vacío (BUCHI Rotavapor^{TM} R-210). Se realizó un tamizaje fitoquímico para determinar los tres principales grupos de compuestos secundarios (compuestos fenólicos, compuestos nitrogenados y terpenos). También se realizó un análisis de Espectroscopía Infrarroja (FTIR) y Espectroscopía Ultravioleta-Visible (UV) para saber cuáles eran los grupos funcionales presentes. Se utilizó Past 4.02 para generar una gráfica de abundancia relativa de los compuestos secundarios presentes, y se aplicó el índice de Whittaker para evaluar el porcentaje de diferencia en la composición fitoquímica de las diferentes partes vegetales. Con estos datos se logró identificar que la caracterización fitoquímica semicuantitativa es variable para cada parte, presentando mayor abundancia de compuestos nitrogenados (alcaloides) y compuestos fenólicos (taninos, flavonoides). No se observó presencia de glicósidos cardiotónicos, ni de gomas y mucilagos. Esta información resalta que *T. catappa L.* es un potencial recurso para la salud, siendo de gran valor etnobotánico, farmacológico y para la industria alimenticia en Panamá.

Palabras clave: Etnobotánica, funciones bioactivas, metabolitos secundarios, planta medicinal, productos naturales, *Terminalia catappa.*

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1 Introduction

Panama is one of the countries with the greatest biological diversity worldwide (Olmedo et al., 2018; López and Mainieri, 2019), with 8 of the 200 recognized eco-regions around the world with 21 times more plant species per km^2 than Brazil (ANAM, 2010). About 10000 species of vascular plants have been described in the country (Correa et al., 2004), but this list has increased in recent years with reports of described species and new distribution ranges in Panama (Flores et al., 2016, 2017, 2018; Ortiz et al., 2019).

The Panamanian flora presents reservoirs of natural products with great value for bioprospecting new therapeutic treatments with high economic and medicinal potential (Gupta, 2004, 2008; Leija et al., 2014). Therefore, it is important and necessary to increase further chemical, biological and toxicological studies of the country's plants.

One of the first works on ethnobotany in Panama was conducted by Esposito et al. (1985), including the first phytochemical characterizations of leaves and stem in river almond (*Terminalia catappa L.*), finding the presence of tannins. Muhammad and Mudi (2011); Mena et al. (2015) mentioned the importance in traditional and medicinal uses (e.g., tonic-astringent properties), in countries such as Costa Rica, Colombia and others. In Panama, locals of Los Santos report their use as a medicinal plant (Torres et al., 2017). This species is native to Southeast Asia and was introduced in Panama (Correa et al., 2010; Farnum and Murillo, 2015; Farnum and Yángüez, 2015; Jiménez and Espino, 2020).

Secondary compounds (e.g., triterpenic acids, anti-inflammatory) of ethanolic extracts of *T. catappa* leaf have been reported (Fan et al., 2004). Some flavone glycosides [6-C-(2''-O-galloyl)-b-D-glucopyranoside] have also been isolated from ethanolic extracts of the dry leaf with bioactive antioxidant functions (Lin et al., 2000). Aphrodisiac functions have been reported to the seed, with the presence of alkaloids, oils, amino acids and peptides (Ratnasooriya and Dharmasiri, 2000). Similarly, it has been observed that dry leaf extract inhibits the growth of *Bacillus subtilis*, a soil bacterium that can act as a biofungicide (Lahlali et al., 2013), and *Staphylococcus aureus*, a bacterium in our body that could cause infections if not controlled (Lowy, 1998).

Hepatoprotective bioactive functions against acute liver damage induced by carbon tetrachloride (CCl_4) and D-galactosamine, hepatocyte injury induced by (D-Galn) have also been determined from chloroform extracts of leaves. This active function is related to the presence of triterpenes (Gao et al., 2004). Researchers have established that the study of the different parts of T. catappa L. is pivotal for bioprospecting and creating possible drugs for diseases (Muhammad and Mudi, 2011; Chanda et al., 2013; Jiménez and Rebolledo, 2015; Calderón et al., 2013).

There are few studies carried out in Panama on *T. catappa L.* and upon analyzing the potential of this species for future studies focused on bioprospecting secondary bioactive compounds, the aim of this research is to conduct the first preliminary characterization of the ethanolic extracts of new leaves, green and mature fruits in *T. cattappa L.* in Panama.

2 Materials and Methods

2.1 Collection Area

Vegetable parts of *T. catappa* were collected in the community of Bella Vista, Limones Community, Punta Burica, Panama (8° 2′7.15"N, 82°53′22.38"W) (Figure 1). This community separates the Pacific coast of Panama with Costa Rica. The human population living in this area has fragmented forests for socio-economic activities, such as grasslands and oil palm monoculture (Miranda, 2013). The area has a humid tropical climate, with an annual rainfall of 4247.8 mm and an annual average temperature of 26.4-27°C. According to Holdridge, the original dominant vegetation in the region is the Tropical Wet Forest (Miranda, 2013).

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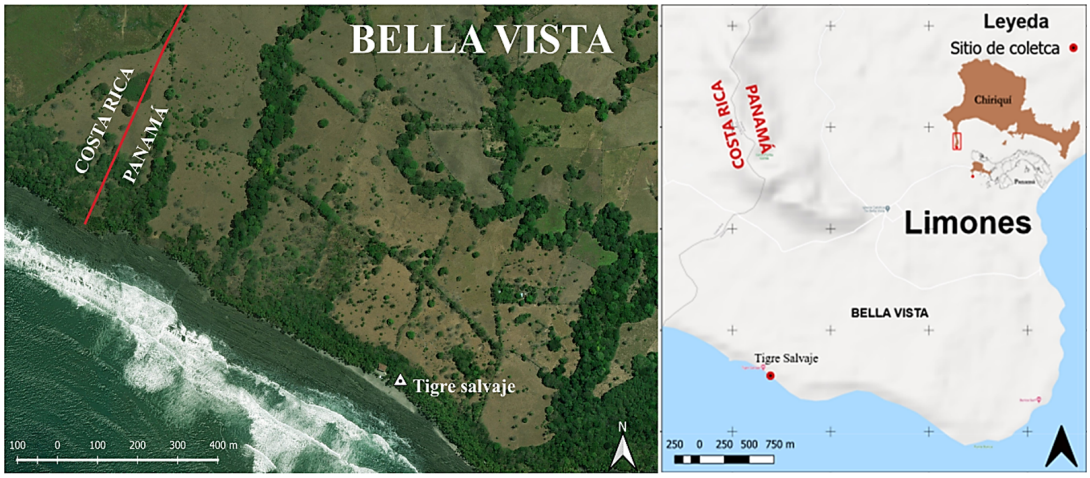

Figure 1. *T. catappa L.* collection area.

2.2 Plant Material Collection and Preparation

Samples were collected during dry to rainy transition (May-June, 2017). New leaves (GL) were collected, which were closest to the base of the bud and are green, and differ from dried ones because they change from red-orange to brown. Samples of green fruits (GF) and ripened fruits (RP) were also collected. GF is green and RF is yellow, and are qualitatively differentiated (Figure 2). The different plant parts were properly pressed and taken to the Herbarium of the University of Autonomous Chiriquí (UNACHI), where they were subsequently identified by botanical specialists.

Direct treatment of almond GL, GF and RF was performed, which consisted of placing each part separately in 95% of ethanol for one day. The ethanolic extract of these parts was obtained by gravity filtering. A filter paper was placed in a funnel to separate the solid part of the liquid. This extract was then concentrated in a vacuum rotavapor (BUCHI Rotavapor^{TM} R-210) for further analysis (Hostettmann et al., 2008).

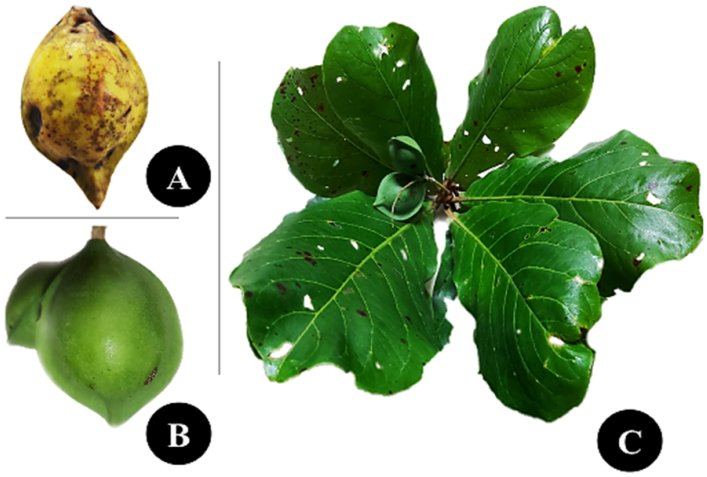

Figure 2. Photographic illustration of the vegetable parts of the almond tree (*T. catappa L.*) A. Ripened fruit, B. Green fruit, C. New leaf and green fruit.

2.3 Phytochemical characterization

Different qualitative and semi-quantitative tests were performed to determine the three large groups of secondary compounds (phenolic compounds, nitrogenous compounds and terpenes). Qualitative signals were identified by precipitates, color change, and foam formation, when the reagent corresponding to the type of metabolite intended to be characterized was added. The semiquantitative ones were evaluated as follows: Absence (–), mild presence (+), mean presence (++), abundant presence (+++), according to the intensity of the qualitative signals mentioned above. Triplicates were performed for each test with reagent and extract control samples, avoiding related false positives.

Nitrogen compounds (alkaloids) were characterized with Draguendorff, Mayer, Wagner and Hager. Each subgroup of terpenes was characterized with the corresponding reagent, terpenoids with Rosenthaler, phytosterols with Liebermann-Buchard and triterpenes with Salkowski. Ferric chloride at 5% ($FeCl_3$) was used in the identification of tannins; flavonoids were characterized with concentrated H_2SO_4 and 5% NaOH. For the identification of saponins, an aqueous solution of the extracts was prepared and agitated, and the formation of foam indicates their presence. The identification of cardiotonic glycosides was done with the Legal test. For the identification of gums and mucilage, 2 mL of the extract was dissolved in 2.5 mL of distilled water, and 5 mL of 95% alcohol was added with constant agitation (Rocha de Albuquerque, 2000; Hostettmann et al., 2008).

Infrared Spectroscopy (FTIR) and UV-Visible Spectroscopy (UV) analysis were performed on the raw extract of each part to determine which functional groups were present and are consistent with the base structures of the screening-detected compounds (Hostettmann et al., 2008).

2.4 Data analysis

The Past 4.02 program was used to graph the relative abundance of the secondary compounds present in each of the extracts of the studied plant parts. The Whittaker index was applied to assess the percentage difference in the phytochemical composition in different plant parts. This analysis was assessed for the presence (1) and absence (0) of the secondary compound groups (Whittaker, 1960).

3 Results and Discussion

This study extends the preliminary phytochemical information of *T. catappa L.* in Panama, based on the qualitative phytochemical characterization of the different parts of *T. catappa L.* It was possible to identify the three large groups of secondary compounds in the plant, presenting similarities in the composition of secondary compounds. The semiquantitative characterization or abundance of these compounds changes in each part, with greater abundance of nitrogen compounds (alkaloids) and phenolic compounds (tannins, flavonoids).

The presence of terpenoids was detected, but in very low abundance, and absence of phytosterols was observed. There was also no presence of cardiotonic glycosides, or gums and mucilage (Table 1, Figure 2). This preliminary characterization is similar to other reports. The abundant presence of phenolic compounds or polyphenolic acids is a characteristic of leaves of this plant (Tanaka et al., 1986), such as gallic acid, which is considered to be one of the most abundant (Marrero and Morales, 2016).

Table 1. Qualitative and semi-quantitative characterization of the different parts of *T. catappa L.*

Secondary compounds	New leaf	Ripened fruit	Green fruit
Alkaloids	+++	+	+
Phytosterols	-	-	-
Terpenoids	+	+	-
Tanins	+++	+	++
Flavonoids	+	++	++
Carbohydrates	+	+++	+
Saponins	+++	+++	-
Gums and Mucilages	-	-	-
Cardiotonic Glycosides	-	-	+

Legend: absence (–), mild presence (+), mean presence (++), abundant presence (+++).

FTIR signals for the extract of almond GF (*T. catappa L.*) were aromatic compounds (1770-2010 cm^{-1}), OH of phenolic compounds (1066 cm^{-1}), and C=O-C of aromatic ethers (1229 cm^{-1}), indicating a possible presence of polyphenolic acid groups, gallic tannins, saponins and flavonoids. As for GL, these show C-H (2946 cm^{-1}) and C=H (1662 cm^{-1}) pressures, which could be possible indicators of terpenoid chains or phytosterols. Similarly, RF and GF show harmonic signals, which indicate aromatic compounds (1770-2010 cm^{-1}) along with OH signals, which indicate possible phenolic compounds.

In UV spectroscopy of the extracts of GL and GF of the almond (*T. catappa L.*) peaks of 255nm, 294nm, 377nm were obtained, which represent aromatic, nitrogenous compounds that could be tannins, alkaloids and flavonoids; saponins are found at wavelengths of 666 and 662 nm, because of their complex skeletal structures.

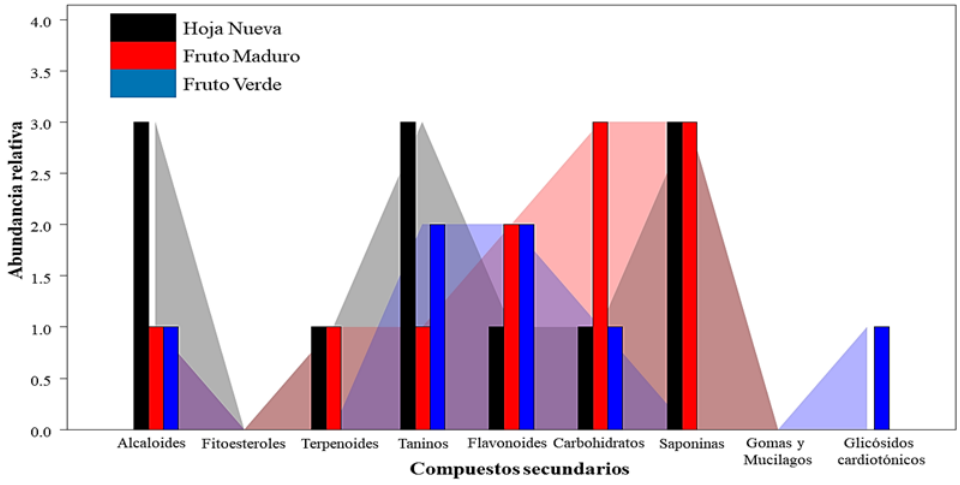

Figure 3. Relative abundance with respect to the semi-quantitative characterization of secondary compounds.

Research confirms that the abundance of polyphenolic compounds is related to bioactive functions, such as antioxidants of great interest at the pharmacogenetic level (Tanaka et al., 1986; Masuda et al., 1999; Lin et al., 2001; Marques et al., 2012). One of the studies that test the possible effectiveness of phenolic compounds (Punicalagin) as antioxidants is the one presented by Chen et al. (2000), where Pu-

nicalagin suppresses the generation of bleomycin-induced intracellular free radicals, identified as superoxide and hydrogen peroxides, avoiding genotoxicity. It has also been reported that punicalagin and punicalin have antihepatotoxic activity on the toxicity induced by acetaminophen (Paracetamol) in rats' liver (Lin et al., 2001).

The Whittaker index only showed a 23.53 % difference between the parts of the plants. GL with RF presented the same groups of secondary compounds. GF with respect to GL and RF presented 27.27 % difference in phytochemical composition. The green fruit presented five groups of secondary compounds, comprising nitrogen compounds (alkaloids) and phenolic compounds (tannins and flavonoids). There was no presence of terpenoids. In RF, six groups belonging to the three major groups were identified. GF presented cardiotonic glycosides with greater abundance of tannins; however, ripened fruit presented terpenoids, with high abundance of carbohydrates and low tannins.

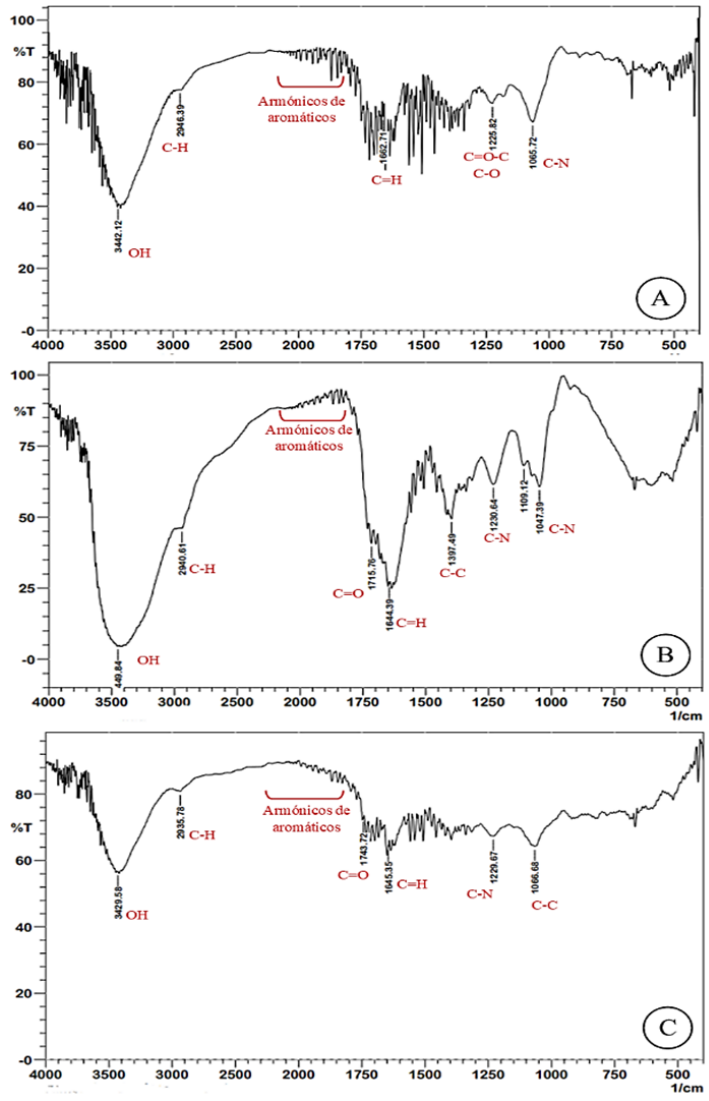

Figure 4. FTIR of the different plant parts of *T. catappa L.* A. New leaf B. Ripened fruit C. Green fruit.

Variations in fruit are common in maturation processes where higher carbohydrate or sugar content is produced; there is also an increase in the production of volatile or aromatic compounds (terpenoids) and a decrease in others, in this case tannins (Arrieta et al., 2006). The presence of flavonoids is also important as they give color to the vegetable parts and indicate maturation. In the case of almond fruit, it decreases the green color (chlorophyll) and increases the yellow color associated with a type of flavonoid (anthocyanin) (Ronald, 2011).

These variations agree with what was reported above, where a considerable increase in carbohydrates is observed in the mature state, being one of the predominant compounds, followed by terpenoids and phenolic compounds (Ratnasooriya and Dharmasiri, 2000; Santos et al., 2016). These carbohydrate values have shown that almond fruit has a high energy value; similarly, they have been reported to provide basic substances for the growth and maintenance of body functions (Lima, 2012). At present, it is related with the presence of saponins. Therefore, they have been used in various products as raw material in the enrichment of existing formulations in the food industry (Nagappa et al., 2003; Lima, 2012; Chanda et al., 2013).

4 Conclusions

In this study, the first phytochemical composition was achieved of the ethanolic extracts of green leaves, green fruits and ripened fruits of *T. catappa L.* in Panama. The semi-quantitative characterization or abundance of phytochemical compounds changes for each part, and nitrogen compounds (alkaloids) and phenolic compounds (tannins, flavonoids) were observed with greater abundance. Low abundance of terpenoids and absence of phytosterols, cardiotonic glycosides, gums and mucilage were detected.

Considering the phytochemical composition of *T. catappa L.* reported in other studies and its similarity with what was found in this work, it can be considered as a potential resource for the health, since it has a great ethnobotanic and pharmacological value in the food industry of Panama.

Acknowledgments

The authors thank the School of Chemistry and Natural Products and Biotechnology Research Center (CIPNABIOT) of the Autonomous University of Chiriquí (UNACHI) by having provided the facilities, equipment and reagents of the laboratories to carry out phytochemical analyses. They also thank the teachers and researchers of the School of Chemistry-UNACHI for the teaching, training and support provided in the acquisition of knowledge to carry out this research. Milagros Centeno-Beitia and Karoline Montezuma for the collection of samples. Ph.D. Pedro Méndez Carvajal for the recommendations in the manuscript.

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Natural Products

Evaluation of PLA active biodegradable films incorporated of essential oils to inhibit microbial adhesion

Evaluación de películas biodegradables activas de PLA incorporada de aceites esenciales para inhibir adhesión microbiana

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Abstract

An evaluation of incorporated polylactic acid (PLA) active biodegradable films of essential oils to inhibit microbial adhesion was performed using a 2_V^{5-1} fractional exploratory design, combining the factors, amount of PEG 400 plasticizer (10 and 20 %), amount of cinnamon essential oil (0.5 % and 1 %), amount of oregano essential oil (0.5 % and 1 %), *Salmonella* spp. concentration (10^3 CFU mL^{-1} and 10^4 CFU mL^{-1}) and *Staphylococcus aureus* concentration (10^3 CFU mL^{-1} and 10^4 CFU mL^{-1}). The results of microbial adhesion inhibition test showed that the maximum inhibition percentage reached 73.82 ± 0.35 % corresponding to experiment 7 (bce), which contains 10 % of PEG 400, 1 % of cinnamon essential oil, 1 % of oregano essential oil, 10^3 CFU mL^{-1} of *Salmonella* spp. concentration, 10^4 CFU mL^{-1} of *Staphylococcus aureus* concentration. Statistical analysis determined that there is strong significant evidence ($p - value = 0.0283$) that *Staphylococcus aureus* concentration influences the inhibition percentage to microbial adhesion; as well as that the cinnamon essential oil- *Salmonella* spp. interaction has little significant evidence ($p - value = 0.0711$) that influences the inhibition percentage. Inhibition results greater than 60 % have the highest concentration of *Staphylococcus aureus* as a common factor. PLA active biodegradable films with a higher inhibition percentage can potentially be used in the food industry as a barrier mechanism to avoid bacterial contamination.

Keywords: Biopolymer, food, barrier, infectious agent.

Resumen

La evaluación de películas biodegradables activas de ácido poliláctico (PLA) incorporado de aceites esenciales para inhibir la adhesión microbiana se realizó mediante un diseño exploratorio fraccionario 2_V^{5-1}, combinando los factores, cantidad de plastificante PEG 400 (10 y 20 %), cantidad de aceite esencial de canela (0,5 y 1 %), cantidad de aceite esencial de orégano (0,5 y 1 %), concentración en unidades formadoras de colonias (UFC) de *Salmonella* spp. (10^3 y 10^4 UFC mL^{-1}) y concentración de *Staphylococcus aureus* (10^3 y 10^4 UFC mL^{-1}). Los resultados del ensayo de inhibición a la adhesión microbiana mostraron que el porcentaje máximo de inhibición alcanzó el $73,82 \pm 0,35\,\%$, correspondiente al experimento 7 (bce), el cual contiene 10 % PEG 400, 1 % aceite esencial de canela, 1 % aceite esencial de orégano, 10^3 UFC mL^{-1} de concentración de *Salmonella* spp. y 10^4 UFC mL^{-1} de concentración de *Staphylococcus aureus*. El análisis estadístico determinó que existe evidencia significativa (valor $p = 0,0283$) que indica que la concentración de *Staphylococcus aureus* influye en el porcentaje de inhibición a la adhesión microbiana; así como también, que la interacción del aceite esencial de canela-*Salmonella* spp. tiene poca evidencia significativa (valor $p = 0,0711$) que influye en el porcentaje de inhibición. Los resultados de inhibición están en función del tipo de bacteria, siendo mayor para las Gram positivas. Los resultados de inhibición superiores al 60 % tienen como factor común la concentración más alta de *Staphylococcus aureus*. Por lo tanto, las películas biodegradables activas de PLA con mayor porcentaje de inhibición pueden usarse potencialmente en la industria alimentaria como mecanismo de barrera para evitar contaminación bacteriana.

Palabras clave: Biopolímero, alimento, barrera, patógenos.

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1 Introduction

The use of biodegradable packaging to replace polyolefins with a low degradability rate is one of the main challenges of the food industry, as are efforts to reduce the number of people affected by foodborne diseases by encouraging the application of biocontrols and other barrier mechanisms to ensure food safety for consumers (Tauxe et al., 2010; Jahid and Ha, 2012), especially in minimally processed or fresh food, considering for example, outbreaks of illnesses caused by *Salmonella* spp. have been linked to contamination of cucumbers, fresh papaya, cut melon and pineapple, chicken, eggs, raw tuna, ground beef, ground turkey meat, and many other food (Centers for Disease Control and Prevention, 2020).

The World Health Organization (WHO), estimates that foodborne diseases affect one in ten people globally, and cause 420,000 deaths per year, with the main etiological agents being *Norovirus, E. coli, Campylobacter* spp., and non-typhoidal *Salmonella* (WHO, 2016). The Centers for Disease Control and Prevention estimates that each year in the United States, 48 million people are affected by foodborne illness, out of which 128,000 are hospitalized and 3,000 die (Centers for Disease Control and Prevention, 2019). Out of the total affected, 9.4 million people (20%) become ill from 31 known foodborne pathogens, and 38.4 million (80%) are affected by unspecified agents that are in food whose symptoms start with acute gastroenteritis. The estimated figures indicate that foodborne illnesses are caused by viruses (59%), bacteria (39%), and parasites (2%). The pathogens causing most of the illnesses are *Norovirus* (58%), *Salmonella* spp. (11%), *Clostridium perfringens* (10%), *Campylobacter* spp. (9%), and *Staphylococcus aureus* (3%). Of the total estimated hospitalizations, 64% are caused by bacteria, 35% by *Salmonella* spp., and 15% by *Campylobacter* spp. Of the total estimated deaths, 64% are caused by bacteria, of which 28% correspond to *Salmonella* spp., 19% to *Listeria monocytogenes*, and 6% to *Campylobacter* spp. (Scallan et al., 2011).

The use of essential oils is an option to improve the properties of biodegradable films or packaging, providing antibacterial properties against pathogens that cause foodborne diseases (Calo et al., 2015). Among these essential oils are oregano oil (*Origanum vulgare*) and cinnamon essential oil (*Cinnamomum zeylanicum*), both categorized as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (FDA, 2020)

Cinnamon essential oil is considered useful for food preservation due to its antioxidant, antifungal and antibacterial properties. These properties are a consequence of its Cinnamaldehyde (94.65% - 65%), D-Limonene (3.55%) and Eugenol (1.8%) content (Burt, 2004; Cardoso-Ugarte et al., 2016; Echegoyen and Nerín, 2015). Studies on the mechanism of action and minimum inhibitory concentrations against *Yersinia enterolitica* (0.075 mg mL^{-1}), *Listeria monocytogenes* (2.5 mg mL^{-1}), *Bacillus cereus* (2.5 mg mL^{-1}), *Escherichia coli* (5 mg mL^{-1}), *Salmonella enterica serovar Typhimurium* (5 mg mL^{-1}) and *Staphylococcus aureus* (5 mg mL^{-1}), have determined that the release of intracellular potassium ions, reduction of metabolic and replication activity resulted as a consequence of the essential oil (Duan and Zhao, 2009; Silveira et al., 2012).

Oregano essential oil has a high antioxidant and antimicrobial power, which favors the preservation of the chemical, sensory, nutritional and microbiological quality of food, due to the effect of its main components Carvacrol (0.5-88.7%), Thymol (3.1-82%), 1,8-Cineole (0.1-14%), p-Cymene (2.7-28.0%) (Franz and Novak, 2009; Ventura et al., 2011; Teixeira et al., 2013; Rostro-Alanis et al., 2019). Gram-negative bacteria, *E. coli, Pseudomonas aeruginosa, Vibrio cholerae Salmonella enterica serovar Typhimurium, Salmonella choleraesuis*, and Gram-positive bacteria, *S. aureus, Listeria monocytogenes* and *B. cereus* have been used to evaluate the antimicrobial activity of oregano essential oil and its active principles, and out of these only *Pseudomonas aeruginosa* has shown high resistance (Albado Plaus et al., 2001; Oussalah et al., 2007; Emiroğlu et al., 2010; Sung et al., 2013; Calo et al., 2015). This bactericidal action is due to its active principles that increase the permeability of the cytoplasmic membrane of microorganisms, causing cell death (Lambert et al., 2001; Rhayour et al., 2003; Sung et al., 2013; Calo et al., 2015).

A good synergistic antibacterial effect of Cinnamaldehyde and Carvacrol was demonstrated by Ye et al. (2013), especially against *E. coli* and *S. aureus*; thus, the use of essential oils containing these compounds can lead to the development of bio-

polymers with high bactericidal activity for food preservation.

Regarding the creation of biodegradable films, the semicrystalline biopolymer polylactic acid (PLA), due to its characteristics of transparency, safety, biodegradability and ease of processing is presented as a suitable polymer for food packaging (Jamshidian et al., 2010; Lalanne et al., 2011; Burgos et al., 2013; Blanot, 2017; Salazar et al., 2014; Ruellan et al., 2015).

PLA biofilms with essential oils (bergamot, lemongrass, rosemary, clove, myrtle, thyme, cinnamon, garlic, and oregano) have been elaborated with positive results both in rheological, thermal, antioxidant and antibacterial aspect (Ahmed et al., 2016; Qin et al., 2017; Yahyaoui et al., 2016; Anuar et al., 2017; Scaffaro et al., 2018; Zeid et al., 2019), obtaining biofilms with adequate transparency, more flexible, less permeable to water vapor and oxygen, without significantly affecting the thermal degradation profile of PLA, and with antimicrobial activity against Gram-positive and Gram-negative bacteria.

However, the evaluation of the incorporation of these essential oils has been individually, so it is necessary to establish possible combinations of essential oils in biopolymers to obtain better barrier and control effects against pathogenic microorganisms that develop biofilms on different surfaces and cause food diseases, considering possible positive effects on the synergy of the essential oils used and the interactions between them and the other components of the polymer (Calo et al., 2015).

Due to the worldwide need to produce biodegradable packaging and because pathogenic microorganisms use food and packaging as transport to affect the health of consumers, this study aims to evaluate the antimicrobial activity of biodegradable active PLA films with essential oils of oregano and cinnamon.

2 Materials and Methods

Polylactic acid (PLA-Ingeo^{TM} Biopolymer 2003D) was acquired from Nature Works®Co. LLC (USA). The plasticizer polyethylene glycol (PEG 400) and the chloroform solvent were purchased from Loba Chemie Pvt. Ltd (India). Absolute ethanol was obtained from Sigma-Aldrich (United States).

The essential oils of oregano (*Origanum vulgare*) and cinnamon (*Cinnamomum zeylanicum*) were purchased from Nowfoods (Bloomingdale, IL, USA), are 100 % pure, and were obtained by the steam distillation method. According to technical specifications, oregano essential oil (specific gravity: 0.937-0.955 g mL^{-1} at 20°C; refractive index: 1.498-1.521 n_D; ≈75 % Carvacrol) was extracted from dried flower herb, and cinnamon essential oil (specific gravity: 1.010-1.030 g mL^{-1} at 20°C; refractive index: 1.573-1.591 n_D; ≈60 % Cinnamaldehyde) was extracted from dried inner bark of the plant.

Culture media and reagents for microbiological tests were purchased from Difco (USA). The pathogenic bacteria used in this research, *Staphylococcus aureus* (ATCC 25923) and *Samonella* spp. (ATCC 14028) were obtained from the strain bank of the Biotechnology Research Center of Ecuador (CIBE, Ecuador).

2.1 Determination of the component solubility with PLA

PLA solubility with chloroform, PEG 400, Carvacrol (main component of oregano essential oil) and Cinnamaldehyde (main component of cinnamon essential oil) was determined as described by Ruellan et al. (2015). Equation 1 and Equation 2 are used to determine the distance (Δδ) of the compound with PLA and the relative differential energy (RED), respectively. The Hansen solubility parameters needed to apply the equations were consulted in the user manual described by Hansen (2007).

$$\text{Distance}(\Delta\delta) = ((\delta_{\text{d comp}} - \delta_{\text{d PLA}})^2 + (\delta_{\text{p comp}} - \delta_{\text{p PLA}})^2 + (\delta_{\text{h comp}} - \delta_{\text{h PLA}})^2)^{0,5} \quad (1)$$

$$RED = \frac{Distance}{Ratio} \quad (2)$$

Where, δ_d, δ_p and δ_h are the components of the solubility parameters.

2.2 Minimum inhibitory concentration of essential oils

The minimum inhibitory concentration (MIC) of each essential oil was determined by the methodology described by Pazmiño et al. (2020), where the MIC was determined by microdilution assay in nutrient broth and supplemented with nutrient agar of 0.15% (w/v) concentration; the amount of 50 μL of this broth was distributed from the second to the twelfth well in each row of a 96-well polypropylene microtiter plate (HDM Cia. Ltda., Ecuador).

A 4% dilution of each essential oil was prepared with bacterial agar and nutrient broth. Independently, 100 μL of each prepared dilution was poured into the first well of each row of the microtiter plate; and then 50 μL in scalar dilution were transferred from the second to the eleventh well, thus obtaining an amount of essential oil between 2% (v/v) and 0.0019% (v/v) from the first well to the eleventh well. The twelfth well did not receive the essential oil and was considered as a growth control. Then, 50 μL of *Salmonella* spp. suspension of 10^6 - 10^7 colony forming units (CFU mL^{-1}) were added to each well, except to the eleventh well, which was considered as witness. The assay was done in triplicate, and the same procedure was performed with *S. aureus* (10^6 - 10^7 CFU mL^{-1}). The plate was incubated at 37°C for 18 h.

2.3 Experiment design

To evaluate the inhibition of microbial adhesion on the plastic film, the following factors were considered: amount of PEG 400 (A), amount of cinnamon essential oil (B), amount of oregano essential oil (C), concentration of *Salmonella* spp. (D) and concentration of *S. aureus* (E), each factor evaluated at two levels (Table 1). For this purpose, an exploratory fractional design 2_v^{5-1} with a generating ratio E = ABCD and in two blocks (AB=CDE) (Table 2) was programmed and analyzed in RStudio Version 0.99.486. Sixteen experiments were carried out, each with its replication.

Table 1. Levels of each factor to be assessed in the experimental design

Factor	Low level (-1)	High level (1)
Amount of PEG 400 (%)	10	20
Amount of cinnamon essential oil (%)	0,5	1
Amount of oregano essential oil (%)	0,5	1
Concentration of *Salmonella* spp. (CFU mL^{-1})	10^3	10^4
Concentration of *Staphylococcus aureus* (CFU mL^{-1})	10^3	10^4

2.4 Development of biodegradable active films based on PLA and essential oils

PLA pellets were dried at 40°C for 48 hours, so that the moisture content before processing was 0.29% measured on a thermobalance (KERN, Germany, Mod MLB50-3). The plate casting method was performed with the methodology described by Pazmiño et al. (2020). The amount of 0.45 g of PLA and PEG 400 (10 or 20% with respect to PLA) were dissolved in 10 mL of chloroform. After dissolving the resin in the solution at room temperature, the essential oils were added in the corresponding amount (0.5% or 1% with respect to PLA) while stirring for a few minutes until dissolved. The solution was then poured into Petri dishes, adding $5 \pm 0{,}5$ g of solution in each dish, and finally left for 24 h at room temperature inside the extraction chamber for the solvent to evaporate. Films without addition of essential oils (control films) were also prepared. The films obtained had a thickness between 27 to 30 μm, measured by a micrometer (Qualitest, United States, Mod MRG-25).

2.5 Inhibition adhesion percentage of microorganisms on active biodegradable films

The inhibition percentage to microbial adhesion was performed by modifying the method applied by Coronel-León et al. (2016). According to the experimental design, 1 mL of each microorganism

concentration (10^3 o 10^4 CFU mL^{-1}) was inoculated on the plastic film, with incubation at 37°C for 24 hours. At the end of the incubation period, each film was washed twice with 1 mL of distilled water to remove residues. 1 mL of absolute ethanol was added, placing the plates in the extraction chamber for 24 hours to evaporate the alcohol. The plastic film was cut into a rectangle (8.75 cm^2), and placed on a slide where the crystal violet reagent (500 μL) was added and left for one minute. Then, it was rinsed with distilled water to eliminate the excess of the reagent. Once dried with paper towel, it was cut into 8 pieces to introduce them in a test tube, adding 2 mL of absolute ethanol. Subsequently, 200 μL of each solution was deposited in the wells of the 96-well microplate to measure the concentration of the reagent by UV-Vis absorption spectrum in the Synergy^{TM} HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, United States) with an absorbance of 595 nm at 24.8°C.

The inhibition percentage to microbial adhesion (%) was calculated with Equation 3. Where, A_c represents the absorbance of the solution in the well corresponding to the plastic film according to the experimental design, and A_0 represents the absorbance of the control film solution (absence of essential oil). The experiment was performed in triplicate, and the results are presented as an average.

$$\% = [1 - (A_c/A_0)] \times 100 \quad (3)$$

Table 2. Fractional exploratory design 2_V^{5-1} with a generating ratio E = ABCD in two blocks (AB=CDE)

Experiment	Factors					AB	Totals	Blocks
	A	B	C	D	E			
1	-1	-1	-1	-1	1	1	e	1
2	1	-1	-1	-1	-1	-1	a	2
3	-1	1	-1	-1	-1	-1	b	2
4	1	1	-1	-1	1	1	abe	1
5	-1	-1	1	-1	-1	1	c	1
6	1	-1	1	-1	1	-1	ace	2
7	-1	1	1	-1	1	-1	bce	2
8	1	1	1	-1	-1	1	abc	1
9	-1	-1	-1	1	-1	1	d	1
10	1	-1	-1	1	1	-1	ade	2
11	-1	1	-1	1	1	-1	bde	2
12	1	1	-1	1	-1	1	abd	1
13	-1	-1	1	1	1	1	cde	1
14	1	-1	1	1	-1	-1	acd	2
15	-1	1	1	1	-1	-1	bcd	2
16	1	1	1	1	1	1	abcde	1

3 Results and Discussion

3.1 Solubility of components with PLA

The values obtained with Equation 1 and Equation 2 are shown in Table 3. Based on the criterion that distances less than 5 (J cm^{-3})$^{0.5}$ indicate a better solubility of the compound with the polymer, it can then be stated that the compounds have a good solubility with PLA, especially Cinnamaldehyde from cinnamon essential oil. The RED value close to zero indicates a better compatibility of the compounds with the polymer, and a value greater than 1 indicates a non-miscibility of the compound with the polymer, and according to the results, all the compounds are completely miscible.

Table 3. Distance results and RED value of Carvacrol, Cinnamaldehyde, PEG 400 and chloroform, in relation to PLA.

Compound	Hansen solubility parameters (J cm^{-3})$^{0.5}$			Distance	RED*
	δd	Δp	Δh	$\Delta\delta$	
PLA[a]	18.60	9.90	6.00	—-	—-
PEG400[a]	17.90	4.20	14.20	10.01	0.94
Carvacrol[b]	19.00	4.50	10.80	7.24	0.68
Chloroform[b]	17.80	3.10	5.70	6.85	0.64
Cinnamaldehyde[b]	19.40	12.40	6.20	2.63	0.25

*PLA Radius = 10.7 (Ruellan et al., 2015).
a: (Ruellan et al., 2015); b: (Hansen, 2007), values of carvacrol as an isomer of thymol.

3.2 Minimum inhibitory concentration of the essential oil

The results show that both essential oils inhibit Gram-positive *S. aureus* and Gram-negative *Salmonella* spp. bacteria (Table 4). The MIC 0.016% (v/v) of oregano essential oil for *Salmonella* spp. is lower than the MIC of cinnamon essential oil for the same bacteria; while the MIC of both essential oils against *S. aureus* is equal, being 0.031% (v/v).

The MIC of oregano essential oil for *Salmonella* spp. is lower compared to the results of Oussalah et al. (2007), where the MIC for *Salmonella enterica serovar Typhimurium* was 0.05% (v/v), and also compared to the results of Pesavento et al. (2015), where the MIC found for *Salmonella enteritidis* and *S. enterica serovar Typhimurium* was 0.125% (v/v). The MIC results of oregano essential oil for *S. aureus* are within the range of values reported by Nostro (2007), where the MIC of oregano essential oil on several strains of this microorganism were between 0.015-0.062% (v/v) and in certain strains up to 0.125% (v/v).

The MIC of cinnamon essential oil for *Salmonella* spp. and *S. aureus* is higher than that found by Sheng and Zhu (2014), where the MIC of cinnamon essential oil extracted from *Cinnamomum cassia* variety was 0.025% (v/v) for both *S. enterica serovar Typhimurium* and *S. aureus*. The MIC results of oregano essential oil and cinnamon essential oil, expressed in mg mL^{-1} in (Table 4) and which were obtained in this research are higher compared to the results found in other investigations, where the MIC of oregano essential oil ranged from 0.16 - 0.2 mg mL^{-1} for *Salmonella spp.* and 0.4-0.9 mg mL^{-1} for *S. aureus*, and the MIC of cinnamon essential oil ranges from 0.2 - 0.5 mg mL^{-1} for both *Salmonella* spp. and *S. aureus* (Chang et al., 2001; Becerril et al., 2012; Boskovic et al., 2015; Martucci et al., 2015; Cui et al., 2019).

Table 4. Minimum inhibitory concentration (MIC) of cinnamon and oregano essential oils for *Salmonella* spp. and *Staphylococcus aureus*.

Essential oil	Active ingredient	MIC *Salmonella* spp.		MIC *Staphylococcus aureus*	
		(%) (v/v)	mg mL^{-1}	(%) (v/v)	mg mL^{-1}
Cinnamon	Cinnamaldehyde	0.063	64.26	0.031	31.62
Oregano	Carvacrol	0.016	15.14	0.031	29.33

The data described above demonstrate the antimicrobial activity of oregano and cinnamon essential oils. Carvacrol from oregano essential oil damages the cell wall and membrane, increases permeability, thus leading to cell lysis (Lambert et al., 2001; Rhayour et al., 2003; Lv et al., 2011; Cui et al., 2019). Cinnamaldehyde from cinnamon essential oil presents different mechanisms of action on its own or along with Carvacrol, collapsing the bacterial structure, as well as disrupting metabolic processes such as cellular energy generation, cytoplasmic material formation and cell division (Kwon et al., 2003; Burt, 2004; Becerril et al., 2007; Bouhdid et al., 2009; Ye et al., 2013; Calo et al., 2015).

3.3 Inhibition adhesion percentage of microorganisms on active biodegradable films

The results of the inhibition percentages to microbial adhesion in the active biodegradable films are detailed in Table 5. The results obtained showed that the maximum inhibition percentage reached 73.82 ± 0.35% corresponding to experiment 7 (bce) of the experimental design, and there is a significant difference with the other results, except with 13 (cde). Experiment 7 (bce) comprised 10% amount of PEG 400, 1% cinnamon essential oil, 1% oregano essential oil, 10^3 CFU mL^{-1} concentration of *Salmonella* spp, 10^4 CFU mL^{-1} concentration of *S. aureus*. This inhibition percentage to adhesion is higher than that reported in studies performed with Lichenysin (4000 μg mL^{-1}) in *S. aureus* (68.73%) (Coronel-León et al., 2016), and with Rhamnolipids (1%) in *S. aureus* (67.7%), and it was not significant in *Salmonella enteritidis* (Do Valle Gomes and Nitschke, 2012).

Table 5. Microbial adhesion inhibition rates of active biodegradable films.

Experiment	PEG 400 (%)	Cinnamon essential oil	Oregano essential oil	*Salmonella* spp. (UFC mL^{-1})	*Staphylococcus aureus* (UFC mL^{-1})	Inhibition of microbial adhesion
1 (e)	10	0.5	0.5	10^3	10^4	$55.04 \pm 1.52^{c,d}$
2 (a)	20	0.5	0.5	10^3	10^3	31.46 ± 2.96^{e}
3 (b)	10	1	0.5	10^3	10^3	$54.71 \pm 0.55^{c,d}$
4 (abe)	20	1	0.5	10^3	10^4	$60.73 \pm 0.39^{b,c}$
5 (c)	10	0.5	1	10^3	10^3	43.49 ± 0.83^{d}
6 (ace)	20	0.5	1	10^3	10^4	48.04 ± 1.96^{d}
7 (bce)	10	1	1	10^3	10^4	73.82 ± 0.35^{a}
8 (abc)	20	1	1	10^3	10^3	47.24 ± 2.40^{d}
9 (d)	10	0.5	0.5	10^4	10^3	58.21 ± 1.34^{c}
10 (ade)	20	0.5	0.5	10^4	10^4	66.11 ± 1.55^{b}
11 (bde)	10	1	0.5	10^4	10^4	$59.83 \pm 1.56^{b,c}$
12 (abd)	20	1	0.5	10^4	10^3	66.34 ± 0.53^{b}
13 (cde)	10	0.5	1	10^4	10^4	$67.73 \pm 0.66^{a,b}$
14 (acd)	20	0.5	1	10^4	10^3	42.93 ± 0.49^{d}
15 (bcd)	10	1	1	10^4	10^3	$51.46 \pm 1.95^{c,d}$
16 (abcde)	20	1	1	10^4	10^4	49.60 ± 2.07^{d}

Values for inhibition percentage of microbial adhesion are means ± SD.
Values followed by different letters are significantly different ($p \leq 0,05$).

The inhibition percentage achieved in experiment 7 (ecb) reveals the high affinity between the radicals of the active ingredients of essential oils, especially oregano essential oil, and the cell membranes of Gram-positive bacteria, causing structural alterations and loss of cell viability (Bouhdid et al., 2009; Lv et al., 2011). Thus, the higher the concentration of this type of microorganisms on a surface, the higher the inhibition percentage.

Studies of *in vitro* evaluations or food matrices (especially meat products) of PLA and other biopolymers (alginate, soy protein) with oregano essential oil and cinnamon essential oil, both employed at 1% independently, showed good results in the microbiological control of Gram-positive and Gram-negative bacteria (Oussalah et al., 2006; Emiroğlu et al., 2010; Anuar et al., 2017); however, these studies do not specify the inhibition percentage to establish more precise comparisons, because they employed inhibition halo measurements.

The results also indicate that experiments 4 (abe), 10 (ade), 12 (abd), and 13 (cde), show inhibitions to microbial adhesion of 60.73 ± 0.39%, 66.11 ± 1.55%, 66.34 ± 0.53%, and 67.73 ± 0.66%, respectively; also showing that the common factor between them and experiment 7 (bce) is the *S. aureus* concentration (10^4 CFU mL^{-1}), except in experiment 12 (abd). There are no significant differences between 7 (bce) and 13 (cde), and there is differen-

ce between the components because they differ in the amount of cinnamon essential oil and the concentration of *Salmonella* spp. with 13 (cde), having the lowest amount of cinnamon essential oil and the highest concentration of *Salmonella* spp. than in 7 (bce). However, as discussed below, at the lower amount of cinnamon essential oil when the concentration of *Salmonella* spp. is higher, the results of the inhibition percentage of microbial adhesion are as high as when 1% cinnamon essential oil is used. This situation contributes to the fact that there is no significant difference between 7 (bce) and 13 (cde).

A model was made with the data from the experiment corresponding to design 2_V^{5-1}, with two blocks of eight observations as specified by resolution V, that included the main effects and the two-way interactions, except for the PEG 400 - cinnamon essential oil (AB) interaction, because it was misled with the blocks. According to the results obtained from this initial model, the PEG 400 - *S. aureus* interaction was removed (p-value > 0.1). In the same way we proceeded with the following models, where the interactions PEG 400 - oregano essential oil, PEG 400 - *Salmonella* spp., cinnamon essential oil - oregano essential oil, cinnamon essential oil - *S. aureus*, *Salmonella* spp. - *S. aureus*, oregano essential oil - *S. aureus*, and oregano essential oil - *Salmonella* spp. were gradually eliminated, because these interactions were not statistically significant either. After eliminating the interactions, the results of the applied model are shown in Table 6.

Table 6. Main effects and two-way interactions with their corresponding effect value and p-value.

Factor or interaction	Effect	p-value
PEG 400	-6.48	0.1422
Cinnamon essential oil	6.34	0.1498
Oregano essential oil	-3.51	0.4029
Salmonella spp.	5.96	0.1727
Staphylococcus aureus	10.63	0.0283
Cinnamon essential oil-*Salmonella* spp.	-8.28	0.0711

There is strong significant evidence (p-value = 0.0283) that *S. aureus* concentration factor influences the inhibition percentage of microbial adhesion (Figure 1) with an effect value of 10.63% between the mean inhibition percentage when *S. aureus* concentration is low (49.48%) and the mean inhibition percentage when *S. aureus* concentration is high (60.11%). Hence, the inhibition percentages of microbial adhesion higher than 60% have the higher concentration of *S. aureus* as a common factor. The cinnamon essential oil - *Salmonella* spp. interaction has little significant evidence (p-value = 0.0711) that influences the inhibition percentage. There is no significant statistical evidence of their influence on the inhibition results regarding the other factors and interactions.

In the graph of cinnamon essential oil - *Salmonella* spp. interaction (Figure 2) is observed that when the amount of cinnamon essential oil and the concentration of *Salmonella* spp. are the lowest (Figure 2), the mean of the inhibition adhesion percentage is 44.51%, and when the amount of cinnamon essential oil is the highest and the concentration of *Salmonella* spp. is still the lowest, the mean of the inhibition percentage is 59.12%; when the amount of cinnamon essential oil is the lowest and the concentration of *Salmonella* spp. is the highest, the mean of the inhibition adhesion percentage is 58.74%, and when the amount of cinnamon essential oil is the highest and the concentration of *Salmonella* spp. is still high, the mean of the inhibition percentage is 56.81%.

Thus, the greatest effect is observed at cinnamon essential oil concentration of 0.5% with an increase in the inhibition percentage of 14.24% when the concentration of *Salmonella* spp. goes from low level (10^3 CFU mL^{-1}) to a high level (10^4 CFU mL^{-1}), while there is a reduction in the inhibition percentage of 2.32% at cinnamon oil concentration of 1% when the concentration of *Salmonella* spp. also changes. When the concentration of cinnamon essential oil goes from 0.5% to 1% and a low *Salmone-*

lla spp. concentration, the inhibition percentage increases by 14.62%, while at high concentration of *Salmonella* spp. the inhibition percentage reduces by 1.94%, which could indicate a tendency to decrease in the inhibition percentage at higher concentrations of *Salmonella* spp.

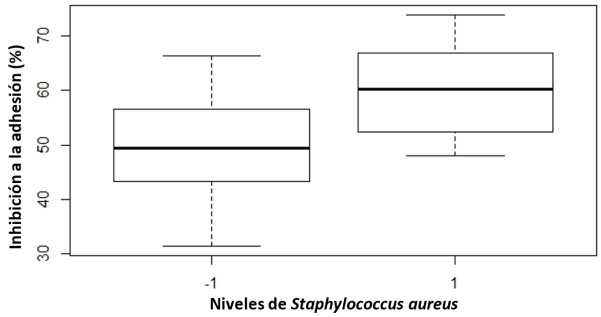

Figure 1. Adhesion inhibition percentage to different concentrations of *Staphylococcus aureus*.

Considering these two factors, it is also evident that the highest inhibition percentage of microbial adhesion 59.12% occurs when the cinnamon essential oil is at 1% and the *Salmonella* concentration is 10^3 CFU mL^{-1}; and the lowest inhibition percentage 44.51% occurs when the cinnamon essential oil is at 0.5% at the same *Salmonella* spp. concentration. Then, Figure 2 indicates that 1% of cinnamon essential oil has greater inhibition effectiveness for the different concentrations of *Salmonella* spp., represented in this research as Gram-negative bacteria.

The results of the cinnamon essential oil - *Salmonella* spp. interaction demonstate that oregano essential oil is less effective against Gram-negative bacteria because it has a lower affinity with the cell membrane of a thin layer of peptidoglycan, opening the action of cinnamon essential oil and its active ingredient on this type of bacteria (Becerril et al., 2007), which are metabolically more difficult to fight because of the external film they develop as a protection mechanism (Cabrera et al., 2007).

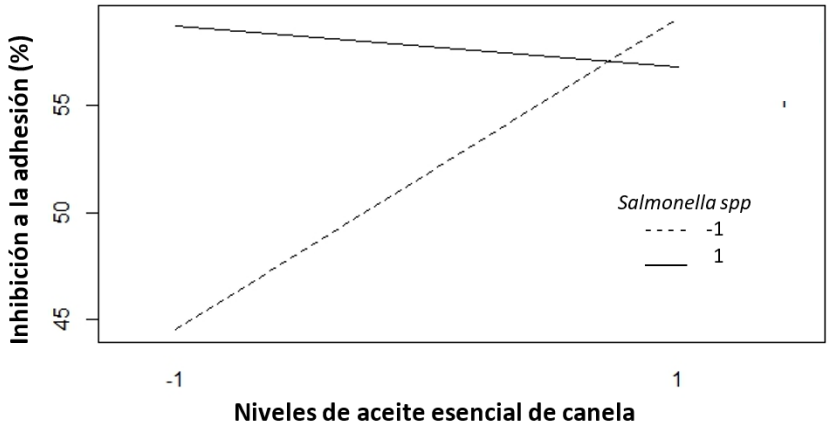

Figure 2. Cinnamon essential oil-*Salmonella* spp. interaction.

4 Conclusions

Biodegradable active films of PLA with oregano and cinnamon essential oils were obtained, with good solubility between the components. The inhibition percentage to microbial adhesion on the active biodegradable films is a function of the representative Gram-positive bacteria model for this research. Statistical analysis determined that there is strong significant evidence that the concentration of *S. aureus* influences the inhibition percentage of microbial adhesion. Thus, inhibitions higher than 60% have higher concentration of *S. aureus* as a common factor.

In addition, the amount of 1% cinnamon essential oil has greater inhibition effectiveness for the different concentrations of *Salmonella* spp. despite registering a slight inhibition decrease when going from the lowest to the highest *Salmonella* spp. concentration.

Biodegradable active PLA films with higher inhibition percentage to microbial adhesion can potentially be used in the food industry as an environmentally friendly alternative to increase additional barrier mechanisms to prevent contamination with *Salmonella* spp. and *S. aureus*, and reduce the number of people infected by these microorganisms.

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Conservation

Modified preservation and fungalysin description for *Batrachochytrium dendrobatidis*

Preservación modificada y descripción de la fungalisina para *Batrachochytrium dendrobatidis*

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Abstract

Batrachochytrium dendrobatidis is a pathogenic fungus causing chytridiomycosis, a cutaneous affection resulting in reduction on Anura populations around the world, because of fungalysins, the most important virulence factor. The *B. dendrobatidis* maps (http://www.bd-maps.net/) is the dataset of information about the *B. dendrobatidis* -related isolates. An alternative available for reconstitution of *B. dendrobatidis* strains from cryopreserved cells include methods relying on the World Organization for Animal Health (OIE) protocol. Most recently, much of the interest in the *B. dendrobatidis* research has focused on its DNA sequencing, especially *B. dendrobatidis* JAM81 and *B. dendrobatidis* JEL423 genomes. OBJECTIVE. To evaluate a modified form from OIE protocol for *B. dendrobatidis* strain criopreservation, and *in silico* analysis of *B. dendrobatidis* fungalysin. OIE protocol was modified using *B. dendrobatidis* EV001, focused on cryopreservant concentrations, antibiotics and recovering to −80°C, and microscopic viability evaluation on Tryptone Gelatin hydrolyzed agar. Besides, Bioinformatics was used for the determination of biochemical characteristics from a *B. dendrobatidis* JAM81 fungalysin. Modified OIE protocol was useful by viability, recovering *B. dendrobatidis* EV001 strain. The characterization of *B. dendrobatidis* JAM81 fungalysin showed a molecular weight of 85 kDa, isoelectric point of 8.33, and tertiary structure among others. This indicated that the protein is a metalloproteinase, it has a PepSY domain for protease inhibition, and a catalytic domain that destroy protein barriers.

Keywords: *Batrachochytrium dendrobatidis*, anura, pathogen, zoospore, cryopreservation, fungalysin.

Resumen

Batrachochytrium dendrobatidis es un hongo patógeno que causa quitridiomicosis, una afección cutánea que resulta en la reducción de las poblaciones de anuros en todo el mundo, debido a las fungalisinas, su factor de virulencia más importante. Los mapas de *B. dendrobatidis* (http://www.bd-maps.net/) contienen la información sobre los aislamientos relacionados con *B. dendrobatidis*. Una alternativa disponible para la reconstitución de cepas de *B. dendrobatidis* es a partir de células criopreservadas, los métodos para esto se basan en el protocolo de la Organización Mundial de Sanidad Animal (OIE). Recientemente, gran parte del interés en la investigación de *B. dendrobatidis* se ha centrado en la secuenciación de su ADN, se encuentran disponibles los genomas de *B. dendrobatidis* JAM81 y *B. dendrobatidis* JEL423. OBJETIVO. Evaluar una forma modificada del protocolo de la OIE para la criopreservación de cepas de *B. dendrobatidis* y el análisis *in silico* de su fungalisina. Se modificó el protocolo de la OIE utilizando la cepa de *B. dendrobatidis* EV001, enfocado en los ítems de concentraciones de criopreservantes, antibióticos y recuperación, desde −80°C, y evaluación de viabilidad microscópica en agar hidrolizado con triptona y gelatina. Además se utilizó Bioinformática para la determinación de características bioquímicas de la fungalisina de la cepa de *B. dendrobatidis* JAM81. El protocolo modificado de la OIE resultó útil para la recuperación de la viabilidad de la cepa *B. dendrobatidis* EV001. La caracterización de la fungalisina de *B. dendrobatidis* JAM81 mostró un peso molecular de 85 kDa, punto isoeléctrico de 8,33 y estructura terciaria característica que indicó que la proteína es una metaloproteinasa, tiene un dominio PepSY para la inhibición de proteasas, y un dominio catalítico asociado a destrucción de barreras proteicas.

Palabras clave: *Batrachochytrium dendrobatidis*, anura, patógeno, zoospora, criopreservación, fungalisina.

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1 Introduction

There are several possible reduction causes of the amphibian population, including the following: commercial consumption; introduced species introduction, land usage, pollutants, climate change, and pathogens. There are more than 4,000 species concerning the anurans, and America tropical areas have high diversity of anurans (32% are in South America), e.g., Cali region (Colombia) had a high index of anuran diversity (35 species), Pichincha area (Ecuador) has a richness of anuran species (20 species), and Bolivar region (Venezuela) reported 26 species (Duellman, 1988). Colombia has a richness of anuran species (Flechas et al., 2017; Lynch et al., 1997), but there are more than 50 threatened species (Fisher and Garner, 2020; Spitzen-van der Sluijs et al., 2017; Rueda-Almonacid et al., 2004). Infectious diseases are one of the factors affecting the status of anurans, and among these is the chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis*. The fungus has harmful effects on frogs and toads across nations. *B. dendrobatidis* has an affinity with keratinized dermis (Lindauer et al., 2019; Green et al., 2010; Vredenburg et al., 2010; Berger et al., 1998). *B. dendrobatidis* seems to affect anurans according to species, alkaloid synthesis, or relationship between skin and environmental temperature. *B. dendrobatidis* proliferates during the rainy season, and water ecosystems stimulate the pathogen presence in the host (Richmond et al., 2009). The disease has been reported in a total of six areas in Colombia: Amazonas, Boyacá, Cauca, Cundinamarca, Nariño and Valle del Cauca, these regions have a tropical climate with little variation in temperature and high humidity (Flechas et al., 2017; Vásquez-Ochoa et al., 2012; Velásquez et al., 2008).

B. dendrobatidis has two stages: a zoospore stage which is motile and flagellated, where zoospores appeared to initiate a colonization of skin frog. Once inside a host, zoospore can develop a thallus which develops into a zoosporangium (or sporangia), containing the next generation of spores, renewing the cycle. Early zoosporangium is present in viable cells causing hyperkeratosis and hydric-electrolite balance disruption, but matured zoosporangium is predominant in the outer keratinized stratums. The discharge tubes are turned towards the skin frog surface, releasing zoospores into the environment (Rosenblum et al., 2010). *B. dendrobatidis* genome shows a high change rate in lineages, which has promoted theories of their pathogenic evolution: NPH (the novel pathogen hypothesis) which is based on an invasive spread model into new ecosystems or hosts, and EPH (the endemic pathogen hypothesis) which is focused on *B. dendrobatidis* disease as a consequence of an altered association among pathogen-anura-environment (Scheele et al., 2019; Rosenblum et al., 2009).

The method of *B. dendrobatidis* cryopreservation was described by the World Organization for Animal Health (OIE) (OIE-World Organization for Animal Health, 2012). This method is based solely on the microorganism culture for an increase in zoospore and zoosporangium concentration; hence, Dimethyl sulfoxide (DMSO) 10% and fetal bovine serum (FBS) 10% at -80 °C must be used. During thawing, cultures are to be subjected to 43 °C, lactose and antibiotics.

The evidence suggests that enzymatic activity of *B. dendrobatidis* directly influences its degree of pathogenicity. The *B. dendrobatidis* penetration into frog epidermal cells probably requires digestive enzymes. A reference strain of *B. dendrobatidis* is cultured and used to produce proteases, which degrade casein and gelatin. Knowledge on genetic bases, and interactions at molecular level lead to gene expression for serin proteases and fungalysin metallopeptidase, two pathogenesis-related genes identified in other fungus (Rosenblum et al., 2010). *B. dendrobatidis* synthesizes fungalysin metallopeptidases, a class of proteins that are essential for microorganims such as *Trichophyton sp.* and *Microsporum sp.* Fungalysin metallopeptidase gene of *B. dendrobatidis* has high expression levels in zoosporangium (McDonald et al., 2020; Rosenblum et al., 2009). This work evaluates changes to OIE protocol for *B. dendrobatidis* cryopreservation and subsequent recovery of this microorganism and *in silico* description of its fungalysin.

2 Materials and Methods

2.1 Strain

The *B. dendrobatidis* strain EV001 was used, which was donated by Universidad de los Andes (Bogotá-Colombia). *B. dendrobatidis* EV001 represents the

first strain isolated in Colombia (from Ubaqué, Cundinamarca), which was recovered from *Rheobates palmatus* (Flechas et al., 2013). The *B. dendrobatidis* culture medium used is a mixture of tryptone and hydrolysed gelatin (TGh) (tryptone 1%, hydrolysed gelatin 0.4%, agar 1%). Tryptone 1% broth has been used for zoospore production. *B. dendrobatidis* EV001 cells were examined under a microscope using lactophenol cotton blue and Congo red stains.

2.2 *Batrachochytrium dendrobatidis* cryopreservation

A modified OIE preservation method was applied to *B. dendrobatidis* EV001. A 2.0 mL portion of 1-week-old tryptone medium inoculated with *B. dendrobatidis* EV001 was diluted with 13 mL of a fresh tryptone medium in a 25 cm^2 cell culture flask and incubated at room temperature for 4 days by gentle agitation. A scraping of the flask walls was done, then the product was centrifuged at 1700 g for 10 min. The supernatant was eliminated, and the pellet was suspended in 0.6 mL of tryptone broth containing DMSO 10% and freezed at -80 °C. A month later, the frozen pellet were placed in water bath at 43 °C for 30 s and transferred to TGh medium without lactose or antibiotics. A randomized design with three replications was used.

2.3 *Batrachochytrium dendrobatidis* fungalysin

A bioinformatic analysis of fungalysin-like virulence factor was done using the corresponding amino acid sequence from *B. dendrobatidis* JAM81 genome deposited in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (NCBI ID: XP_006675712.1). A comparison between the *B. dendrobatidis* JAM81 fungalysin and a fungalysin from *Aspergillus* (Protein Data Bank ID: 4k90) was made with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). ProtParam tool from ExPASy (http://www.expasy.org/) was applied for theoretical description of the *B. dendrobatidis* fungalysin (isoelectric point, molecular weight, hydropathicity, positive residues and aliphatic index). Functional domains were determined using the Conserved Domain Database (CDD) from NCBI. A three dimensional (3D) model of the *B. dendrobatidis* JAM81 fungalysin was calculated by homology modelling using Swiss-model (https://swissmodel.expasy.org/), the model was submitted for refinement to 3Drefine (http://sysbio.rnet.missouri.edu/3Drefine/index.html) and validated, applying QMEAN (https://swissmodel.expasy.org/qmean/). The figure was visualized using Jmol (https://www.bioinformatics.org/firstglance/fgij/).

3 Results

3.1 *B. dendrobatidis* EV001 morphology

B. dendrobatidis EV001 viability was evidenced by means of growth in TGh medium and its morphological structures. *B. dendrobatidis* EV001 shows granular and cream colonies (Figure 1A), and a morphology-like pan can be seen when we examine a Lactophenol Cotton Blue preparation under the microscope, a characteristic of chytridiomycetes (Figure 1B).

B. dendrobatidis EV001 was grown on TGh medium for morphology determination involving zoospores, zoosporangium, discharge tube, thalli or rhizoids (Figure 2 and 3) to establish *B. dendrobatidis* development stages (Figure 4).

3.2 *B. dendrobatidis* cryopreservation method

The *B. dendrobatidis* cryopreservation method based on OIE protocol (OIE-World Organization for Animal Health, 2012) was modified as shown in Table 1. Viability of *B. dendrobatidis* EV001 was confirmed into TGh medium (Figure 5).

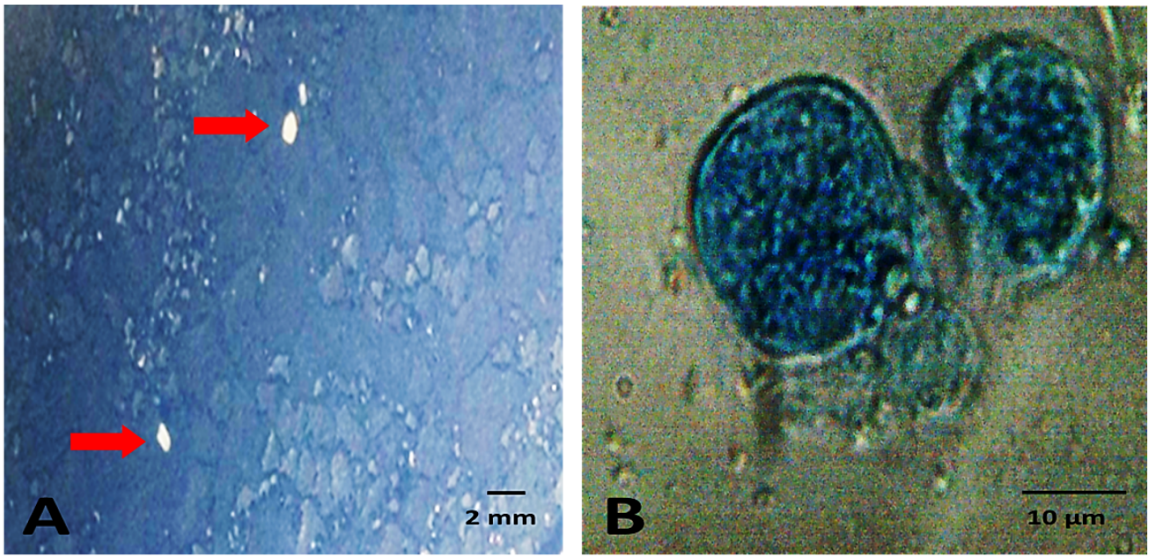

Figure 1. Macroscopic and microscopic morphology of *B. dendrobatidis*. (A) *B. dendrobatidis* EV001 granular breadcrumb colonies on TGh medium (arrows). (B) *B. dendrobatidis* EV001 pan morphology of the fungus (light microscopy at a 40× lens); they typically have 10-40 μm in diameter (Berger et al., 2005).

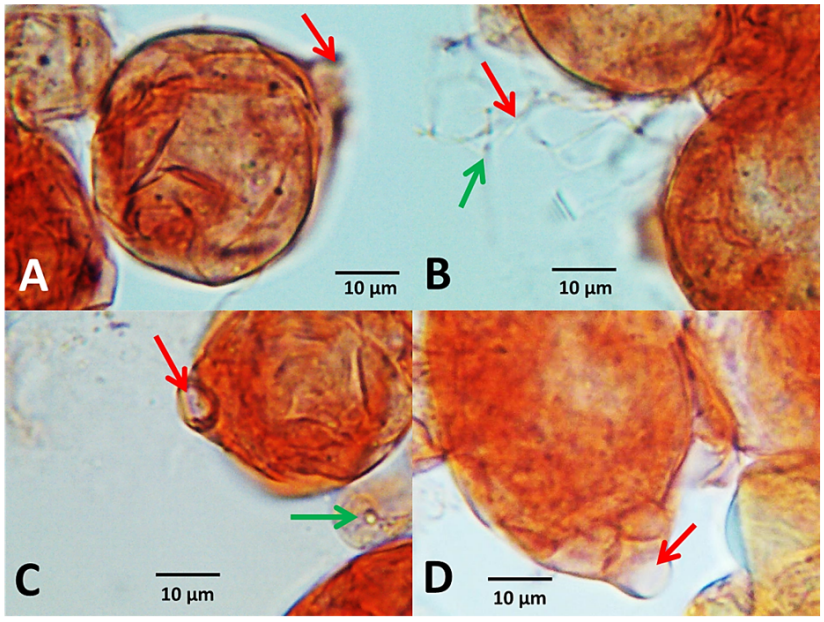

Figure 2. *B. dendrobatidis* EV001 morphology on TGh using Congo red stain. (A). *B. dendrobatidis* EV001 zoosporangium and open discharge tube (red arrow). (B). *B. dendrobatidis* EV001 zoosporangium with thalli (red arrow) and rhizoids (green arrow). (C) *B. dendrobatidis* EV001 zoosporangium with open discharge tube (red arrow), which contains cellulose and chitin, and a zoospore (green arrow). (D) Inoperculate *B. dendrobatidis* EV001 zoosporangium (red arrow). Light microscopy at a 100× lens. Zoosporangium typically has 10-40 μm in diameter (Berger et al., 2005).

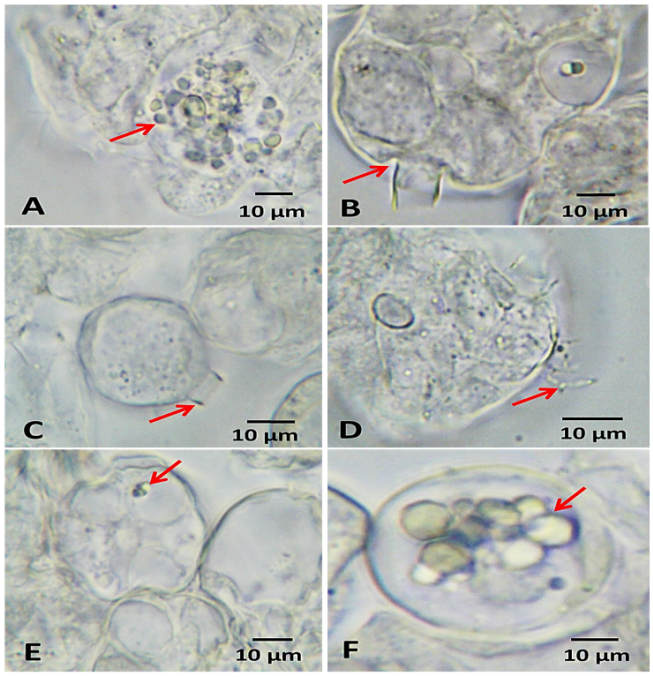

Figure 3. *B. dendrobatidis* EV001 wet mount microscopic examination. (A) *B. dendrobatidis* EV001 zoosporangium and inner zoospores (red arrow). (B) *B. dendrobatidis* EV001 zoosporangium with dictyosomes (red arrow). (C) *B. dendrobatidis* EV001 zoosporangium and discharge tube (red arrow). (D) *B. dendrobatidis* EV001 rhizoid (red arrow) (E) *B. dendrobatidis* EV001 zoospore (red arrow) (F) *B. dendrobatidis* EV001 plasmalemma and inner saccules (red arrow). Light microscopy at a 40× lens. Zoospores typically have 0.7 to 6.0 μm of diameter (Berger et al., 2005).

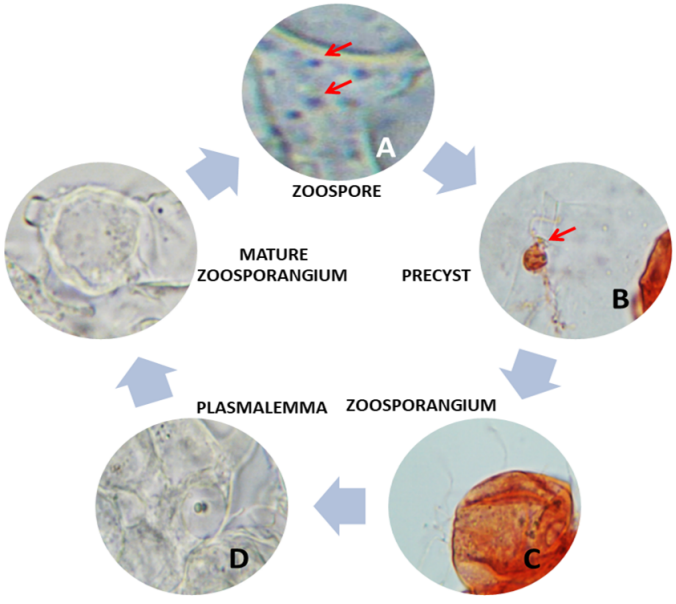

Figure 4. *B. dendrobatidis* EV001 life cycle. (A) *B. dendrobatidis* EV001 zoospores (red arrows). (B) *B. dendrobatidis* EV001 thalli-forming zoospore (precyst) (red arrow). (C) *B. dendrobatidis* EV001 zoosporangium with thalli and rhizoids. (D) *B. dendrobatidis* EV001 plasmalemma, saccules and zoospores. (E) *B. dendrobatidis* EV001 zoosporangium before release of zoospores. Light microscopy at a 100× (A, B, C) and a 40× (D, E) lens.

Table 1. Proposal for modification of OIE method (OIE, 2012) dedicated to the *B. dendrobatidis* cryopreservation using *B. dendrobatidis* EV001.

CRYOPRESERVATION	
OIE protocol	**Changes**
Take 2 ml of culture broth with active growth (one week). Add 13 mL of new broth in a 25 cm^2 flask. Incubate for 3-4 days. Cultures must have a lot of active zoospores, zoosporangium on the broth. Scrape the flask walls and centrifuge to 1700 g per 10 min.	None
Pour off the supernatant	Supernatant was inactivated with hypochlorite 2.5 % (Supernatant contains active zoospores)
Suspend the sediment in 1 ml 10 % DMSO and 10 % FBS in broth and freeze.	0.6 ml of 5 % and 10 % DMSO were used, and FBS was not used. Freezing process was applied to -80 °C for a month
THAWING	
Place the vials directly from liquid nitrogen into water (43 °C) place the vials. Agitate for 30 seconds.	None
Pour the content onto the TGhL agar supplemented with lactose (TGhL) and antibiotics.	The contents of the vial were poured directly into TGh medium. No antibiotics or lactose were used.
Pipette liquid cryoprotectant onto the sample (in the Petri dish).	It has not been applied in this work.
Put on 1 ml of weak saline solution (0.001 M KH_2PO_4, 0.0001 M $MgCl_2$, 0.00002 M $CaCl_2$) into the transferred sample and incubate. Examine the cultures over 7-10 days for movement of zoospores.	To avoid contamination risks, all of the process is designed to be as non-intrusive as possible and reduce the amount of manipulation needed until the colonial growth was observed.

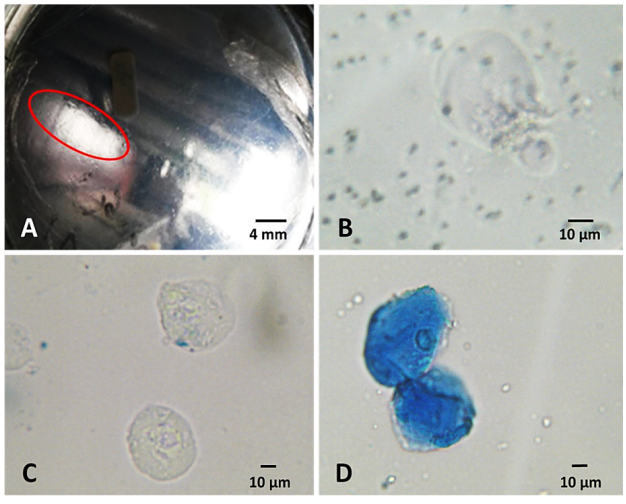

Figure 5. *B. dendrobatidis* EV001 after cryopreservation. A. TGh medium with translucent colonies of *B. dendrobatidis* EV001 are observed in the medium 20 days after thawing. B. *B. dendrobatidis* EV001 zoosporangium (red arrow) and zoospores. C. *B. dendrobatidis* EV001 sporangium with discharge tube. D. Lactophenol blue, *B. dendrobatidis* EV001 zoosporangium, zoospore (lactophenol cotton blue). Light microscopy at a 40× lens

3.3 Bioinformatic analysis of fungalysin

According to the bioinformatic analysis, the predictions show that the *B. dendrobatidis* JAM81 fungalysin has an average life greater than 20 hours, a molecular weight of 85 kDa, an isoelectric point of 8.33, an aliphatic index of 59.68 (it has a high temperature tolerance similar to other proteins), a hydropathicity of -0,625 (hydrophilic protein). *B. dendrobatidis* fungalysin was modeled using a standard fungalysin of *Aspergillus* as template (PDB: 4k90) (Figure 6) (identity: 40.75%, query cover: 45%, E value: 1e-82). 3D theoretical model corresponds to 368 residues (46.8%) from F237 to G604 (Figure 7). The alpha-helices in the model are formed by 126 residues (34.23%), 46 residues have a beta-sheet conformation (12.5%) and 196 residues form loops (53.26%). A HEXXH signal is between residues 407 and 411, and the EXXXD signal is located between residues 437 and 441. Based on the results from CDD, *B. dendrobatidis* JAM81 fungalysin has a PepSY domain (propeptide peptidase) which goes from I164 to the V230, and the catalytic region is between residues Y239 and A590 (Fernández et al., 2013).

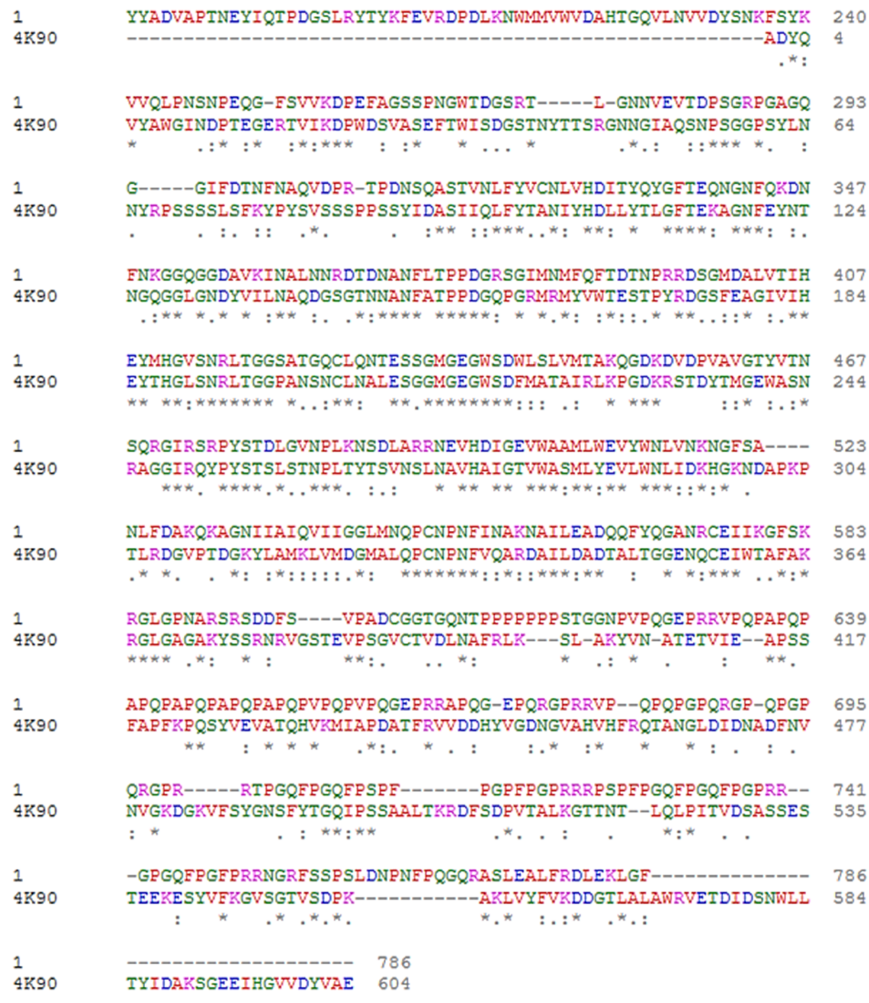

Figure 6. Sequence alignment of *B. dendrobatidis* JAM81 fungalysin and *A. fumigatus* fungalysin. Number 1 represents *B. dendrobatidis* JAM81 fungalysin and 4k90 is the sequence of *A. fumigatus* fungalysin. Asterisks indicate identity, two points are conserved substitutions and a point is a semiconserved substitution. Colors represent amino acid features according to Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

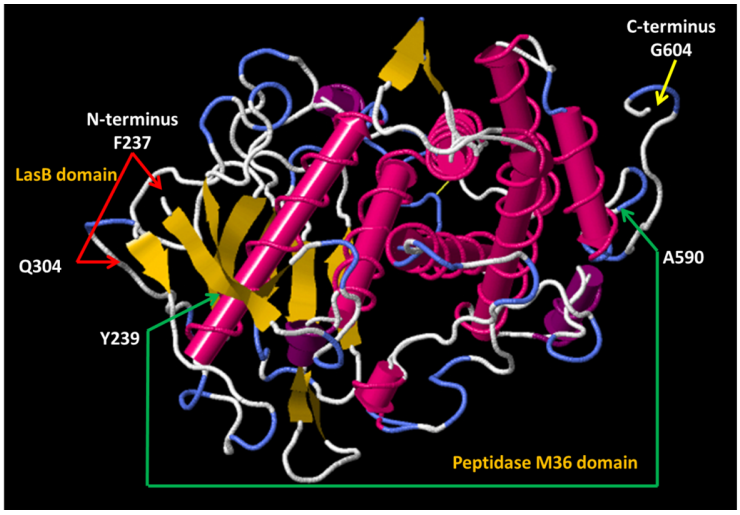

Figure 7. 3D model of *B. dendrobatidis* JAM81 fungalysin. The N-terminus end (F237) and the C-terminus residue G604 (yellow arrow) are indicated. Yellow arrows are beta sheets and pink tubes are helices. Two domains are determined, the LasB domain (F237-Q304, red arrows) is related to Zinc-dependent metalloproteases, and the peptidase M36 domain (Y239-A590, green arrows) is typical of fungalysins.

4 Discussion

4.1 *B. dendrobatidis* EV001 morphology

B. dendrobatidis has a first stage, consisting of a flagellate zoospore that colonizes anura skin. Subsequently, *B. dendrobatidis* zoospores change to spherical thalli that produce new asexually reproduced zoospores inside skin epidermal cells of frog, and then infective zoospores are released into skin surface (Greenberg et al., 2017; Kilpatrick et al., 2010). It is important to analyze this microorganism insofar as it is relevant to understand emergent pathogens that are rapidly disseminated (cosmopolitan), in addition to their aggressive nature toward anurans (Rosenblum et al., 2009). *B. dendrobatidis*-anura interactions are not fully understood (De León et al., 2019).

It should be noted that *B. dendrobatidis* zoosporangium has a refractive structure and abundant presence of zoospores in wet mount analyses (Robinson et al., 2020; Berger et al., 1998). The lactophenol cotton blue slides were similar to those raised in wet slides. Lactophenol cotton blue is a staining agent based on methyl blue and lactophenol; the first component serves as dye of fungal chitin, and lactophenol (phenol, lactic acid, glycerol, water) is a mountant (Leck, 1999). Congo red stain has proven to be a valuable tool for *B. dendrobatidis* identification, which acts similarly to lactophenol cotton blue by staining fungal chitin (Slifkin and Cumbie, 1988). *B. dendrobatidis* EV001 features correspond to the observations made by experts: zoospore presence, refractive zoosporangium or discharge tube among others (Briggs and Burgin, 2004; Berger et al., 1998).

4.2 *B. dendrobatidis* cryopreservation method

At the same time, analyses for the cryopreservation such as the testing of aseptic practices using techniques like storage at cryogenic temperature with 5% DMSO were applied. One month later, the thawing procedure was developed using TGh medium. It was determined that agitate by inverting the container (lid down) was positive for *B. dendrobatidis* EV001 growth, together with culture sealing flasks and their maintenance under aseptic laboratory conditions. Other cryoprotectants were evaluated: 10% skin milk, 10% DMSO with TGhL broth, and 10% DMSO with TGhL broth and 10% FBS. These assays were done using freezer at -80 °C and liquid nitrogen. An evaluation highlighted a better recovery with DMSO and FBS at 4-5 days using freezing at -80 °C, the outcome was similar for liquid nitrogen at 7-14 days (Boyle et al., 2003).

It should be emphasized that antibiotic usage was not employed to avoid microbial contamination, which will reduce artificial selection (Brem et al., 2013); these results can often be achieved with strict use of aseptic conditions involving a biological safety set and previous material sterilization among other procedures. Growth medium can also be contaminated due to tryptone, which is the best nutritional source for microorganisms. Therefore, *Penicillium sp.* and *Bacillus sp.* contamination was observed in our work. Other authors have previously described that *B. dendrobatidis* handling needs a regular examination of flasks and vials for minimizing the impact of contamination variables, besides it was necessary to develop an appropriate set of constant passages (at least every 14-21 days in agar, at least every 4-5 months in broth, storage at 4-6 °C) (Boyle et al., 2003).

The use of lactose is suggested (Flechas et al., 2012), but was not necessary in our study. TGh medium is ideal because *B. dendrobatidis* grow best in tryptone or peptonized milk, and does not require additional sugars; *B. dendrobatidis* synthesizes proteases, which can degrade casein and gelatin (Piotrowski et al., 2004).

On the other hand, weak saline solution replaces pond water as natural environment of *B. dendrobatidis* (Boyle et al., 2003). In this work, weak saline solution was omitted with no effects on *B. dendrobatidis* recovery. Thus, it is said that when environmental and physico-chemical conditions are modified for this microorganism, as has occurred in culture media, changes in zoosporangium size may occur (Garcés et al., 2003). Concerning the observations of stages regarding *B. dendrobatidis* cycle, young zoosporangium formation was observed (germination process), besides discharge tube in 1% tryptone broth related to humidity for zoospore release can be determined, and longer tubes can be viewed in liquid media; however, zoospore release should be in solid media. Zoosporangium requirements on frog skin can be variable and unpredictable in occurrence; however, *B. dendrobatidis* can be viable using 1% tryptone broth, and their rhizoids can be observed with congo red (Berger et al., 2005). This approach can be particularly effective for smaller laboratories, especially those with limited-resource settings, but which can be highly effective in studying *B. dendrobatidis* if good microbiology practices are applied.

4.3 Bioinformatic analysis of fungalysin

B. dendrobatidis fungalysyn-related genes (approximately 25) are expressed primarily in zoosporangium (76%) and only a small percentage is expressed in zoospore (Rosenblum et al., 2008). 3D experimental structure of an *A. fumigatus* fungalysin (AfuMep) has been described, showing relevant parameters for this virulence factors, such as propeptides presence that supress the host proteases (residues 19-245), and C-terminal catalytic domain (residues 245-634) that degrade proteins from extracellular matrix (elastin and collagen). It was found that fungalysin belongs to zinc metalloproteases M36. This paper describes fungalysin as a non-glycosilated protein of 68 kDa, containing a catalytic signal HEXXH for zinc atom, involving 12 alpha-helices (35%) and 22 beta-sheets (12%).

B. dendrobatidis JAM81 fungalysin complies with the M36 family requirements regarding molecular weight (85 kDa), disposal of helices and sheets, HEXXH metalloprotease-like signal, a R470 residue, which is regarded as the important element in folding, and catalytic activity of this enzyme, because elastases and thermolisins have a calcium ion near to zinc ion (16 Å) from catalytic site, with ability to protect the enzyme from autolysis. AfuMep and *B. dendrobatidis* JAM81 fungalysin have a R470 that would protect these virulence factors (Fernández et al., 2013).

The precise mechanisms underlying the chytridiomycosis process are not fully understood, for that reason, several hypotheses have been put forward (Berger et al., 2005). Frog susceptibility to *B. dendrobatidis* is related to how amphibian take their water directly through skin while preserving animal homeostasis, reflecting the fact that *B. dendrobatidis* inhibits sodium channels which are related to chytridiomycosis. It was determined that frog transports sodium from pod to plasma, and epithelial sodium channels and their influence in amphibian kidneys are important in this process, and there is deterioration of the cardiac function before death (Campbell et al., 2012). *B. dendrobatidis* is a nonspecific pathogen, which does not affect all amphibian species equally (Strauss and Smith, 2013) due presumably to skin microbiota, or antimicrobial peptides, and could play an important role in the defense against fungi (Flechas et al., 2013; Berger et al., 1999). *B. dendrobatidis* has alternative hosts and re-

servoirs such as nematodes and river crabs, but infections in other vertebrates are not lethal (Rosenblum et al., 2010; Strauss and Smith, 2013).

5 Conclusion

This work shows a *B. dendrobatidis* EV001 description as an introduction to a modified cryopreservation method, which would be a significant contribution to the laboratories focused on this area. Additionally, a theoretical model of fungalysin is added as a contribution towards understanding the virulence factors of this anura pathogen.

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Sustainable Development

The Sustainable Development Goals in America: Overview

Los Objetivos de Desarrollo Sostenible en América: Panorama

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Abstract

The Sustainable Development Goals (SDG), commit 193 countries to improving environmental, social and economic indicators through their performance on 169 targets by 2030 in order to achieve compliance with the Paris Agreement. However, development dynamics in relation to the SDGs change over time and with this, countries do not identify the strengths and weaknesses in which the reality of their territory is visible, in addition to the indicators they have been addressed individually because its multidimensionality generates divergent results. In this sense, the objective of this review is to analyze the state of evolution of the SDGs in America and particularly in Colombia. To analyze the SDG dynamics in 32 countries of America, we systematize the official indicator data from 2010 to 2019 and United Nations Statistics Division (UNSD) indicators, taking into account variables such as per capita gross domestic product, greenhouse gas emissions and renewable energies. As expected, the countries presented different strength in the development of the indicators related to synergies and antagonisms of the SDGs. Also, it was possible to find a wide distribution in the advancement of SDGs, finding nine countries that managed to meet at least one of the goals, which allows make visible that each of the nations has taken government initiatives to achieve development and quality of life its inhabitants.

Keywords: Quality of life, Sustainable development, Environmental Indicators.

Resumen

Los Objetivos de Desarrollo Sostenible (ODS) comprometen a 193 países en el mejoramiento de indicadores ambientales, sociales y económicas a través de su desempeño en 169 metas para el 2030, con el fin de lograr el cumplimiento del acuerdo de Paris. Sin embargo, las dinámicas de desarrollo en relación con los ODS cambian a través del tiempo y con ello, los países no identifican las fortalezas y debilidades en las que se visibiliza la realidad de su territorio. Además, los indicadores se han abordado de manera individual debido a que su multidimensionalidad generan resultados divergentes. En este sentido, el objetivo de esta revisión es analizar el estado de evolución de los ODS en América y particularmente en Colombia. Para analizar la dinámica de los ODS en 32 países de América sistematizamos los datos de los indicadores oficiales de 2010 a 2019 y los indicadores de la División de Estadística de las Naciones Unidas (DENU), teniendo en cuenta variables como el Producto Interno Bruto PIB per cápita, las emisiones de gases de efecto invernadero y las energías renovables. Como se esperaba, los países presentaron diferentes fortalezas en el desarrollo de los indicadores relacionados con sinergias y antagonismos de los ODS. En este sentido, hubo una amplia distribución en el avance de los ODS, encontrando a nueve países que lograron cumplir por lo menos en un objetivo la totalidad de las metas, lo que permite visibilizar que cada una de las naciones ha tomado iniciativas gubernamentales para lograr el desarrollo y la calidad de vida de sus pobladores.

Palabras clave: Calidad de vida, Desarrollo sustentable, Indicadores Ambientales.

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1 Introduction

Historically, sustainable development as a concept, derives from economics as a discipline. In this sense, at the beginning of the 19th century the main discussion that gave its origin was whether the limited capacity of the earth's natural resources could support the accelerated population growth of humanity, while statistics predicted that the world's human population would continue to increase exponentially and, therefore, natural resources would not be able to meet the needs of the growing population (Mensah, 2019). However, the importance of this application was ignored for years and it was thought that it would create technology that would address this situation. For this reason, in 1972 a concept of sustainable development was first developed in UN Conference on the Human Environment held in Stockholm (Seyfang, 2003). Subsequently, in 1987 in the World Commission on Environment and Development in Norway a clearer concept of sustainable development was consolidated during the construction of the Brundtland commission report that gave rise to United Nations Conference on Environment and Development (UNCED), known as the Rio Earth Summit, in 1992. In World Summit on Sustainable Development (WSSD), known as Rio + 10, was held in Johannesburg, committed the nations to strengthen the advancement of the Millennium Goals and finally in 2012 the United Nations Conference on Sustainable Development (UNCSD) or Rio + 20 was held, which was based on the strengthening of the green economy and the institutional framework of the new SDGs (Allen et al., 2019).

The sustainable development approach is strengthened and made operational globally with the Millennium Development Goals (WHO), after which it has grown on theoretical foundations and local experiences, enhancing it as a fundamental guide to global change (Grigss et al., 2013; Kumar et al., 2016). In this regard, the 2030 agenda, which incorporates a new set of global goals known as the 17 Sustainable Development Goals (SDGs), includes economic, social, political and environmental indicators for the local and regional governments of the 193 countries of the United Nations (Kaltenborn et al., 2019), contrary to previous development agendas, based on economic growth (Kroll et al., 2019).

The implementation and evaluation of 169 goals framed within the 17 Sustainable Development Goals (SDGs) has served to determine their effectiveness at global level (Pradhan et al., 2017), through the open access database of the United Nations Statistics Division, where the SDG indicators were established since 2010 for all countries. With this reference, interest has been aroused in its study, at economic lines such as agriculture (Nasr-Allah et al., 2020), industry (Sangwan and Bhatia, 2020), education (Cebrián et al., 2020), health (Bennett et al., 2020), market (van der Waal and Thijssens, 2020), design (Horne et al., 2020) and technology (Vinuesa et al., 2020), as well as, climate change related in a transversal way (Kaltenborn et al., 2019) to carry out exhaustive analysis on the synergy quantification and interaction within and between the SDGs, as reported by Fuso Nerini et al. (2018) and Nilsson et al. (2018).

In this regard, Nilsson et al. (2016) presented one of the first quantitative methodologies of the SDGs, taking into account that the valuation is specific to each nation and its contexts; therefore, it cannot be generalized. In the same way, Moyer and Bohl (2019), highlight the importance of setting goals to achieve the objectives in favor of new generations' life, for which they propose three political paths that would enable the enforcement of SDGs: technology, lifestyle change, and decentralized governance. Thus, each country must follow a route to generate synergies within and between the goals, or compensate one goal for another, as studied for the countries that make up the G20 and the OECD, through close relationships and antagonisms (Schmidt-Traub et al., 2017).

In most nations, the issue of eradicating poverty was taken as one of the first tasks from the very MDGs, and continues to be one of the main challenges faced by humanity (SDG 1), due to its high synergy with others. Similarly, regarding responsible consumption and production (SDG 12), it has been associated with economic growth (Lusseau and Mancini, 2019). Latin American diversity is also expressed in Colombia, where there are different development levels in all aspects, due to the influence from major cities, the strong regional agricultural aptitude, or the existence of vast natural reserves, as reported by CEPAL (2017), when asses-

sing the provinces through regional competitiveness, according to economic strength, infrastructure and logistics, social welfare and human capital, science, technology and innovation, as well as institutions and public management.

These studies have stuck together an instantaneous and extensive view on the interactions and challenges that humanity must face, which requires the maximum capacity to analyze, enforce and provide feedback through time tracking, making it possible to project the contributions for the 2030 agenda. Therefore, this research seeks to analyze the development stage of the Sustainable Development Goals (SDG) in 32 countries of America, and more specifically in Colombia, through a systematic of bibliometric information, taking into account the near past in order to evaluate its compliance/breakthroughs.

2 Methods

The research is developed through descriptive research, using the systematic research method that according to Aguilera (2014), uses electronic resources such as databases mainly, allowing a more critical view. The SDG of 32 countries of America Index and Dashboards Database (www.sdgindex.org) was used, which provides information at a global level on the Sustainable Development Goals SDGs from 2010 to 2019 and the data available from the United Nations Statistics Division (UNSD; www.unstats.un.org) in which indicators of gender, urban and rural population and groups are addressed. What is more, an exploratory analysis of variables such as per capita gross domestic product (data www.worldbank.org), greenhouse gas emissions and renewable energies (data www.iea.org) was carried out, following the methodology proposed by Schmidt-Traub et al. (2017).

The data from the 2030 agenda of the SDGs for Colombia (www.ods.gov.co) were analyzed in prospective and current terms, making correlations (Pearson) as a threshold to define the synergy and antagonism between a pair of goals, understood as synergy when the result is positive and antagonism when it is negative. However, synergy was taken into account when it was greater than 0.5 and antagonism less than $-0{,}5$, following the methodology proposed by Pradhan et al. (2017). Finally, an analysis of the national research centers and the scholars who have made the greatest academic-scientific contributions to the SDGs in Colombia was carried out.

In addition to that, an analysis of researchers and research groups in Colombia was carried out to demonstrate processes in which higher education institutions and research centers conduct the implementation and formulation of SDGs in the academic and productive areas using the platform https://sba.minciencias.gov.co/Buscador_HojasDeVida/ of the Ministry of Science, Technology and Innovation of Colombia (Minciencias). The statistical program Rstudio version 4.0.0. was used to perform the analysis, using *ggplot2*, *corrplot* and *agricolae* packages.

3 Results and Discussion

3.1 Overview of SDGs in America

America has featured different levels of progress in Sustainable Development Goals, which can be observed in Figure 1, and some countries set out a better perspective in their fulfillment of goals. Thus, the goals that have been achieved in the shortest time in some countries are affordable and clean energy, followed by no poverty, quality education along with decent work and economic growth. Altogether, it is possible to observe a differentiating advance in the partnerships for the goals and a thoughtless advance in peace, justice and strong institutions.

Although countries such as Ecuador, the United States and Peru did not submit progress data on the goals for the 2019 SDGs. It is worth noting that Canada and Costa Rica are the only countries in America that have achieved the highest number of goals; $4-7$ and $1-7$ respectively. Canada, one of the members of the Organization for Economic Cooperation and Development OECD, is the one with the best human development index (Schmidt-Traub et al., 2017), which is apparent from its inhabitants' quality of education. In this regard, Costa Rica has made great efforts to reduce poverty, as highlighted by the UNDP and the Central American Bank for Economic Integration CABEI (CABEI, 2017; Ortiz-Juérez, 2017).

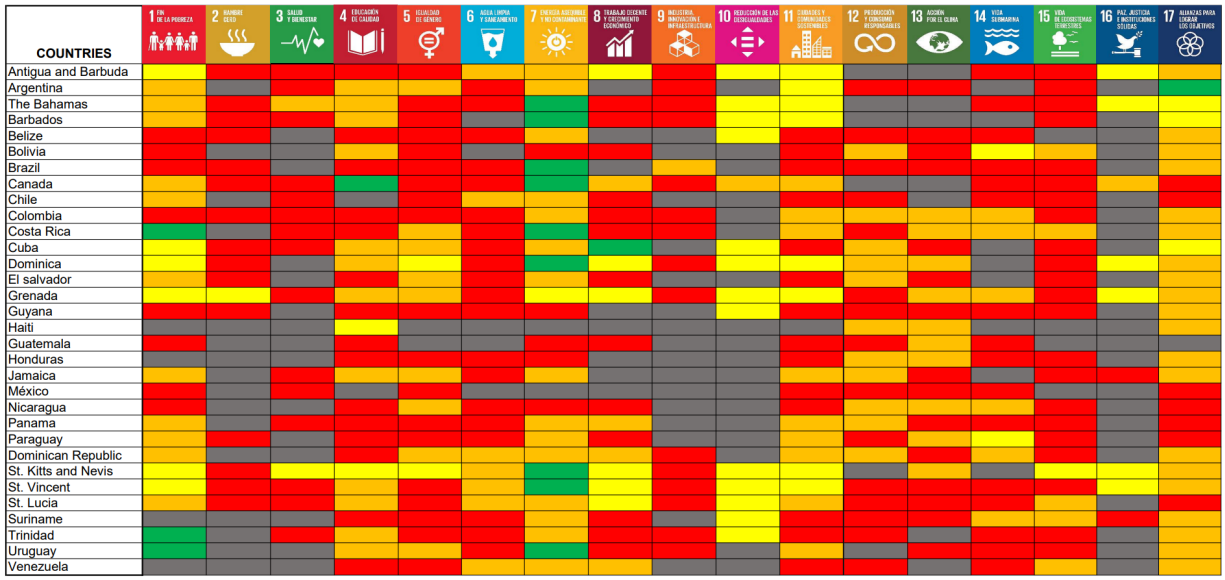

Figure 1. Overview of the analysis of the Sustainable Development Goals in America for 2019. Green: Goals achieved; Yellow: Some challenges remain: Orange: Significant permanence of challenges: Red: Progress has been minimal. Gray: no existing data. Adapted from Sachs et al. (2019).

Colombia's outlook is encouraging in objectives 7, 11, 12, 13, 14 and 17 through the implementation of clean energies, with tax incentives for those who invest in their research and use; aspect that positively impacted communities where infrastructure does not predominate and access to energy is limited (Villada et al., 2017). In the same sense, manufacturing and agricultural production has focused on less use of external sources of energy and resilience capacity (Plazas-Leguizamón and Jurado-Álvarez, 2018). Regarding objective 13, with the national climate change policy for the rural and urban sector, it seeks to mitigate the energy flows from the different productive sectors that give rise to environmental pollution (Minambiente, 2016).

On the contrary, there is a lag in the goals of reduced inequality and peace, justice and strong institutions. These aspects have been relevant in Colombia in regards of the armed conflict that have unleashed subversive groups for more than 5 decades (Rettberg, 2020), which has led to murders, forced displacements, kidnappings, extortion, moral damage and even considerable increases in growing illicit crops to obtain narcotic drugs (Rochlin, 2020).

In respect to Latin American, Haiti is the country with the least progress in meeting SDGs. However, this country shows improvement in education and climate action, as well as in sustainable production and consumption due to the abilities Haitians have acquired after the political and socio-economic crisis, which has allowed them to seek new energy sources and reduce fossil fuel impact on climate change (Perry, 2020).

The well-being of people is one of the most representative indicators in sustainable development, and has been a useful variable in the rural sector to evaluate human living, as well as sustainable livelihoods (Rasul, 2016). This aspect is well highlighted in the goal of decent work and economic growth, which in the case of America, where countries present a level of development corresponding to 3.1% (Figure 2A), 18.7 (Figure 2B), 12.5 (Figure 2C) and the highest with 37.5% (Figure 2D). This result is due to the fact that the unemployed exceeded 192 million persons for 2017, while the number for 2019 was 35 million more (Organización Internacional de Trabajo, 2018).

The assessment of economic development, climate change and the search for energy strategies have become determining aspects in the growth of regions, mainly in the rural sector. In this regard,

Figure 3 shows the behavior that the countries have had during 2019 in correspondence to GDP per capita, the emission of greenhouse gases and the use of renewable energies, emphasizing in the first two an agglomeration of a large part of the countries in the American continent and a slight dispersion of the rest.

The GDP per capita is an indicator related to the citizens' quality of life in many cases, which in the case of Venezuela, the economic, social, political and even environmental crisis has led to great effects on this factor, giving negative digit results (Figure 2A). This is reflected in low wages, rising inflation, high prices of basic products and a dramatic reduction in purchasing power, leaving as a result that 87% of Venezuelans live in absolute poverty (Caraballo-Arias et al., 2018).

For Colombia, GDP per capita is above countries such as Canada, Brazil, Argentina and Mexico, but far below Antigua and Barbuda, as well as the Dominican Republic. In general, the countries of the American continent are characterized by presenting a grouping in this variable, with a linear performance, very similar to purchasing power parity (PPP) in countries that make up the OECD (Schmidt-Traub et al., 2017).

In the case of greenhouse gas emissions in relation to support in research and development of clean and renewable energy, the work carried out in El Salvador, Honduras and Costa Rica stands out their economic support is greater than the emission of CO_2, while in Colombia, investment has been lower. The investment of US$ 1036.8 per Kt of CO_2 released to the environment is much lower in Peru (US$ 7,160.2 / Kt CO_2) and Chile (US$ 1692.8 / Kt CO_2). However, according to CO_2 per capita emission, it is higher in North American countries such as the US$ (16.24 Tons per capita) and Canada (15.64 Tons per capita), while in South America; Venezuela (4.99 Tons per capita), Chile (4.69 Tons per capita), and Argentina (4.62 Tons per capita), being these the largest contributors (Ritchie and Roser, 2020).

Among the sectors with the highest pollution levels is the generation of energy and heat, whose CO_2 release is 42%; used in industrial activity (17%), residential (11%), services (8%) and others (4%) (Hannan et al., 2019). These aspects are relevant when it comes to consolidating new energy strategies, with less environmental impact and benefit for the most underprivileged in rural areas. According to SDG 7, this benchmark contemplates a favorable outlook for the Bahamas, Barbados, Brazil, Canada, Costa Rica, Dominica, Saint Kitts, Saint Vincent and Uruguay, which are countries that have already reached the goals (Sachs et al., 2019), according to Figure 2C.

In Colombia, access to energy is still an unsatisfied service and, therefore, life quality of the affected inhabitants is adversely affected by the use of fuels with higher polluting impact such as gasoline, oil, petroleum diesel, ethanol, mineral coal, wood, manure, polyethylene and polystyrene (Franco et al., 2008; Herran and Nakata, 2012).

3.2 Overview of the Sustainable Development Goals in Colombia

Global change in economic and environmental areas has an impact on inhabitants' daily life and on their productive, social and human dynamics; important aspects in the sustainable development of the regions. In Colombia, social, political and ecological problems have marked the pace of development, with lags in some areas more than others, reflected in poverty, hunger, low access to health, education and basic home services (Nhamo and Mjimba, 2020).

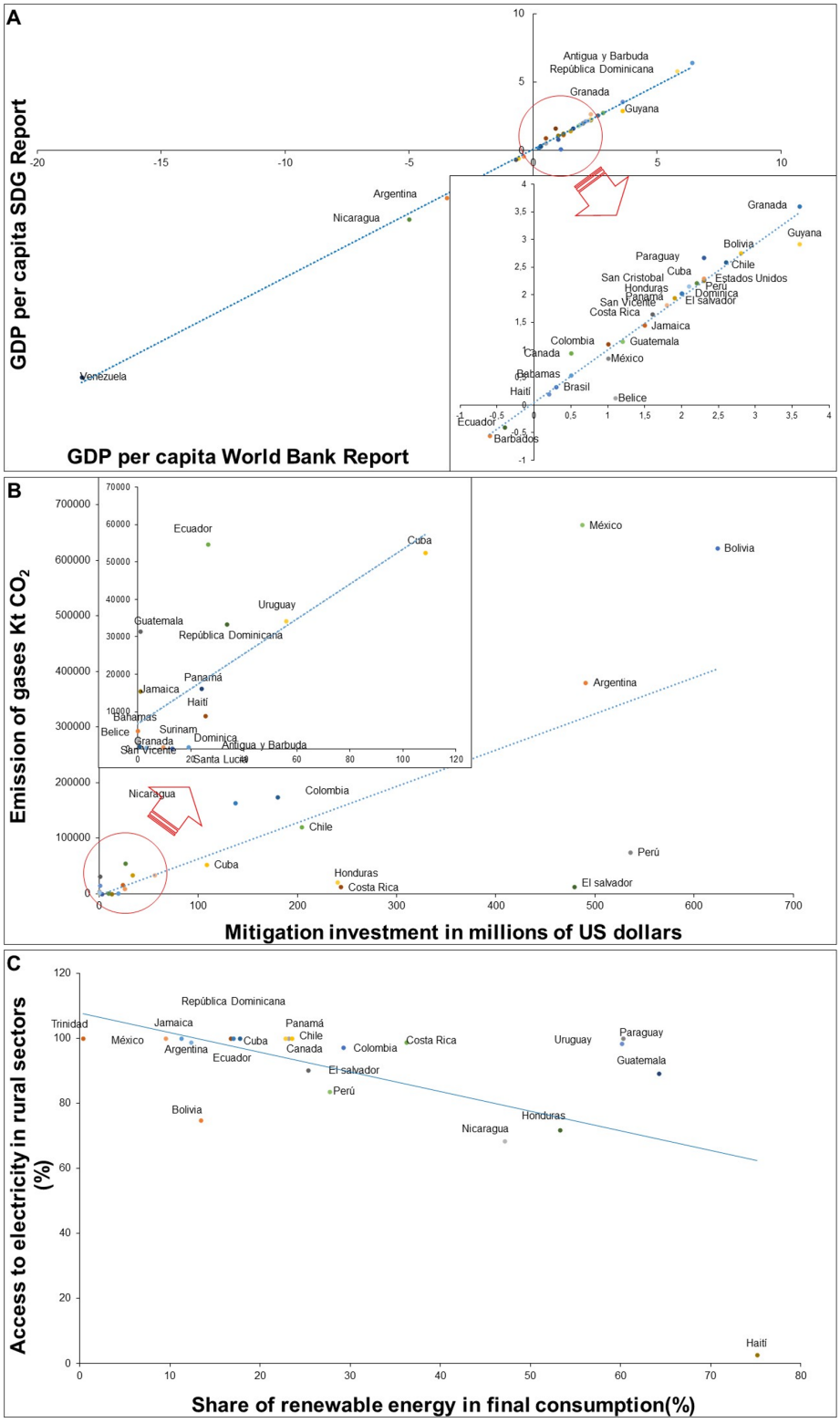

Figure 2. Relationship of SDG indicators with other indices. A: per capita analysis of countries in the American continent. B: Analysis of greenhouse gas emissions versus investment in mitigation by countries. C: Analysis of the availability of energy and the implementation of new sources.

However, throughout history, the national government has consolidated different strategies for the implementation of territorial development plans (TDPs) and even through the National Council on Economic and Social Policy (CONPES); aspects that have been contemplated in Decree 280 of 2015 and in Law 1955 of 2019. On the other hand, international organizations such as the United Nations (UN), the Economic Commission for Latin America and the Caribbean (ECLAC), and the German Corporation for International Cooperation (Gesellschaft für Internationale Zusammenarbeit GIZ) have carried out forums, meetings, sessions, workshops and summits, in which it is intended to support nations through the SDGs in Colombia; with different development nuances from implementation to current advance (Figure 3).

Figure 3. Timeline, national and international progress regarding the SDGs in Colombia. Adapted from DPN. HLPF: High Level Political Forum. DNP: National Development Plan. TDP: Territorial Development Plan. CONPES. Adapted from Cancillería (2012)

In this sense, Colombia has achieved autonomy in the search for strategies to fulfill the SDGs in 2030. However, the progress in the consolidation of goal indicators is still present in large gaps, and generates uncertainty to achieve the SDGs in the territories. This is how the National Planning Department DPN gathers all goal indicators for SDGs in Colombia. Data is mostly from 2016 and 2018, and according to the Colombian Ministry of Foreign Affairs, this is because the Sustainable development paradigm has not achieved the economy and society operate in an excessive and poorly balanced vertical vision, making holistic links between topics difficult (Cancillería, 2012).

In this sense, the unitary and decentralized condition of the state of Colombia ensures that subnational governments have prioritized the SDGs in regional and local planning instruments, committing resources and implementing concrete actions (Aldeanueva and Cervantes, 2019).

According to data submitted in the sustainable development reports for the years 2017, 2018 and 2019, Colombia ranks between 67 and 88 in the advancement of SDGs in recent years, whereas in the last report, it is recognized that Colombia has achieved 69.6% of goals. In this sense, the implementation of SDGs brings along synergies and antagonisms, in which some processes and people benefit as recognized by Fuso Nerini et al. (2018). In Figure 4, the degree of relationship between the SDGs in Colombia is outlined, and a higher percentage is observed in the synergy (26.7% greater than 0.9) of the ODS than the antagonism (10.4% less than 0.9) according to Pearson's Correlation Coefficient, which indicates favorability in its implementation, otherwise it would run the risk of generating perverse results, where vertical development is more important than holistic and interdisciplinary (Nilsson et al., 2016).

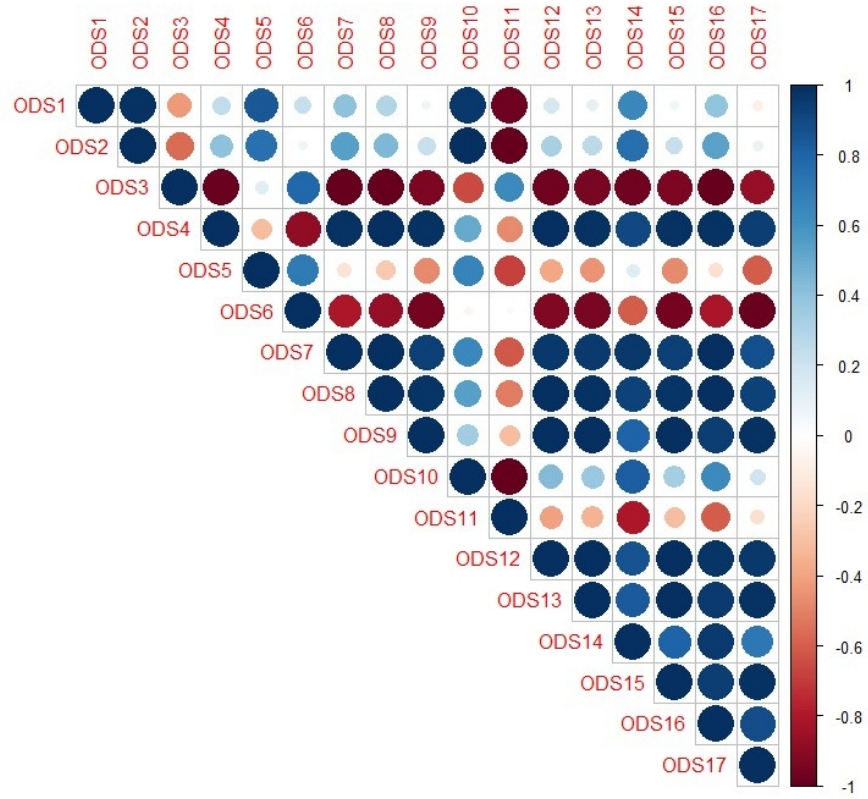

Figure 4. Correlation of SDGs for Colombia in the periods of 2017, 2018 and 2019.

In this context, the most dynamic objectives in the synergy are: (a) Quality education, the importance of which lies in the recognition of knowledge, implementation and even its innovation in the lines of greater social dynamism (Cebrián et al., 2020); (b) Affordable and non-polluting energy with a view to new models that generate less impact and favor the resilient development of the environment (Hannan et al., 2019); (c) Decent work and economic growth, the result of which has been the international benchmark on the social well-being of individuals and the strength of nations, making it one of the pillars that requires the greatest strategic demand (Rai et al., 2019) and finally (d) Industry, innovation and infrastructure, based on the consolidation of resilient infrastructure, which allows sustainable industrialization through the innovation of products and processes (Perea-Hinestroza, 2016).

Additionally, Figure 5 shows an updated panorama (2017, 2018 and 2019) of the SDGs in Colombia, which recognizes their dynamics over time. However, the progress of the objectives varies over the years and in many cases, there is a setback in their fulfillment as observed in the final report for objective 1, 2, 5, 6 and 10. This aspect reflects gaps related to the lack of logistics, investment, support and even external dynamics (van der Waal and Thijssens, 2020).

In the same sense, there is a lag in the objectives of industry, innovation and infrastructure, as well as in the reduction of inequality, which is worrying since they do not exceed the goals of the objectives by 31.8% and 21.7% respectively, and in 2020 they would be severely affected because of the secondary consequences that the Covid-19 virus would bring, which triggered the increase in unemployment, the change in the economy, the crisis of health systems and the lack of food (Miller et al., 2020; Spinelli and Pellino, 2020), which combined with the increase in climate change indices, among which is the unpredictability of dry and rainy seasons in tropical and subtropical areas, will favor the rise in world crisis indices (Marengo et al., 2014).

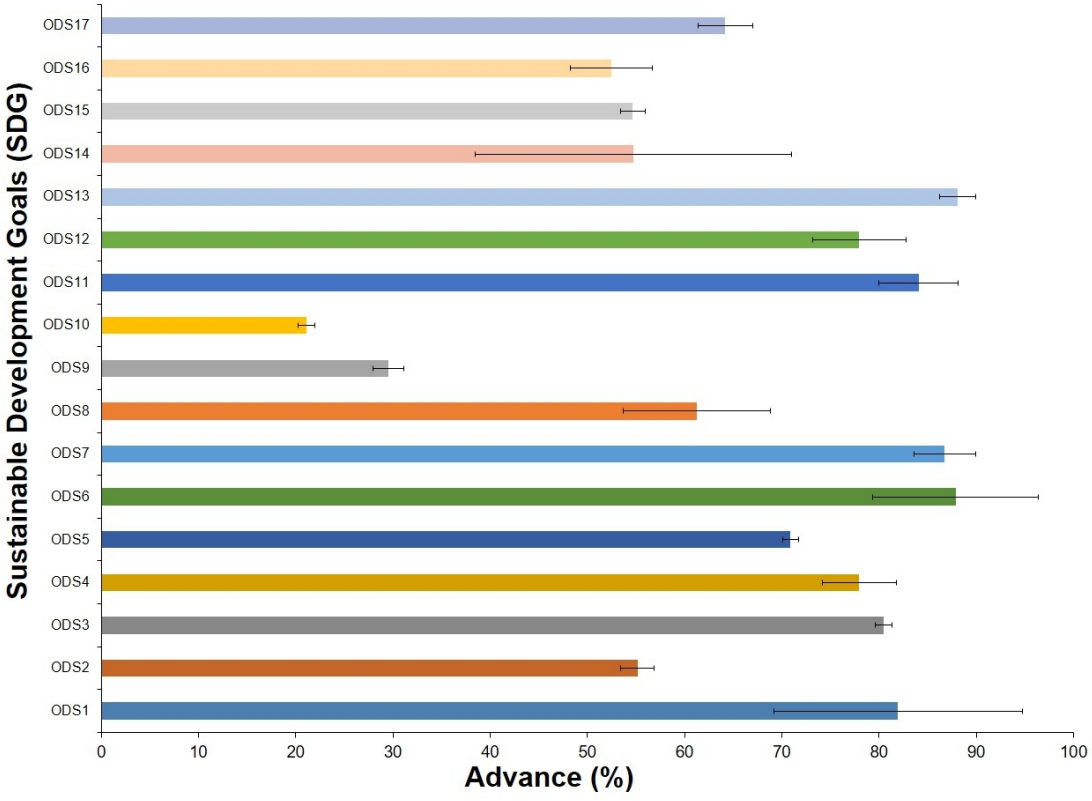

Figure 5. Progress of SDGs in Colombia during the last 3 years. Bars indicate standard deviation.

3.3 Scientific academic context

The panorama of sustainable development goals in Colombia has been strengthened by government entities, headed by the presidency of the republic, who in 2018 made official through the National Council on Economic and Social Policy CONPES - 3918, the implementation strategy for the SDGs throughout the national territory, whose first task was their incorporation into the Territorial Development Plans TDP. This initiative had a high incorporation in 24% of the Colombian Provinces, among which are Cesar, Córdoba, Santander, Boyacá, Cundinamarca, Quindío, Nariño, and Caquetá, while a general and very general incorporation of 38% and 38%, respectively (DPN, 2018).

In this sense, the articulation in work programs developed since the fulfillment of the Millennium Development Goals (MDGs), favored the continuous work for the SDGs among the ministries, agencies and civil society, despite the fact that the entire MDG targets for 2015 were not achieved, which brought along a greater commitment to achieve in 2030.

Thus, the Ministry of Science, Technology and Innovation consolidated in 2017 the first advance in the relationship between knowledge and the SDGs (Chavarro et al., 2017), in which they analyze the goals that were met and remained to be met in the MDGs, the strategies to be implemented for the SDGs, as well as the current panorama and the relationship between goals. In 2018, the National Policy on Science and Innovation for Sustainable Development (Resolution 0614 of 2018) entitled "Green Book 2030" was consolidated, in which the voices of citizens, businessmen, and national and international academics were compiled, whose theme focuses on the holistic application of science as a tool for knowledge and technology, as a support strategy, taking society as the main actors, and politics as an interdisciplinary aspect to achieve sustainable and resilient development with the environment (COLCIENCIAS, 2018).

Faced with this situation, different research centers and higher education institutions have come together, and with this, they have undertaken projects that seek to involve the SDGs in the productive sector, supported by the interdisciplinarity of science with academic and scientific actors who conduct knowledge to be applied in different fields, in order to contribute to the fulfillment of the goals (Perea-Hinestroza, 2016).

In this regard, research centers and higher education institutions have advanced in particular studies that contribute to the SDG indicators; an aspect that can be seen in the Gruplac and Cvlac of Colombian researchers (Figure 6).

Table 1. Research groups developing research on SDGs.

Research Group	Research area
Studies in urban and business sustainability -SuyE	Urban-regional sustainability
	Sustainable regional development
	Sustainability of organizations
Sustainable Regional Development	Territorial management
	Social capital
Research Group of the Latin American School for Cooperation and Development -GIELACID	National cooperation and financing for the development
	Society and strategic development sectors
Group of Legal Research Universidad de Medellín	Law and society
Phytosanitary and Biological Control Unit	Bioactive substances for agriculture
	Bioprospecting and microbial ecology
Environmental Management and Modeling Research Group (GAIA)	Modeling of environmental systems
	Biological treatment of waste and sewage
	Applied environmental microbiology
	Ecology of coastal aquatic systems
GINEI Infectious Diseases Research Group	Infectious disease epidemiology
Applied Geoinformatics	Geoinformatics and climate change
Raimundo de Peñafort	Fiscal sustainability
	Democratic sustainability

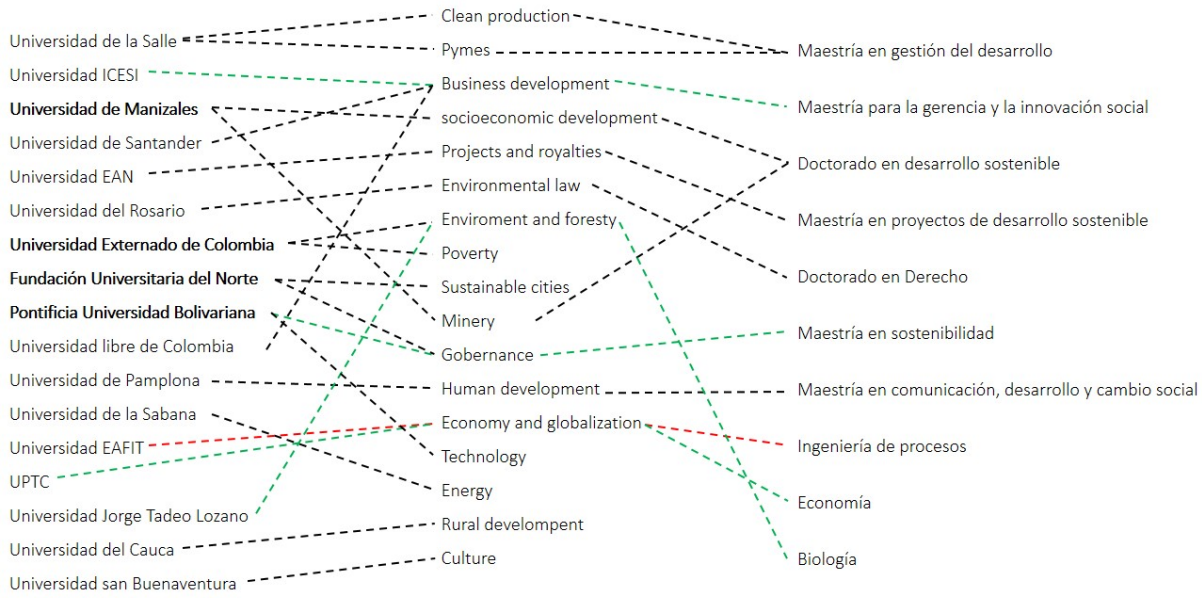

Figure 6. Analysis of the research work developed by national universities, strategic areas and academic programs.

Universidad de Manizales, Universidad Externado de Colombia, Fundación Universitaria del Norte, and Pontificia Universidad Bolivariana carry out more than one process that involves the implementation of SDGs, within projects that link the academic programs, and whose purpose has been highlighted by researchers who have a track record in the subject, and research groups that offer support with SDGs in academic-scientific work. However, there are research groups (Table 1), which, through their lines of research, route sustainable development projects, supported by students, academics and scientists, who deliver knowledge to the productive sector or civil society (Rodríguez-Rojas et al., 2019).

4 Conclusions

When analyzing the evolution of the Sustainable Development Goals (SDGs), the contributions of alliances on economic, social, political and environmental aspects at the local level are highlighted, for which it is necessary to strengthen interdisciplinary strategies in order to achieve transdisciplinary contributions for the benefit of local communities, new generations and the territories, through the integration of issues on affordable and clean energy, poverty, quality education, decent work and economic growth.

Among the actors participating in work teams, the leadership of the academic and research processes is required, focused on the holistic application of science as a knowledge and technology tool. Moreover, as a support tactic, the active participation of local communities is essential for their contextualization, in order to contribute to the particular resilience processes, evidenced by people's livelihood components and well-being, as indicators of sustainable development. To this end, the importance of intergenerational interaction is highlighted as a strategy for the evolution of generational renewal at the academic, research and rural community levels; thus, addressing economic progress with emphasis on the relationship between climate change, technology, lifestyle, decentralized governance, poverty-inequality and responsible production, according to the regional agricultural vocation and its natural reserves to counteract problems on the energy flows of the productive sectors, causes of environmental pollution, through the use of clean energy.

In this way, it is possible to show the need to address economic development, climate change and the search for energy strategies for the rural sector, through the consolidation of teams from different areas of knowledge as support for research on social, technical, political and environmental problems, as they are the causes of the backwardness of the localities, as evidenced by the levels of poverty, loss of security and sovereignty food, in the same way inequity for quality access to rights such as health, education and basic services.

Sustainable development is the contextualized added of social, environmental, cultural and economic indicators, which in a systemic way have been made visible through significant local experiences, where the resilience of natural resources is relevant for the benefit of the community.

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Climate Change

Aptitude of Coffee (*Coffea arabica L.*) and Cacao (*Theobroma cacao L.*) crops considering climate change

Aptitud de los cultivos de café (*Coffea arabica L.*) y cacao (*Theobroma cacao L.*) considerando escenarios de cambio climático

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Abstract

The areas of current and future aptitude for cocoa (*Theobroma cacao L.*) and coffee (*Coffea arabica L.*) crops were evaluated, in climate change scenarios in the Soconusco region, Chiapas, Mexico and the areas where the aptitude of the land increased or decreased were defined. The above provides information to recommend its management and conservation. Two methodologies were used: weighting of factors and limiting factors; both based on the optimal and extreme tolerance values of each crop for each edaphoclimatic requirement. First one, for the determination of potential areas for the establishment of crops; the second one, for differentiation of the combinations of environmental variables that took place within the study area. Climate change scenarios were evaluated and the distribution and aptitude areas were determined. Also, a comparison was made between current and future suitability for both crops to quantify the impact of climate change. The results indicate that the future aptitude of the land will have a decrease in the potential areas for the establishment of both crops that range between 4.5 and 4.8% for coffee and from 7.2 to 9.3% for cocoa.

Keywords: Biodiversity, Chiapas, Socunusco, Agroforestry systems, Sustainability.

Resumen

Se evaluaron las zonas de aptitud actual y futura para los cultivos de cacao (*Theobroma cacao L.*) y café (*Coffea arabica L.*), en escenarios de cambio climático en la región del Soconusco, Chiapas, México y se definieron las áreas donde aumentó o disminuyó la aptitud de la tierra. Lo anterior permite tener información para recomendar su manejo y conservación. Se emplearon dos metodologías: ponderación de factores y factores limitantes; ambas con base en los valores óptimos y extremos de tolerancia de cada cultivo para cada requerimiento edafoclimático. Con la primera, se determinaron las áreas potenciales para el establecimiento de los cultivos; con la segunda, se diferenciaron las combinaciones de las variables ambientales que tuvieron lugar dentro del área de estudio. Se evaluaron escenarios de cambio climático y se determinó la distribución y superficies de aptitud. Además, se realizó una comparación entre aptitud actual y futura de ambos cultivos para cuantificar el impacto del cambio climático. Los resultados indican que la aptitud futura de la tierra tendrá una disminución de las áreas potenciales para el establecimiento de ambos cultivos de entre 4,5 y 4,8 % para café y de 7,2 a 9,3 % para cacao.

Palabras clave: Biodiversidad, Chiapas, Socunusco, sistemas agroforestales, sustentabilidad.

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1 Introduction

The Mexican tropics have agro-ecological conditions which are suitable to have perennial crops, such as cocoa (*Theobroma cacao L.*) and coffee (*Coffea arabica L.*), which are species that are naturally distributed in the middle strata of humid warm forests; however, the situation in the world's forests is worrying and difficult, due to many factors such as uncontrolled deforestation, the increase in the agricultural frontier, extensive livestock, climatic changes that affect the increase in the number of forest fires, increase of pests and diseases, floods and population growth, among others (Roa-Romero et al., 2009).

Climate change is the result of meteorological events that influence the functioning of natural systems, observed in alterations in the biodiversity of ecosystems, in productivity and food sources and, consequently, in the life of humans. There is a growing interest in understanding the processes that make up climate change and their influence on the dynamics of the carbon cycle in natural ecosystems, since plants are essential to the global balance of this gas due to their ability to absorb CO_2 through photosynthesis, storing it as cellulose or transforming it into another type of organic compound (Yepes and Buckeridge, 2011; Fatichi et al., 2016; Baumgartner et al., 2018; Beaumont et al., 2019; Guisan et al., 2014).

Regarding Chiapas, the cocoa cultivated area is 18 426 ha, with a production of 9 869 t with an average yield of 535,6 $kg \cdot ha^{-1}$ (SIAP, 2018). According to Avendaño et al. (2011) it represents an important source of income with 33.0% of national production, so that 11 069 producers in Chiapas depend economically on this crop. In most cases, cacao farmers cultivate cacao with experience and knowledge of traditional agriculture and do so with a peasant economy approach (Nájera, 2012), even though Avendaño et al. (2013) indicate that cocoa plantations have economic, social, cultural and environmental value. These assessments are corroborated with the results obtained by Hernández et al. (2015), who point out that cocoa production in Chiapas is managed by small producers, with plots smaller than 2 ha (58.7%) and 41.3% with larger plots. Most of the producers are male (80%) and 19.3% female, who are also housewives and take care of their children, tripling their working days.

Cocoa crop faces a crisis due to environmental, technological, economic, social and phytosanitary factors (Díaz et al., 2013; Hernández et al., 2015; Suárez-Venero et al., 2019); however, it presents encouraging future, since there is a national and international potential market that reports a demand growth of 2.5% per year, led by the consumption of cocoa that is related to industrial demand to produce mainly chocolates (ICI, 2013). There are agroclimatic conditions in Chiapas to increase production, which implies increasing the cultivated area. For this reason, it is necessary to identify areas with high productive and economic potential by generating information that becomes a necessary element for decision-making (Espinosa-García et al., 2015).

Coffee cultivation was introduced in Mexico in 1796, and consolidated as an important crop in the economic, environmental and social context that is part of the agro-productive culture of subtropical and tropical regions. Chiapas is the main coffee producer (var. robusta and arabiga), with a sown area equivalent to 252.743,77 ha, with approximately 180 856 coffee producers and 367.874,15 t of production in 2019 (SIAP, 2018). The coffee areas in Chiapas are characterized by their environmental, technical, economic and sociocultural differences, which influence grain production (Medina-Meléndez et al., 2016). Coffee is currently produced in 11 states of the Republic. Chiapas, Veracruz, Puebla and Oaxaca account for 89.7% of total national production in 85% of the total area sown, with 83% of national producers (SIAP, 2018). Internationally, Mexico is the eighth coffee largest producer (Organización Internacional del Café, 2018).

Hence the importance of generating climate change scenarios that represent thermal variation in the study region. Regional vulnerability reveals the differential effects of climate on society and its productive activities; for this reason, it is necessary to study the causes and distribution of the impacts of climate change on agro-productive systems based on the complex interaction of environmental, social, economic and political factors involved in each region or geographic area (Torres et al., 2011).

Studies based on the increase in temperature due to the effect of climate change show that cof-

fee cultivation will suffer a significant geographical redistribution in Mexico and other producing countries (Paes, 2010; Haggar and Schepp, 2012; Ovalle-Rivera et al., 2015); in this sense, there are few studies on cocoa farms; however, the scenario is not different. Regarding factors and limiting factors for agricultural production, agro-ecological zoning is a practical tool that defines areas by combining similar characteristics of climate, soil and biophysical potential for agricultural production. This combination is observed in areas with limitations and potentialities, which allows it to be a reference to improve the existing situation, increasing production or the area of the crop, or limiting the degradation of natural resources (Suárez-Venero et al., 2019).

Climate variability is the main factor responsible for annual fluctuations in crop production (Comisión Económica para América Latina y el Caribe, 2010). Therefore, the purpose of this research is to evaluate the current and future suitable areas (2018-2040) for cocoa (*Theobroma cacao L.*) and coffee (Coffea arabica L.) crops in climate change scenarios in the Soconusco region, Chiapas, Mexico and define the regions where availability to have information to recommend their management and conservation increases or decreases.

2 Materials and methods

2.1 Area of study

The socio-economic region of Soconusco has an area of approximately 4.605,4 km^2, equivalent to 6.28% of the state territory and is composed of 16 municipalities: Acacoyagua, Acapetahua, Cacahoatan, Escuintla, Frontera Hidalgo, Huehuetan, Huixtla, Mapastepec, Mazatan, Metapa, Suchiate, Tapachula, Tuxtla Chico, Tuzantan, Union Juarez and Villa Comaltitlan. According to the Population and Housing Census (INEGI, 2010), this region had a population of 664,437 inhabitants, which represented 16.9% of the state total.

2.2 Crop requirements

The edapho-climatic requirements involved in the development and productivity of each crop were defined with the aim of assigning levels of importance to each of the variables to be considered. A table was created for each crop with their corresponding requirements and were classified into different proficiency levels: very suitable, marginally suitable and unsuitable, using as a reference the lower and upper ranges of each of the requirements tolerated by each crop (Table 1 and 2).

Mapping was handled and generated using ArcGIS 9.3 software. The layers used for the classification of proficiency levels according to Tables 1 and 2 were obtained from the following sources. 1) The precipitation and temperature maps were obtained from the UNAM Center for Atmospheric Sciences (Gómez et al., 2008). 2) Terrain elevation and slope maps were generated using ArcGIS 3D Analysis and Spatial Analyts tools based on INEGI (1994) 1:50.000 scale digital elevation models, available online on the official website. 3) The soil map was classified from the dominant soil map of INEGI (1994) scale 1:1.000.000.

Table 1. Environmental requirements for *Coffea arabica L.* and fitness levels with respect to environmental variables.

	Fitness level				
Requirement (variable)	Not suitable	Marginally suitable	Very suitable	Marginally suitable	Not suitable
Average annual precipitation (mm)	<750	750-1400	1400-2200	2200-4200	>4200
Average annual temperature (°C)	<10	10-14	14-28	28-34	>34
Height (masl)	<1000	1000-1200	1200-1700	1700-2800	>2800
Slope terrain (%)			<30	30-40	>40
Soils (texture)	Light (sandy)		Loams (medium)		Heavy (clay)

Source: Adapted from INIFAP (2018); Pérez-Portilla and Geissert-Kientz (2006).

Table 2. Environmental requirements for *Theobroma cacao L.* and fitness levels with respect to environmental variables.

	Fitness level				
Requirement (variable)	Not suitable	Marginally suitable	Very suitable	Marginally suitable	Not suitable
Average annual precipitation (mm)	<1000	1000-1500	1500-2500	2500-3000	>3000
Average annual temperature (°*C*)	<18	18-22	22-28	28-32	>32
Height (masl)	<5		5-400	401-601	>601
Slope terrain(%)			0-15	15-30	>30
Soils (texture)	sandy		alluvial		Clayey, burdensome

Source: Adapted from INIFAP (2018); SIAP-SAGARPA (2019).

2.3 Zoning by weight

From the above requirement matrices, current aptitude maps were generated by requirement for both crops, assigning the following weight to each of the variables: precipitation= 0.4, temperature= 0.2, height= 0.2, slope= 0.1, and soils= 0.1. Then, new weighted values were assigned to each proficiency level for each variable or map: 4, 2, and 0 for very suitable, over positioned and algebra were used in maps to obtain the current potentiality ranges. Maps were classified into three equal intervals and were again assigned the key nomenclature very suitable, marginally suitable, and not suitable (Figure 1).

2.3.1 Crop zoning by limiting factors

Zoning by limiting factors was performed using a layer overlay with the Geoprocessing tool. Maps were reclassified according to the tables of crop requirements at their different suitable levels. For this, it was necessary to assign keys to each suitable level and generate a nomenclature that allowed to know the incidence of each factor when superimposing the maps (Table 3).

Using the assigned keys, layer overpositioning was performed in order to obtain a single map that showed all possible combinations (agro-ecological cells) between proficiency levels of each of the indicators studied as shown in Figure 2.

2.3.2 Climate change scenarios

Temperature and precipitation, and in general, the water cycle were the variables through which climate change was evident. Thus, the output data of atmospheric-oceanic general circulation models (GCMs) of these two parameters were necessary for developing a future crop fitness scenario, considering such a change. The output data from two GCMs, HadleyCM3 and GFDLR30, were used to map climate change. Climate change models were obtained from the UNAM Center for Atmospheric Sciences for scenario A2 and B2 at the time horizon of 2030 (Gómez et al., 2008). The same steps, nomenclature and criteria used in determining the current (base scenario) fitness by weight and constraints were used for preparing future fitness maps with climate change. Whenever the temperature and precipitation variables were modified, the tables of climatic requirements were applied again to obtain possible future scenarios. Figure 3 describes the general process of this study.

3 Results and discussion

3.1 Current fitness by weight for the two crops

Once the algebra of maps was done, the ground surfaces of the different fitness levels for coffee were 37.8% for very suitable, 61.0% for marginally suitable and 1.1% for not suitable. On the other hand, the different fitness levels for cacao were: 59.2; 24.3 and 16.5% very suitable, marginally suitable and unfit; respectively (Figure 4).

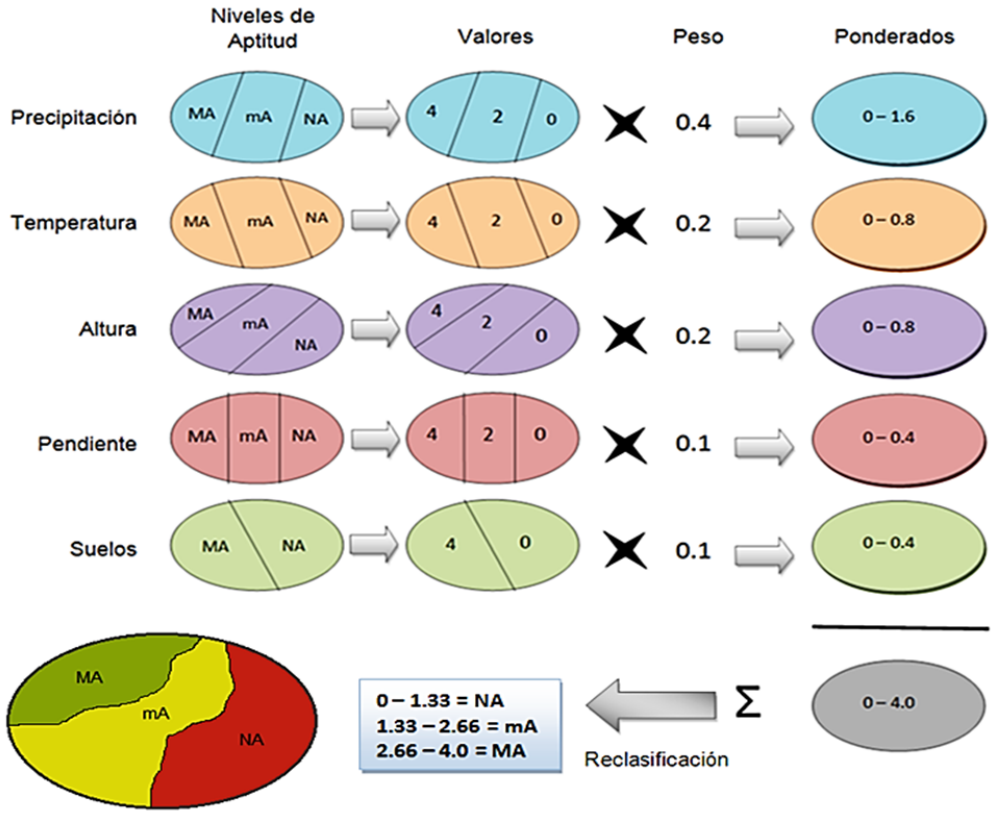

Figure 1. Factor weighting method with nomenclature and assigned values.

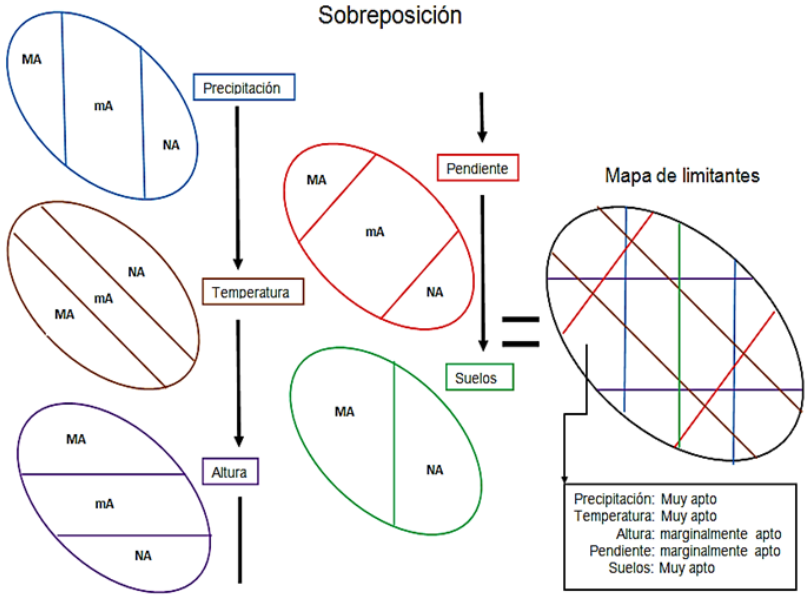

Figure 2. Limitation method with nomenclature and description of agro-ecological cells.

Figure 4 is interpreted as a distribution of areas: (1) where it is advisable to establish crops (highly suitable); (2) in appropriate areas but which require more management for their development (marginally suitable); and finally, (3) where it is not recommended to establish crops because the land does not have the necessary potential for its production.

3.2 Current suitability for coffee and cocoa crop constraints

Constraint maps for both crops showed the variability of different units, perfectly identifiable by the environmental conditions that occurred in each of them, so the color schemes used were neither hierarchical nor exclusive. Likewise, each unit was described by a unique formula. In the case of the current suitability for growing coffee, 24 different units were presented, although the first seven covered more than 75% of the area of the region. On the other hand, the current suitability for cacao presented 36 different classes, of which the first six covered more than 50% of the total area (Figure 5).

The suitability by constraints is totally qualitative and represents a complement to what was obtained during weighting, because it allows to identify the incidence of each of the variables considered in the agro-ecological zoning. It can be seen in Figures 5a and 5b that the red colored areas are limited by a greater number of variables and can be identified using the nomenclature described in Table 3.

Table 3. Nomenclature used for determining formulas.

Variable	Very suitable	Marginally suitable	Not suitable
Precipitation	P_{MA}	P_{mA}	P_{NA}
Temperature	T_{MA}	T_{mA}	T_{NA}
Height	A_{MA}	A_{mA}	A_{NA}
Slope	Pe_{MA}	Pe_{mA}	Pe_{NA}
Soil	S_{MA}	S_{mA}	S_{NA}

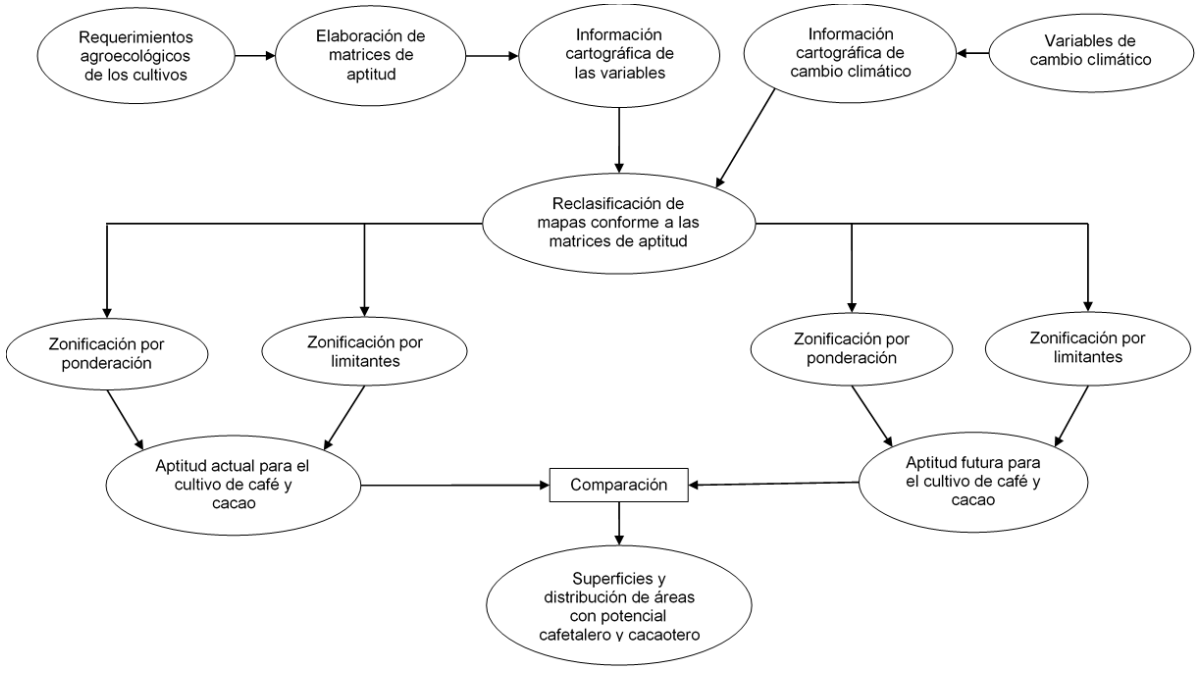

Figure 3. General diagram of the employed method.

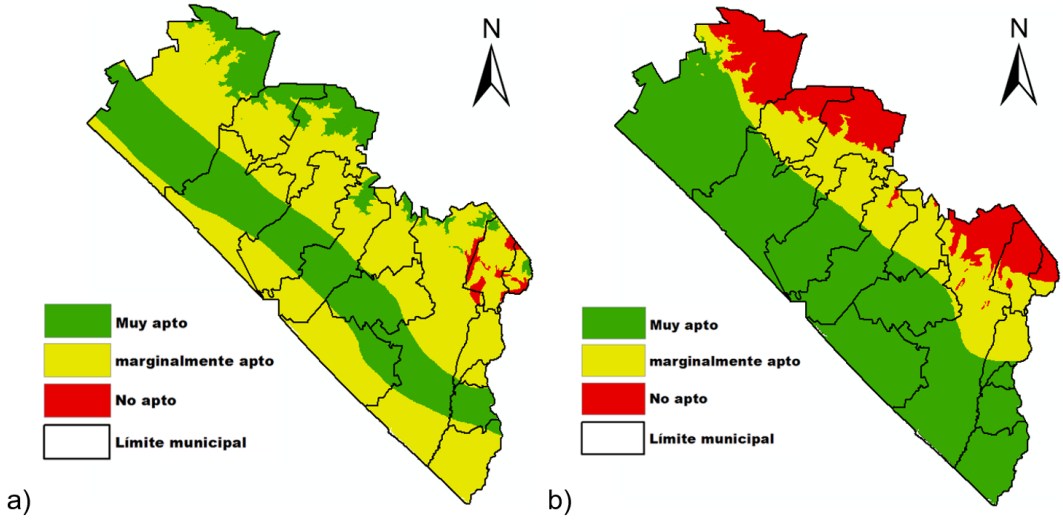

Figure 4. Current land fitness with the weighting method for coffee (a) and cocoa (b) in the Soconusco region, Chiapas, Mexico.

3.3 Climate change scenarios by weighting

Changes in areas with coffee and cocoa potential are presented in Table 4, considering that the total area of the study region was approximately 590 thousand ha. As shown in Table 4, there were no percentage changes between scenarios (A2 and B2) for either model, even though a difference was observed in the precipitation output and temperature values of each model. The above was due to the fact that during the reclassification of the maps, they were assigned the same skill level because the difference between the values was not sufficient to classify them into different skill ranges according to Tables 1 and 2. There was also a minimal change between the two models due to the difference in data processing for each model.

In the case of coffee, the GFDL model showed that the most affected area of the study was the southeast in the municipalities of Frontera Hidalgo, Suchiate, Tapachula, Huehuetan. Also, the municipalities Mapastepec and Acapetahua in the northwest of the region lost potential in areas near the coast. Potential-free areas were also reduced in Cacahoatan and northern Tapachula; the decrease in potential-free areas was mainly due to the higher temperature rise, which allowed a better development of the crop from 450 to 550 meters above sea level. On the other hand, the Hadley model indicated a reduction in suitability in the same municipalities as the previous one, although it also marked an expansion of the potential-free zones in the north of Tapachula and Tuxtla Chico and the east of Cacahoatan, due to a minimal increase in precipitation at lower altitudes in those areas. The Hadley model showed a less encouraging sceneraio (Figure 6).

Table 4. Area (%) by suitability range for coffee and cocoa crops with climate change scenarios in the Soconusco region, Chiapas, Mexico.

	Coffee			Cacao		
Scenarios	Very suitable	Marginally suitable	Not suitable	Very suitable	Marginally suitable	Not suitable
Base	37.8	61.0	1.1	59.2	24.3	16.5
GFDL A2	33.3	66.3	0.4	52.0	31.7	16.3
GFDL B2	33.3	66.3	0.4	52.0	31.7	16.3
Hadley A2	33.0	64.9	2.1	49.9	33.5	16.6
Hadley B2	33.0	64.9	2.1	49.9	33.5	16.6

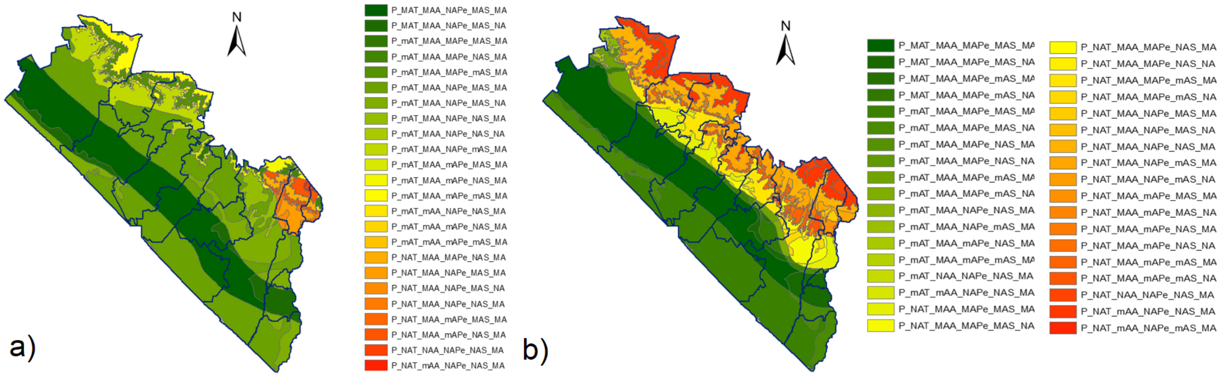

Figure 5. Current land suitability with the constraint method of coffee (a) and cocoa (b) in the Soconusco region, Chiapas, Mexico.

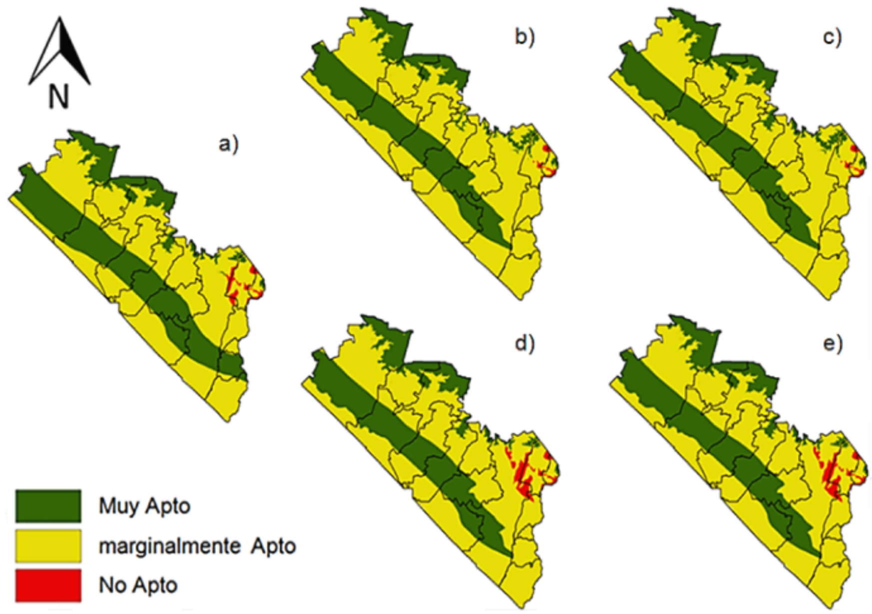

Figure 6. Zoning for coffee cultivation: A) Current fitness and; Climate change scenario readiness for 2030: b) GFDL A2, c) GFDL B2, d) Hadley A2 and e) Hadley B2.

The most important changes in municipalities occurred in Acapetahua, Huehuetan, Mapastepec, Metapa, Suchiate, Tapachula and Frontera Hidalgo, the latter with a fitness reduction of up to 82.5% of its surface area. However, the GFDL model showed small positive changes such as the fitness increase of up to 4% in the municipality of Cacahoatan, (Table 5).

For cocoa, the GFDL model indicated that cocoa potential reduced significantly in the southeast in the municipalities of Suchiate, Frontera Hidalgo, Metapa, Tuxtla Chico, Tapachula and Huehuetan; as well as the coastline of Mapastepec, Acapetahua and, in a smaller proportion, Villa Comalitlan. On the other hand, the potential-free areas were reduced in the north of the municipalities Huixtla and Tuzantan, due to the increase in the temperature from 1250 to 1500 meters/3ft in the upper zones. The Hadley model indicated the same trend as the previous one, reducing potential areas in the same municipalities, although with a higher proportion (2.1%) and reducing potential-free areas in the municipalities of Huixtla and Tuzantan; in addition to an increase in the latter in the north of the municipality of Mapastepec, also due to the increase in

temperature, but in this case in low areas (Figure 7). Again, the Hadley model was less favorable in its prediction.

Similarly, the most significant changes in municipalities were observed in Acapetahua, Huehuetan, Mapastepec, Metapa, Tapachula, Tuxtla Chico, Tuzantan, Villa Comaltitlan, Frontera Hidalgo and Suchiate, the latter two with a fitness reduction of up to 38% of their surface area. In this case, there were no positive changes in fitness for either of the two models (Table 6).

All A2 experiments in the Pacific Climate Impact Consortium Special Report on Emission Scenarios (IPCC, 2013) reported that on average there will be an increase of $2^{\circ}C$ in the average temperature of the study region compared with the current one by the mid-80 of this century. On the other hand, the annual precipitation was more erratic in terms of its prediction, since it showed positive and negative percentage changes, although most models indicated a significant increase, including the Hadley model.

Table 5. Comparison of areas (%) at the municipal level of the base scenario with climate change scenarios for coffee cultivation in the Soconusco region, Chiapas, Mexico.

Municipality	Total area (ha)	Base scenario			GFDL A2 and B2 Model			Hadley A2 and B2 Model		
		Very Suitable	Marginal suitable	Not suitable	Very Suitable	Marginal Suitable	Not suitable	Very suitable	Marginal suitable	Not suitable
Acacoyagua	26.160.90	30.3	69.7	-	30.3	69.7	-	30.3	69.7	-
Acapetahua	55.293.90	53.7	46.3	-	47.3	52.7	-	47.3	52.7	-
Cacahoatán	17.996.90	3.8	76.6	19.6	7.8	85.2	7.0	3.2	76.5	20.3
Escuintla	39.464.80	38.6	61.4	-	38.6	61.4	-	38.6	61.4	-
Frontera Hidalgo	11.493.40	84.3	15.7	-	1.8	98.2	-	1.8	98.2	-
Huehuetán	30.644.40	62.3	37.7	-	50.0	50.0	-	49.0	51.0	-
Huixtla	43.295.10	40.4	59.6	-	40.4	59.6	-	40.4	59.6	-
Mapastepec	120.802.30	57.4	42.6	-	52.3	47.7	-	52.3	47.7	-
Mazatán	37.277.50	28.0	72.0	-	28.0	72.0	-	28.1	71.9	-
Metapa	3.054.30	1.8	98.2	-	-	100.0	-	-	100.0	-
Suchiate	22.997.90	4.8	95.2	-	0.2	99.8	-	0.2	99.8	-
Tapachula	95.371.20	26.0	72.1	1.9	23.7	76.3	-	20.9	72.0	7.1
Tuxtla Chico	13.073.20	2.2	97.5	0.3	-	100.0	-	-	95.4	4.6
Tuzantán	20.435.30	8.7	91.3	-	8.7	91.3	-	8.7	91.3	-
Unión Juárez	5.716.00	12.1	64.1	23.8	14.5	62.4	23.1	9.4	66.7	23.9
Villa Comaltitlán	45.800.30	33.8	66.2	-	33.8	66.2	-	33.8	66.2	-

As for O'Connor et al. (2020) concerns due to climate change correspond to the accelerated loss of biodiversity and variation in wildlife distribution; Rice (2018) suggested that there is a decrease in the available space for agriculture, and thus a reduction in the supply of food. In order to see the impact of climate variability in the case of coffee cultivation, it is necessary to know the distribution in the world. This crop can be found as traditional polyculture, commercial polyculture and monocultures. Each form of crop management has advantages and disadvantages. According to Janissen and Huynh (2018) and Torres et al. (2020) traditional polycultures and wild crops reduce leaf perspiration and the speed of night and day heating, resulting in less stress of the plant due to sudden temperature changes.

From an environmental point of view, these systems allow the conservation of a large number of plants and animals, preserving native ecosystems. On the contrary, the commercial polyculture retains the species associated with the crops that generate monetary profits for coffee farmers. Monoculture and commercial polyculture are the most productive in the short term. However, they are the crops most sensitive to pests because they provide a more conducive environment for the growth of pathogens.

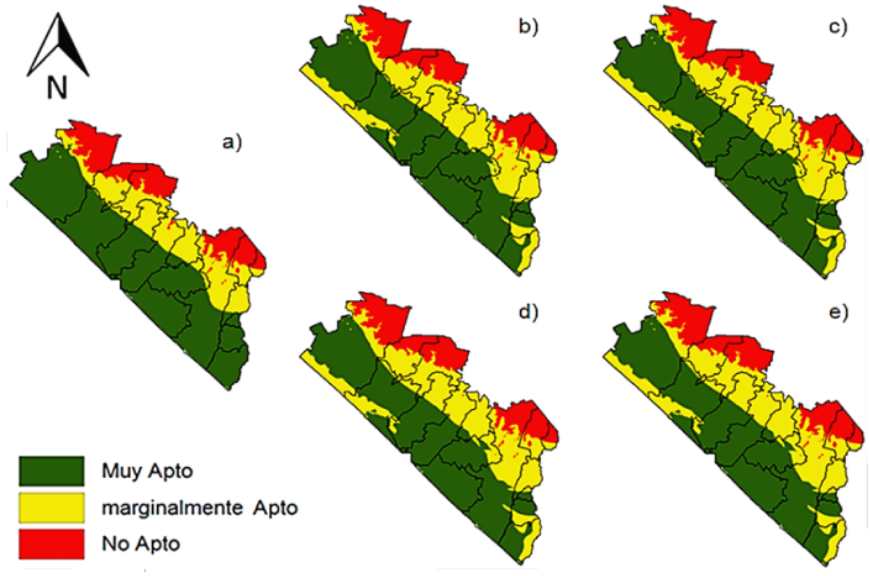

Figure 7. Zoning for cocoa cultivation: A) Current fitness and; Climate change scenario fitness for 2030: b) GFDL A2, c) GFDL B2, d) Hadley A2 and e) Hadley B2.

Table 6. Comparison of areas (%) at the municipal level of the base scenario with climate change scenarios for cocoa cultivation in the Soconusco region, Chiapas, Mexico.

		Base scenario			GFDL A2 and B2 Model			Hadley A2 and B2 Model		
Municipality	Área total (ha)	Very suitable	Marginal suitable	Not suitable	Very suitable	Marginal suitable	Not suitable	Very suitable	Marginal suitable	Not suitable
Acacoyagua	26.160.91	1.1	59.5	39.4	1.1	59.9	39.0	0.5	60.5	39.0
Acapetahua	55.293.91	95.7	4.3	-	77.9	22.1	-	75.2	24.8	-
Cacahoatán	17.996.89	-	39.3	60.7	-	39.4	60.6	-	39.4	60.6
Escuintla	39.464.77	3.2	43.9	52.9	3.2	44.1	52.7	2.6	44.7	52.7
Frontera Hidalgo	11.493.41	100.0	-	-	61.5	38.5	-	61.5	38.5	-
Huehuetán	30.644.37	89.1	10.9	-	84.3	15.7	-	75.1	24.9	-
Huixtla	43.295.09	63.7	34.9	1.4	63.6	36.3	0.1	62.5	37.4	0.1
Mapastepec	120.802.31	64.5	8.6	26.9	54.5	18.7	26.8	52.3	19.7	28.0
Mazatán	37.277.47	100.0	-	-	99.5	0.5	-	99.5	0.5	-
Metapa	3.054.27	99.9	0.1	-	91.9	8.1	-	76.3	23.7	-
Suchiate	22.997.88	100.0	-	-	61.2	38.8	-	61.2	38.8	-
Tapachula	95.371.22	51.2	30.2	18.6	48.6	32.8	18.6	46.4	35.0	18.6
Tuxtla Chico	13.073.17	16.9	83.1	-	10.9	89.1	-	6.2	93.8	-
Tuzantán	20.435.30	18.2	80.8	1.0	14.5	85.5	-	12.2	87.8	-
Unión Juárez	5.716.02	-	32.3	67.7	-	32.4	67.6	-	32.4	67.6
Villa Comaltitlán	45.800.34	70.0	30.0	-	66.6	33.4	-	65.4	34.6	-

3.4 Limiting climate change scenarios for coffee and cocoa cultivation

Like the current skill (base scenario) by constraints, future fitness maps are intended to show how the variability of existing units will behave, i.e., whether the number of different classes will be reduced or increased due to the effect of climate change. Figure 8 shows that the GFDL model showed 27 different classes for coffee cultivation, three more than in the base scenario. On the other hand, the Hadley model presented 28 different units.

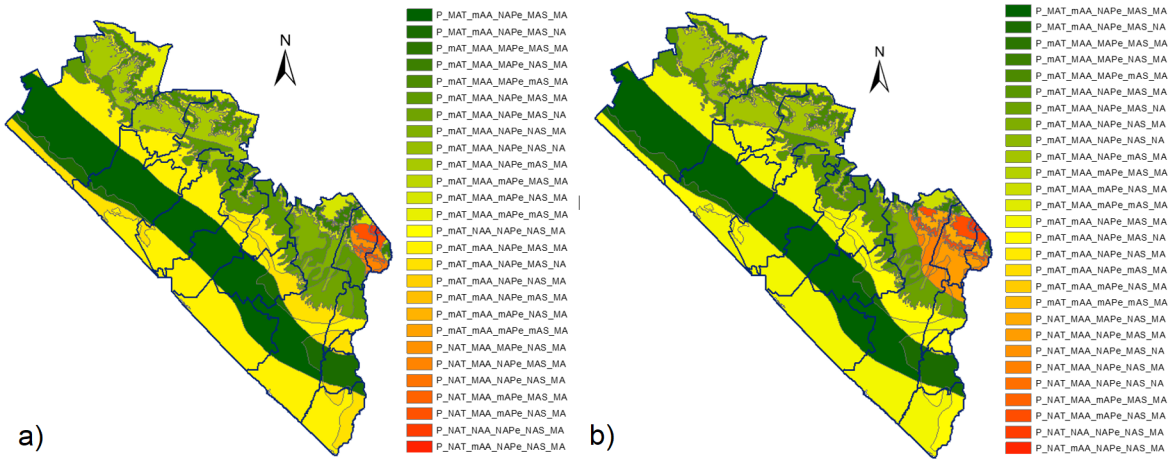

Figure 8. Future suitability by constraints a) GFDL A2 and B2 model, b) Hadley A2 and B2 model for coffee in climate change scenarios in the Soconusco region, Chiapas, Mexico.

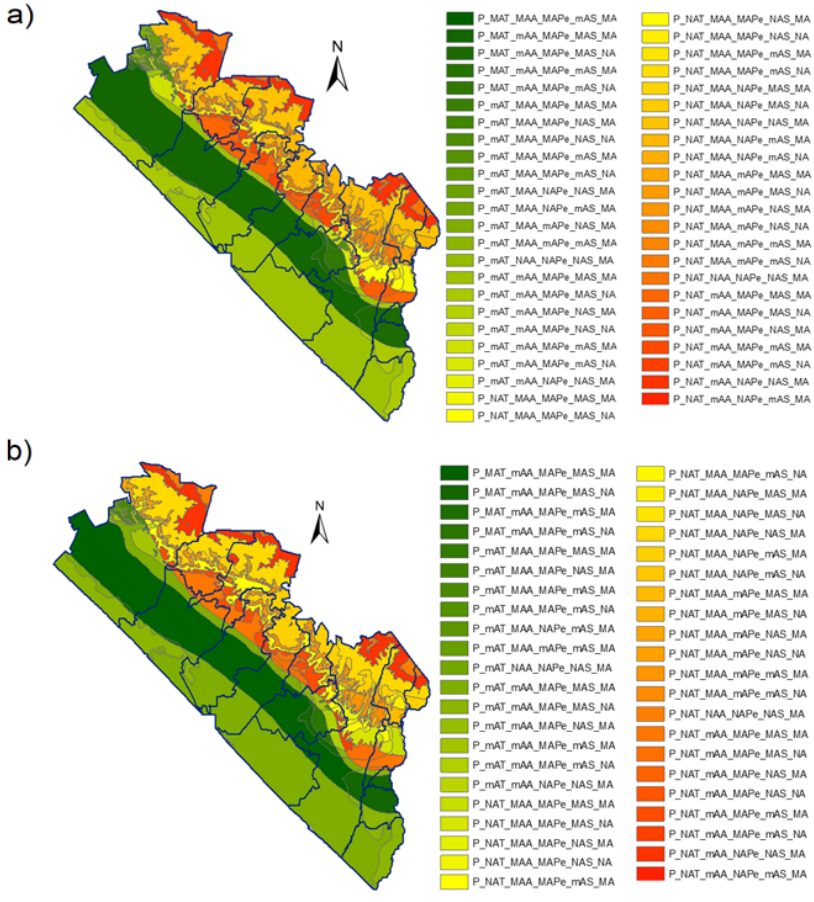

Figure 9. Future suitability by constraints a) GFDL A2 and B2 model, b) Hadley A2 and B2 model for cocoa in climate change scenarios in the Soconusco region, Chiapas, Mexico.

Regarding cocoa, the GFDL model showed 47 different classes, i.e., 11 units more than in the base scenario; the Hadley model featured 43 different classes. Cacao and coffee plantations were suitable for reforesting completely cut areas and can become biological corridors, allowing the repopulation of birds, mammals, reptiles and amphibians, among others, as suggested by Ogata (2007). Agroforestry systems of both coffee and cacao were important tools for the preservation of biodiversity, because they presented different species and forms of plant and animal life, agreeing with what was indicated by Salgado-Mora et al. (2007); these tools also provided many positive advantages, being necessary to characterize the most relevant factors in the socio-economic and environmental aspect to improve the potentialities of the crops studied and to promote themselves as an alternative of sustainable management (Roa-Romero et al., 2009); they can be a "bridge" between agricultural development and preservation, facilitating cooperation and collaboration between farmers and conservationists (Parrish et al., 1999).

4 Conclusions

The GFDL model predicts a reduction of 4.5 and 5.3% for coffee and cocoa cultivation in areas with very high potential, and an increase of 4 and 7.4% in areas with marginal potential; while the potential-free areas have a reduction of 0.7% and 0.2%, respectively; the latter case represents a positive impact of climate change, even if it is less than the one produced in areas with very high potential. Regarding the Hadley model, it predicts a reduction of 4.8% for coffee and cocoa in areas with very high potential, and an increase of 3.9 and 9.2% in areas with marginal potential, and an increase of 1.0% and a decrease of 0.1%, respectively, in areas without potential.

The future fitness of both crops will be adversely affected by climate change. The decrease in the area with a very suitable level for the establishment of both crops over time agrees with the reduction in production volume that occurred in the last decade, according to historical production data. By 2030, between 5.5 and 5.8% of potential coffee areas will be lost; while between 7.2 and 9.3% of the potential areas will be lost in cocoa.

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Hydrology

Effect of weir´s theoretical discharge coefficient on discharge measurements in small Andean streams

Efecto del coeficiente teórico de descarga de vertederos sobre la medición de caudales en pequeños ríos Andinos

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Abstract

Andean ecosystems provide important hydrological services for downstream communities. Due to this importance, several hydrological studies have been carried out in recent years, with emphasis on hydrological processes identification and land use change impacts. In several studies, but also for the operation of small-scale irrigation and drinking water projects, small streams have been equipped with compound, sharp-crested weirs for discharge estimation. To transform the water level (stage) into a discharge (water rate), weir equations use theoretical discharge coefficients, which do not necessarily apply under the actual field conditions, mainly site fluviomorphology and weir construction aspects, introducing uncertainty in their measurements. Therefore, this study analyzes the effect of using theoretical coefficients instead of adjusted coefficients in field. The study was conducted on 9 micro-catchments ($0{,}2 - 7{,}53 km^2$) located in the Zhurucay Ecohydrological Observatory in the paramo of southern Ecuador. To calibrate the coefficients, discharge curves were generated by mechanical and salt-dilution gauging methods. Results revealed that the discharge coefficients differed from their theoretical value by up to 15% for triangular (V-notch) weir section (DCvn) and by up to 41% for rectangular weir section (DCr). The DCvn affects 4 times more in low and medium discharges estimation than DCvn in high discharges. On the other hand, salt-dilution method is more precise for medium and high discharges, but at very low discharges, it overestimates discharge up to 10%. Overall, results suggest that it is essential to calibrate the discharge coefficients in the field to avoid errors in hydrological studies.

Keywords: Hydrology, Tropical Andes, Hydrological monitoring, Gauging methods, Ecuador.

Resumen

Los Ecosistemas Andinos proveen importantes servicios hidrológicos para comunidades aguas abajo de los ríos. Debido a esta importancia, se han realizado varios estudios hidrológicos en los últimos años, con énfasis en la identificación de procesos hidrológicos e impactos de cambio de uso de la tierra. En estas investigaciones y para la operación de pequeños proyectos de riego y agua potable, los ríos de montaña se han equipado con vertederos compuestos de pared delgada para estimar los caudales. Para transformar el nivel de agua en caudal, las ecuaciones de los vertederos emplean coeficientes de descarga teóricos, los cuales no necesariamente se ajustan a las condiciones reales de campo, principalmente a la fluviomorfología del sitio y aspectos constructivos del vertedero, complicando sus mediciones. Por ello, este estudio analiza el efecto de utilizar coeficientes teóricos en lugar de coeficientes ajustados en campo. El estudio se realizó en 9 microcuencas ($0{,}2 - 7{,}53 km^2$) ubicadas en el Observatorio Ecohidrológico de Zhurucay, en el páramo del sur del Ecuador. Para calibrar los coeficientes, se generaron curvas de descarga mediante mediciones de dilución de sal y mecánicos. Los resultados revelaron que los coeficientes de descarga difieren de su valor teórico hasta en un 15% para vertederos de sección triangular (DCvn) y hasta un 25% para sección rectangular (DCr). El DCvn afecta 4 veces más en la estimación de caudales bajos y medios que el DCr en caudales altos. Por su parte, el aforo por dilución de sal es más preciso para caudales medios y altos, pero en caudales bajos, este sobrestima un 10%. En general, los resultados sugieren que es esencial ajustar los coeficientes en campo para evitar errores en diferentes estudios hidrológicos.

Palabras clave: Hidrología, Andes Tropicales, Monitoreo Hidrológico, Aforos, Ecuador.

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1 Introduction

More than 50% of the world population obtains water from mountains. The Andes, which cover a continuous mountain chain in Venezuela, Colombia, Ecuador, Peru, Chile, Bolivia and Argentina, are of more than 2 500000 km^2 and are home to more than 85 million inhabitants (around 45% of total country population). In addition, at least another 20 million people living in the Pacific coastal cities of South America also depend on water originated in the Andes (Hofstede et al., 2003; CONDESAN, 2012). Páramo, puna, jalca and montane cloud forest ecosystems are the water towers of the Andes (Ochoa-Tocachi et al., 2016a), and their natural hydrological regulation is considered key for the operation of unregulated (i.e. without human-made reservoirs) drinking water and irrigation systems in high Andean areas (De Bièvre et al., 2003; Hamel et al., 2018).

Several hydrological studies have been carried out in recent years in Andean páramo catchments, ranging from understanding hydrological processes and runoff generation (Mosquera et al., 2015; Correa et al., 2017, 2019; Mosquera et al., 2018; Lazo et al., 2019) to the impacts of agriculture (Buytaert et al., 2005, 2006; Crespo et al., 2010; Ochoa-Tocachi et al., 2016a) and afforestation with exotic species (Buytaert et al., 2007; Crespo et al., 2012; Bonnesoeur et al., 2019, 2018; Marín et al., 2018). Similar studies have been conducted in Andean forests (Tobón, 2008; Roa-García et al., 2011; Crespo et al., 2012). These studies rely on hydrological data gathered in experimental and representative catchments. Such is the importance of hydrological monitoring that has given rise to the Regional Initiative for the Hydrological Monitoring of Andean Ecosystems-iMHEA (Célleri et al., 2010; Ochoa-Tocachi et al., 2018). iMHEA monitors rainfall and discharge in more than 25 micro-catchments (between 0,2 and 10 km^2) distributed throughout the Tropical Andes.

In most of these small research catchments, V-notch weirs (triangular section) are used to measure open channel discharge (Célleri et al., 2010; Crespo et al., 2010; Guallpa and Célleri, 2013; Mosquera et al., 2015; Ochoa-Tocachi et al., 2016b) as they allow converting stage (or head) to discharge with high precision and accuracy through the weir equation. However, it is very difficult to find ideal field conditions for weir construction and for installation of water level sensors (Guallpa and Célleri, 2013). These departures from ideal conditions increase the uncertainty in measurements, because the discharge coefficient of the weir equation is affected. According to several authors (Westerberg et al., 2011; Guallpa and Célleri, 2013), these uncertainties should be critically evaluated before estimating water resources, but in practice are rarely done.

Theoretical discharge coefficients have been determined in laboratory through tests under controlled conditions (Bergmann, 1963). However, when weirs are constructed in small mountain rivers and ravines, field conditions are different from laboratory. Two conditions that are difficult to meet in the field are: 1) water level must be measured at an upstream minimum distance of four times the maximum head over the weir crest; and 2) incoming water flow speeds must be close to zero. The first condition is not fulfilled because, in most cases, the sensor is installed next to the weir crest, so the measurements are affected by the drawdown over the weir; this position is chosen since this is the least likely place where sediments carried in rainy seasons can cause damage to the submerged electronic sensor.

On the other hand, the riverbed steep slope (Mosquera et al., 2015) and peak flow rates do not allow keeping low flow speeds over the weirs. For these reasons, the weir's discharge coefficient will be different from the theoretical values. Nonetheless, no studies have been found so far that evaluate the impact of using theoretical discharge coefficients instead of those determined in the field. According to Birgand et al. (2013); Coxon et al. (2015), there is a literature gap about the potential uncertainty associated with discharge measurements under non-ideal conditions. In this context, the objective of this study is to identify the effect of using theoretical weir coefficients on discharge calculation under non-ideal weir-construction conditions in Andean headwater catchments.

2 Materials

2.1 Study Area

The study was conducted in the Zhurucay Ecohydrological Observatory (Figure 1), which is located

in the paramo ecosystem in southern Ecuador. Zhurucay is a tributary of the Jubones River that flows into the Pacific Ocean (Mosquera et al., 2015; Correa et al., 2019). Zhurucay has a drainage area of 7,53km^2 and an altitudinal range between 3400 and 3900 *ma.s.l*. The average annual rainfall is 1345 mm; rainfall is well distributed during the year and it is characterized by frequent and low intensity events (Padrón et al., 2015). The annual evapotranspiration (ETa) is 622 mm (Ochoa-Sánchez et al., 2019). Vegetation is mainly composed of tussock grasses (72% of the basin) and cushion plants (24%); forest species such as Polylepis and Pines cover the rest of the catchment (Correa et al., 2017; Carrillo-Rojas et al., 2019).

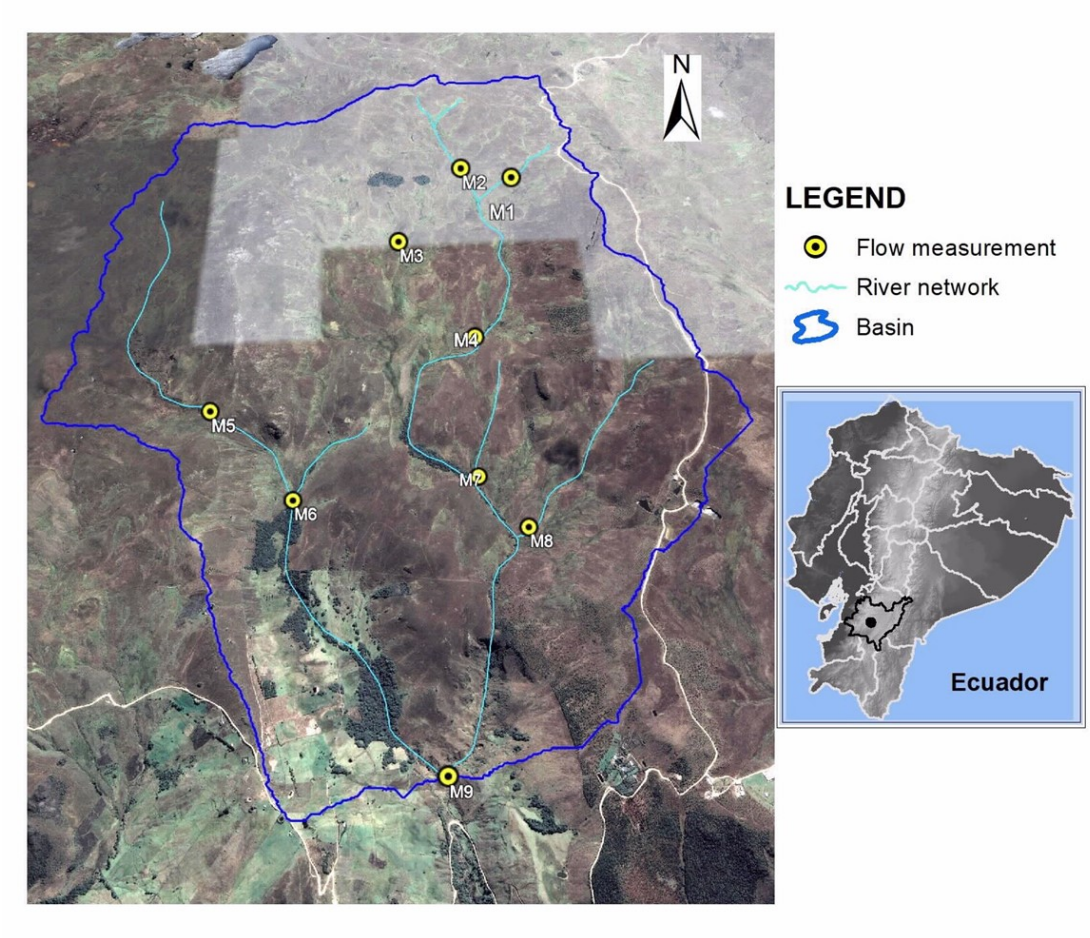

Figure 1. Study site: Zhurucay Ecohydrological Observatory. M9: rectangular weir. M1 to M8: V-notch weirs.

2.2 Weirs

Discharge monitoring in Zhurucay follows a nested approach, using 9 weirs located up or downstream stream the confluences. Those weirs are either compound V-notch sharp-crested weirs (consisting of one v-notch part (triangular section) and a rectangular part, and denoted as M1 to M8) or a sharp-crested rectangular weir (M9). There is free discharge downstream the weirs (i.e., there is air under the sheet of discharge over the weir or the nappe), which is a requirement for using the weir equation (Figure 2). Each weir has a water level sensor with an accuracy of 1 mm, and records data with a 5-min frequency. Compound V-notch weirs were placed in the smaller streams (0,2 to 3,28km^2) as

they allow measuring very low discharges accurately (Célleri et al., 2012). Weir construction and specifications can be found in Bergmann (1963); United States Bureau of Reclamation (2001).

Figure 2. Examples of a rectangular weir (left) and a compound (V-notch-rectangular) weir (right) in the study area.

There are specific weir equations for V-notch and rectangular weirs. For V-notch weirs, when the discharge occurs within the triangular section, we used the Kindsvater equation (Equation 1) given by United States Bureau of Reclamation (2001). For suppressed, sharp-crested rectangular weirs, Kindsvater and Carter (1959) developed equation (Equation 2). The same authors developed an equation to estimate the discharge that passes over a compound weir, consisting of one V-notch part (with a 90-degree notch angle) and a rectangular part (Equation 3).

$$Q = 1{,}37 * h^{5/2} \quad (1)$$

$$Q = 1{,}77 * L * h^{3/2} \quad (2)$$

$$Q = 1{,}37 * (h^{5/2} - (h-H)^{5/2}) + 1{,}77 * B(h-H)^{3/2} \quad (3)$$

For equations 1 to 3: Q is discharge (m^3/s), h is the water head above the bottom of the notch (m), measured at a point at a horizontal distance of 4H upstream the weir, H is the v-notch height (m), B is the combined width (m) of the weir´s rectangular parts, L is the width (m) of the rectangular weir; and 1.37 and 1.77 are the theoretical discharge coefficients for the v-notch (DCvn) and the rectangular section (DCr), respectively. The H of all Zhurucay weirs is 30 cm. For this study, low discharges were considered up to 5,5 l/s ($h < 11$ cm), 5.5 and 67,5 l/s ($11 \leq h < 30$ cm) for medium discharges, and values higher than 67.5 l/s ($h \geq 30$ cm) for high discharges.

3 Methods

Discharge coefficients of equations 1 to 3 were questioned and recalculated. First, water level (stage) and discharge were measured in the field, in several field campaigns, making sure the complete range of flow rate conditions (low to very high) was registered (Section 3.1). Measurements for medium and high flow rates (8 to 10 discharge measurements) were done in February-May period (rainy period), and for low flow rates (5 to 7 measurements) in August and September (dry period). Then, we calculated the effective discharge coefficients and analyzed the impact of using theoretical ones for discharge estimation (Section 3.2).

3.1 Methods for discharge measurements

Three discharge-gauging methods were used to estimate the head-discharge curves: volumetric (volume-time), velocity-area (Current meter) and dilution method. In this section, we provide the details of each method. The dilution method is described in more detail since it is the least common.

3.1.1 Volumetric Method

A 20 liters bucket was used to collect water flowing over the v-notch weirs. The time to fill the bucket was measured by stopwatch. The discharge was estimated as the division of the bucket volume to the time needed to fill the bucket (Hydromatch, 2014). This method was used to measure discharges with water level above the weir bottom of up to 15.5 cm. Several measurements were taken in succession as to have an indication of the accuracy of results.

3.1.2 Velocity-Area Method (Current meter)

The discharge was determined from measuring the stream cross-section area and the average velocity of flow. This method is detailed in the Hydrological Practices Guide of the World Meteorological Organization (WMO, 1994). These measurements were made upstream or downstream of the weir (distance of 5 m approximately), where the water flow conditions were stable. Flow velocity was measured using a current meter propeller revolving about a horizontal axis. Each revolution of the propeller generates an electrical impulse that is recorded by a datalogger. The speed of revolution is proportional to the flow velocity (WMO, 1994). Before undertaking field measurements, the equipment was calibrated in the laboratory according to the factory manuals. This method was used to measure medium to high discharges, when the water head above the weir was higher than 15 cm. The method was not used for low discharges because the low water levels meant the propeller was not submerged totally.

3.1.3 Chemical method (Salt Dilution)

This method relies on the ability of the stream to uniformly dilute the tracer in the water (Rantz, 1982). Mountain rivers and streams have slopes greater than 35 % on average (Mosquera et al., 2015) and turbulent flow. These features allow for the application of the salt dilution method in the study area. Furthermore, according to Kite (1993), this technique should be applied when discharge is lower than 15 m^3/s. In the study area, the maximum measured discharge is $6m^3/s$ at the outlet (M9). Therefore, the method was applied to gauge low, medium and high discharge.

The specific method used was the instantaneous injection (Rantz, 1982). The detailed procedure for the field gauging is described in Frederick and Cobb (1985); Kite (1993); Bronge and Openshaw (1996). This can be summarized in four steps: 1) diluting a known salt quantity in a bucket of water, 2) injecting the solution upstream of the gauging section at a point where the flow is turbulent, 3) measuring the initial Electric Conductivity (EC) at the gauging section and then for every time step (the time step for the present study was 1 second), 4) taking continuous measurements until EC reaches the initial value ($\pm 2\mu S/cm$). When initial–maximum relations of electric conductivities are between 2.5 to 3, discharge measurements are very accurate (Bronge and Openshaw, 1996; Comina et al., 2013).

To calculate discharge, the time-EC curve has to be constructed. The area beneath the curve corresponds to the rise in EC because of the injected salt. The salt equivalent that crosses the section in each time step is then obtained by means of the conversion factor (K). For our case, $K = 2{,}0832$ ($l/mg * \mu S/cm$). The area beneath the curve is determined using equation 4, and the gauging discharge using equation 5. Where A is the area beneath the gauging curve ($\mu S/cm * s$); EC(t) is electric conductivity in the time t ($\mu S/cm$); ECbg is the river's initial electric conductivity ($\mu S/cm$); M is the amount of salt that is injected (mg); K is the conversion factor; and Q is the estimated discharge (l/s).

$$A = \int \left(EC(t) - EC_{bg}(t)\right) dt \approx \sum \left(EC(t) - EC_{bg}(t)\right) \Delta t \tag{4}$$

$$Q = \frac{M}{A/K} \tag{5}$$

In order to make sure that the saline solution that is injected to the river is properly mixed, it is necessary to consider an adequate distance between the injection point and the gauging section. Although there are several equations to calculate this so called mixing length (Kite, 1993; Comina et al., 2013, 2014), it is better to determine this length on the field for every site (Tazioli, 2011). As a rule of thumb, this length should be between 20 and 70 meters, depending on the discharge and the conditions of the flow in the gauging section (Sappa et al., 2015). Besides, the distance between the injection point and the gauging section must be straight and without ponding for the salt not to be separated from the main flow (Hudson and Fraser, 2002). Because every stream of the 9 micro-catchments has

different fluviomorphological properties, different salt amount-discharge relations were used for the flow gauging. Table 1 shows a guide for flow gauging in the páramo ecosystem with the salt dilution method, based on initial and maximum EC ratio (μ_{max}/μ_0) which have to be between 2.5 to 3 for an adequate flow gauging (Comina et al., 2013).

Table 1. Relationship among the salt amount, mixing length and discharge for flow gauging using the salt dilution method in the studied basin.

Salt amount (M)	Mixing length	Discharge (Q)	M/Q	$\frac{\mu_{max}}{\mu_0}$
gr	m	l/s	$\frac{kg}{m^3}$	-
10	13	2.19	4.56	3.17
10	13	3.17	3.15	2.98
14	13	5.31	2.63	3.26
16	15	8.51	1.88	2.68
35	18	14.34	2.44	2.73
36	15	18.89	1.9	3.27
65	18	42.16	1.54	2.94
85	20	51.17	1.66	2.78
148	20	81.75	1.81	3.1
177	25	138.46	1.27	2.29
360	25	384.22	0.93	2.74
420	30	621.65	0.67	2.6
2500	50	1420	1.76	2.8

μ_{max}: max conductivity.
μ_0: initial conductivity.

The determined length for the different streams is between 13 m and 50m. Except from the low flow rate conditions, the lengths were in the 20-70 m range, as suggested by (Sappa et al., 2015). Even though the values shown in Table 1 can be used as a reference to gauge flow rates in basins of similar conditions, it has to be taken into account that the amount of salt to dilute and the mixing length depend on the velocity and turbulence of the flow, on the initial EC, and on the slope and ponding conditions of the water in the stream.

For the total number of flow rate gauging essays that were performed, the average relation was 2.01 kg of salt per m^3, with a minimum of $0{,}67 kg/m^3$ and a maximum of $4{,}56 kg/m^3$. These relations are in agreement with Valdes (2007), who recommended values in the 2-5 kg/m^3 range. On the other hand, (Hudson and Fraser, 2002) shown that the salt quantity is not critical for the application of the salt dilution method. They concluded that if the amount of salt is increased, the area beneath the EC curve would proportionally increase and have a minimum impact on discharge estimations. Nonetheless, they suggest using concentrations of $2 kg/m^3$.

3.2 Discharge coefficient calibration

To calculate the discharge curve for each weir, the best gauging method for different field conditions was determined. For low discharge, the volumetric method was considered as the reference because of the high precision that it has for these conditions. For medium and high discharge, the salt dilution method was considered as the reference because it is very accurate in mountain rivers (Kite, 1993) and its precision is higher than 95% under full mixing conditions (Moore, 2004). To make sure that the results of the present study are robust and to calculate the relative differences among the three described methods, the discharge was gauged using all of them simultaneously.

Once the discharge curves are calculated, the Discharge Coefficients (DC) for each weir can be determined. The DCs were calculated by means of the least squares adjustment method (World Bank y Government of the Netherlands funded, 1999), using the stage-discharge relation for each weir and keeping the exponents of the equations constant (Equations 1 to 3). To obtain the best-adjusted discharge curve to gauging points, the Percent Absolute Bias (PAB) (Equation 6) was used as the statistical index to evaluate the difference between the observations (gauging) and the calibrated discharge curve. Where X is the adjusted data; Y is the observed data; and $\langle X,Y\rangle$ is one-half of the average of the $X+Y$ sum. The criteria to categorize the results was adopted from Tokay et al. (2010), shown in Table 2.

$$PAB = \frac{(1/n)\sum_{i=1}^{n}\|X_i - Y_i\|}{\langle X,Y\rangle} \quad (6)$$

Table 2. Criteria for the Percent Absolute Bias index (PAB).

PAB	Adjustment
< 5	Excellent
$5-10$	Very good
$10-15$	Good
$15-20$	Reasonable
> 20	Poor

4 Results and Discussion

4.1 Comparison among flow rate gauging methods

For low discharge conditions, the salt dilution method slightly overestimates the values when compared to the volumetric method $(0{,}14 l/s)$ (Figure 3 and Table 3 show the average differences among the three gauging methods for 9 micro-catchment). This overestimation might be due to the fact that for low discharge conditions the saline solution does not fully mix in the stream, which is the main condition for an efficient gauging, thus causing this error. As the discharge values rise, turbulence also increases and the difference between the salt dilution and the volumetric methods diminishes to a point in which the volumetric method results in higher values for medium discharge conditions. This is because the precision of the volumetric method decreases for higher discharge, i.e. the container is filled with water much faster and the recording time is shorter ($<$ 2 seconds), which impedes time to be measured accurately and generate measurement errors.

Table 3. Differences among the three flow rate gauging methods.

		Flow rate gauging methods					
	Stage	**Volumetric**		**Velocity-Area**		**Salt Dilution**	
	cm	**l/s**	**Difference* (%)**	**l/s**	**Difference* (%)**	**l/s**	**Difference* (%)**
Low discharge	5	0.73				0.9	23.28
	5.2	0.8				0.9	12.5
	5.4	0.86				0.95	10.46
	6.8	1.52				1.7	11.84
	7.5	1.96				2	2.04
	7.9	2.16				2.05	-5.09
	9.5	4.34				4.5	3.68
	10	5.2				4.73	-9.03
	10.5	5.62				5.31	-5.51
Medium discharge	11	6.81	-0.88			6.87	
	11.5	7.37	5.44			6.99	
	12.5	9.64	8.43	15.62	75.77	8.89	
	13.1	10.8	10.2	17.09	74.45	9.8	
	14.5	13	7.43	18.27	51.06	12.1	
	15.5	15.9	6	22.76	51.79	15	
	17.2			24.93	38.5	18	
	18			26.55	32.1	20.1	
	24.8			54.64	26.5	43.2	
	25.6			57.4	21.1	47.4	
	29.8			88.63	15.4	76.81	

*For low discharges, the difference is with respect to volumetric method while for medium and high discharges is with respect to salt dilution method.

The velocity-area method overestimates the discharge (40% compared to the salt dilution method on average (Figure 3 and Table 3)). This might be due to the fact that the gauged streams do not meet the conditions recommended by the World Meteorological Organization (WMO, 1994), in particular that the flow lines must be parallel along the stream section and that the minimum stage above the weir must be 15 cm. These results are similar to the ones in Gees (1990); Tazioli (2011), who found differences up to 38% and 50% respectively for low and medium discharge conditions. On the other hand, Sappa et al. (2015) concluded that the salt dilution and the velocity-area methods are similar, with small differences between 1% and 8%. However, the differences with our research are that in the aforementioned study, channels with laminar flow were used for the current meter gauging. Based on these re-

sults, the discharge curve for the different weirs was constructed considering data from the volumetric method for low discharge conditions ($Q < 5{,}5$ l/s) and from the salt dilution method for medium ($5{,}5 \leq Q < 67{,}5$ l/s) and high discharges ($Q \geq 67{,}5$ l/s) conditions.

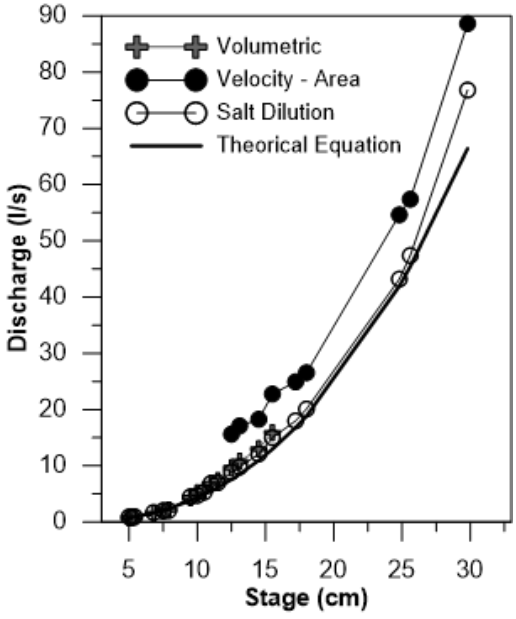

Figure 3. Stage-discharge relation using the three gauging methods, average of 9 weirs.

4.2 Discharge coefficients calibration

Table 4 shows calibrated discharge coefficients for the 9 weirs; five DCr were not calibrated because there were insufficient flow rate gauging data on high discharge in these weirs. The adjustment error for the discharge values obtained in the flow rate gauging are low when compared to the ones calculated using the calibrated discharge curve ($PAB \leq 5\%$ in the Table 4). One the other hand, DCvn vary between 1.19 and 1.56 for the triangular section (theoretical $DCvn = 1{,}37$), and between 1.53 and 3.00 for the rectangular section (theoretical $DCr = 1{,}77$). These differences represent a relative error up to 15% for V-notch weir section (DCvn) and by up to 41.0% for rectangular weir section (DCr) compared with theoretical values (Table 4). The differences between theoretical and calibrated coefficients are because of the specific features of each stream and weir, such as the structure building, the riverbed slope and the cross section.

A more precise hydrograph was obtained by using the calibrated discharge curves. This allows for the determination of the error on water yield of each micro-catchment when using the theoretical equations with the uncalibrated coefficients. Runoff coefficient ($RC =$ input precipitation/outlet runoff) represents the water yield of a catchment. The relative error on the RC is between 2.11% and 15.51% with both negative and positive bias found among this group of weirs, 6.56% on average for all weirs (Table 4). As is observed, when the weirs are not calibrated, the water balance calculation of the micro-catchments is affected.

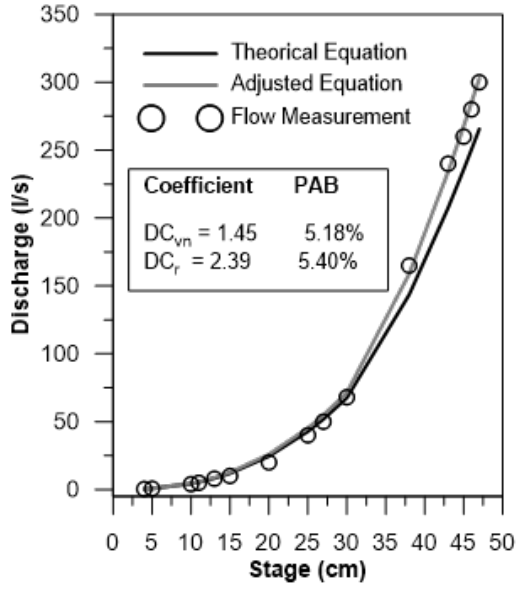

Figure 4. Calibration of discharge curve for compounded weir M4.

A sensitive analysis of discharge coefficients in the hydrograph was carried out (Figure 4). First, the accumulated annual discharge was determined by changing only the adjusted DCvn in the equation 3; then, the same estimation was done by using only the calibrated DCr ($DCvn =$ theoretical coefficient). According to these analyses, for a hydrological year, DCvn affects 4 times more in low and medium discharges than DCr in high discharges. Discharge estimation is more sensitive to DCvn because most of the time (95% of the total records) discharge is lower than $65{,}5 l/s$ ($h < 30$ cm in this study); i.e. flow rate passes only over the triangular section of the weirs. Furthermore, the theoretical equation for the

triangular section is valid only for discharge higher than 1.41 l/s ($h = 6{,}4$ cm) (United States Bureau of Reclamation, 2001), which is higher than the low discharge values observed for the study area (0,70 l/s; $h = 5$ cm), especially for dry conditions.

Table 4. Calibrated discharge coefficients for the weir equations and their effect on the Runoff Coefficients (RC) of each micro-basin.

Weir	Discharge Coefficients						Initial	Corrected RC	Relative Error RC
	DCvn			DCr			mm/mm	mm/mm	%
	Value	PAB	Relative Error DCvn	Value	PAB	Relative Error DCr			
M1	1.235	3.9	10.90%				0.625	0.564	10.82
M2	1.19	3.8	15.10%				0.648	0.564	14.89
M3	1.557	1.95	-12.00%				0.564	0.64	-11.88
M4	1.45	5.18	-5.50%	2.39	5.4	-25.90%	0.623	0.666	-6.46
M5	1.56	3	-12.20%				0.56	0.63	-11.11
M6	1.521	8	-9.90%				0.446	0.491	-9.16
M7	1.3	6.9	5.40%	1.825	2.8	-3.00%	0.873	0.855	2.11
M8	1.417	4.7	-3.30%	3	4.3	-41.00%	0.731	0.776	-5.8
M9				1.533	5.2	15.50%	0.782	0.677	15.51

DCvn = Discharge coefficient for Triangle section (V-notch). *DCr* = Discharge coefficient for rectangular section. *RC* = Runoff coefficient.

5 Conclusions

The present study was focused on determining the discharge coefficients for weirs installed in the field and its importance for discharge calculation. In regard of the different flow rate gauging methods, it can be concluded that the velocity-area method is not applicable in all conditions because of the numerous assumptions it poses that are not generally met for mountain rivers; this caused overestimation of the discharge of 40% on average. The salt dilution method is the best for medium and high discharge conditions, but for low discharge, underestimates 10% on average when compared to the volumetric method, which is more precise for these conditions. The use of this method is particularly convenient because it is low-cost, easy to apply; it has low impact and high precision.

On the other hand, even if the geometry was the same for the compounded weirs, the calibrated coefficients varied between 1.190 and 1.557 for DCvn and between 1.53 and 3.00 for DCr, due to different specific site characteristics. When the theoretical discharge coefficients are used, the error on the micro-catchments water yield varies between 2.11% and 15.51% for the different weirs in a hydrological year. These errors affect directly the closure of the hydrological balance of the micro-catchments. Therefore, we conclude that every weir needs to have a site calibration of the discharge coefficient as a requirement to obtain reliable discharge estimations.

The methodology and the results of the present study will be useful for different water monitoring projects in Andean ecosystems. The values of discharge can be evaluated and corrected in order to minimize the error in hydrological studies and water resources development projects.

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Scientific paper/ Artículo científico

Environmental Pollution

Effectiveness of the use of diatoms in the filtration of waterborne bacteria

Efectividad del uso de diatomeas en la filtración de bacterias de transmisión hídrica

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Abstract

Bacterial transmission to humans can be carried by water and cause enteric diseases, so the objective of this paper is to evaluate the effectiveness of the use of diatoms in the filtration of waterborne bacteria. The study was carried out at Universidad Nacional de Chimborazo, Ecuador; from October 2019 to March 2020, combining culture techniques on blood agar and MacConkey agar, colony quantification and filtration versus filter time and length. 120 dilutions samples of commercial strains of *Escherichia coli* and *Staphylococcus aureus* were used to contaminate the water in such a way that the efficiency of filtration in diatoms of different origins could be observed. The results were contrasted with the ranges established by the World Health Organization and positive and negative controls were carried out on the culture media and water. The diatoms of Guayaquil were the ones that induced a better filtration of the water compared to the diatoms of Palmira. When applying the filter bed of 10 cm of diatoms, a growth of 86 CFU/100ml was obtained in 24 hours, while when the amount of the filter was increased to 20 cm, a decrease in the bacterial load of the water was observed by 21 CFU/100ml in 10 hours. According to the range established by the WHO, bacterial growth decreased, which indicates that diatom filters have the ability to retain bacteria. For this reason, it is presumed that, when combined with additional materials such as activated carbon, their filtering potential would increase.

Keywords: Diatoms, filter, culture, filter bed.

Resumen

La transmisión bacteriana al ser humano puede vehiculizarse por el agua y ocasionar enfermedades entéricas, por lo que el objetivo de la presente investigación es evaluar la efectividad del uso de diatomeas en la filtración de bacterias

hidrotransmisibles. El estudio fue realizado en la Universidad Nacional de Chimborazo, Ecuador, desde octubre de 2019 a marzo de 2020, combinando técnicas de cultivo en agar sangre y agar MacConkey; cuantificación de colonias y filtración frente a tiempo y longitud de filtro. Se utilizaron 120 muestras de diluciones de cepas comerciales de *Escherichia coli* y *Staphylococcus aureus* para contaminar el agua de tal forma que se pudiera evidenciar la eficacia de la filtración en diatomeas de distinta procedencia. Los resultados fueron contrastados con los rangos establecidos por la Organización Mundial de la Salud y se realizaron controles positivos y negativos de los medios de cultivo y agua. Las diatomeas de Guayaquil fueron las que indujeron a una mejor filtración del agua frente a las diatomeas de Palmira. Al aplicar el lecho filtrante de 10 cm de diatomeas, se obtuvo un crecimiento de 86 UFC/100ml en 24 horas, mientras que al aumentarse la cantidad del filtro a 20 cm se observó un descenso de la carga bacteriana del agua en 21 UFC/100ml en 10 horas. De acuerdo al rango establecido por la OMS el crecimiento bacteriano disminuyó, lo que indica que los filtros de diatomeas tienen la capacidad de retener bacterias. Por esto, se presume que, al combinarlas con materiales adicionales como carbón activado, su potencial filtrante se incrementaría.

Palabras clave: Diatomeas, filtro, cultivo, lecho filtrante.

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1 Introduction

Various microbial waterborne outbreaks have been documented around the world due to different factors such as fecal contamination, which is a perfect vehicle until finding a host (Araujo et al., 1997; Rodríguez Miranda et al., 2016). There are examples even in industrialized countries such as the United States, where different cases (Rodríguez Gutiérrez et al., 2015; Lösch and Merino, 2016; Centros para el Control y la Prevención de Enfermedades, 2020; WHO, 2020) of bacterial outbreaks of *Escherichia coli*, *Campylobacter jejuni* and *Legionella* .spp., have been observed, as published by the journal *Quimica Viva* (Córdoba et al., 2010). However, the most significant microbial wasteborne outbreak occurred in Milwaukee, United States; the coccidium *Cryptosporidium parvum* infected about 403 000 people, causing serious complications to immunosupressed patients (Arora and Arora, 2009; Del Coco et al., 2009).

According to the World Health Organization (WHO), at least 2 000 million people worldwide are supplied with drinking water contaminated by feces, and 159 million depend on surface water that is highly likely to be contaminated. In addition, contaminated water is reported to transmit diseases such as diarrhea (Godoy et al., 2011; Cabezas Sánchez, 2018), colera (González et al., 2011; Zelada-Valdés et al., 2015), dysentery (González-Ramírez et al., 2020), typhoid fever (Peranovich, 2019) and poliomyelitis (Cué Brugueras, 2000), causing more than 502 000 deaths from diarrhea annually (WHO, 2019).

These infections usually occur because bacteria can use water as a vehicle until they reach a new host (Ferrari and Torres, 1998; Rodríguez et al., 2015; Hernández et al., 2017; Palomino-Camargo et al., 2018), in addition to the fact that these microorganisms can remain in perfect conditions and survive for weeks to months in water (Marin et al., 2020). Factors such as faulty pipe installations, damaged taps, repairs without the necessary safety measures, containers in poor condition or with poor hygienic-sanitary care and lack of proper maintenance of these facilities are potential reasons for the contamination of drinking water for human consumption (Duran and Torres, 2006), creating the environment for microorganisms to entry and multiply, causing various pathologies (Grupo de Tratamiento de Aguas Residuales Universidad de Sevilla, 2020).

In Ecuador, Palacios (2013), analyzed the condition of drinking water in a Cotopaxi region, and mentioned that fecal coliform contamination reached a prevalence of 35 %, representing a major public health problem, being a source of infection for people with these water sources. In addition, several studies have been carried out in different areas of Chimborazo, which have demonstrated the water pollution of both agricultural irrigation and human consumption. In addition, it was found that the rural communities of the region are the most affected due to the lack of quality water supply since water is available directly from pipes without the corresponding previous treatment (Lara and Martínez, 2019; Tipán and Martínez, 2019). Water is an environment conducive to the proliferation and transport of bacteria, thus being mobilized from one geographical point to another, assuming contamination risk to every living being involved in this cycle.

Considering that it is possible to eliminate most of the microorganisms present in drinking water by using filters (Chulluncuy, 2011; WHO, 2012; Ríos-Tobón et al., 2017), its application is expected to significantly reduce the bacterial load of this resource, thanks to the retention of impurities and microorganisms in it. This post-filtration water has to have a low or no bacterial charge (according to WHO range) to be considered as consumable by humans. The decrease in bacteria in water shows the effectiveness of filters. However, as several materials are required for the development of commercial filters, research on affordable and easy-to-find materials has increased for the manufacture of filters to be able to obtain clean water efficiently and at a low cost (Leal, 2014). Several authors have studied the action of various materials that function as filtering beds, whether sand (Gil et al., 2002; Blacio and Palacios, 2011), charcoal (Silupú et al., 2017; Marín Velásquez et al., 2019), diatoms (Valencia, 2014), etc., and that be highly effective and cost-effective.

The aim of this paper is to verify waterborne bacteria after using diatom filtering beds from the coastal region of Guayaquil and the Andean region of Palmira, located in Ecuador, in order to compare and demonstrate the filtration and purification ca-

pacity of water against bacteria that may be carried on it.

2 Materials and Methods

This research has a quantitative and cross-sectional approach, with an exploratory-descriptive level and an experimental design. The population is constituted by the bacteria found in the water analyzed, based on the pollution risk ranges established by WHO (WHO, 2006), which was carried out with the intermediate risk range. The sample was determined based on the relationship between the filter bed height (centimeters) and the water filtration time (hours).

Data of the positive water control were included as well as positive filter control; results that were similar to each others were done by triplicate, excluding data whose controls indicate contamination, especially negative water control and negative filter control. The study did not require a bioethics permit because it was not conducted in humans.

Due to the experimental design of the research, filters with diatoms (*Bacillariophyceae sensu lato*) of different geographical origin (commercial diatoms from Guayaquil and Palmira) were gradually tested against bacteria of different sizes, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) of commercial crops, bacteria with wide water distribution (Robert Pullés, 2014), and were evaluated after filtration in bacteriological culture media, Tryptic soy agar (TSA) as enrichment medium and specific (5% ram blood agar) and differential media (MacConkey agar). The culture media was prepared according to the specifications indicated by the HIMEDIA® commercial house. Filters were found to be free of contaminants that could alter the results.

To contaminate water, 1/10 dilutions were performed to obtain the desired intermediate range dilution of contamination, 10^{-11} dilution for *S. aureus*, whereas 10^{-13} for *E. coli*. To this, 9 ml of sterile physiological solution and 1 ml of broth containing the contaminating bacteria were added.

Once the dilutions of water contaminated with bacteria were obtained at a known concentration, diatoms were used in the filters. Two assemblies were used, the first with Guayaquil diatoms and the second with Palmira diatoms. The number of diatoms used as a filter bed was 10 cm, 15 cm and 20 cm for each source, respectively; in both cases, filtration time intervals were 30 minutes, 3 hours, 10 hours and 24 hours. During each set time, the culture was performed for the verification of bacterial load.

A glass holder (Figure 1) was used to make the filter, in which the diatoms were introduced separately according to their origin and filter height (10 cm, 15 cm, and 20 cm). The edges were covered with metal mesh and their base was fixed to a tap to control the filtration performed. At the other end of the filter, a drum was arranged which contained the contaminated water for filtering, which was then passed into a sterile flask in which the filtered material was collected for subsequent culture and bacterial quantification.

Figure 1. Diatoms were placed on a glass support which was connected to a faucet at one end and to a drum at the other.

3 Results

Of the 120 samples analyzed, it was found that the difference is minimal between the two classes of diatoms. However, filters made with diatoms from Guayaquil showed a greater filtering capacity compared to those of Palmira. Note in Table 1 that 2 filters were used; Guayaquil filter was the first one and Palmira filter was the second.

As for filter 1, using 10 cm of diatoms has a minimum filtration, yielding a growth of 86 CFU/100ml at 10 hours of waiting while using a diatom distance of 15 and 20 cm a growth of 77 CFU/100ml was obtained at 3 hours and 80 CFU/100ml at 24 hours, respectively, in reference to the strain of *S. aureus*. Regarding *E. coli* strain, when using 10 cm of diatom, growth of 88 CFU/100ml was obtained at 30 minutes of waiting, while using 15 cm of diatom showed a growth of 53 CFU/100ml at 3 hours and 21 CFU/100ml at 10 hours with 20 cm of diatoms.

As for filter 2 (Palmira) using 10 cm of diatoms has a minimum filtration, yielding a growth of 92 CFU/100ml at 10 hours of waiting while using a diatom distance of 15 and 20 cm provided a growth of 94 CFU/100ml at 3 hours and 65 CFU/100ml at 24 hours of waiting for *S. aureus* strain. As for *E. coli*, using 10 cm of diatom showed a growth of 95 CFU/100ml at 30 minutes of waiting while using 15 cm of diatom obtained a growth of 96 CFU/100ml at 3 hours and 65 CFU/100ml at 10 hours using 20 cm of diatoms.

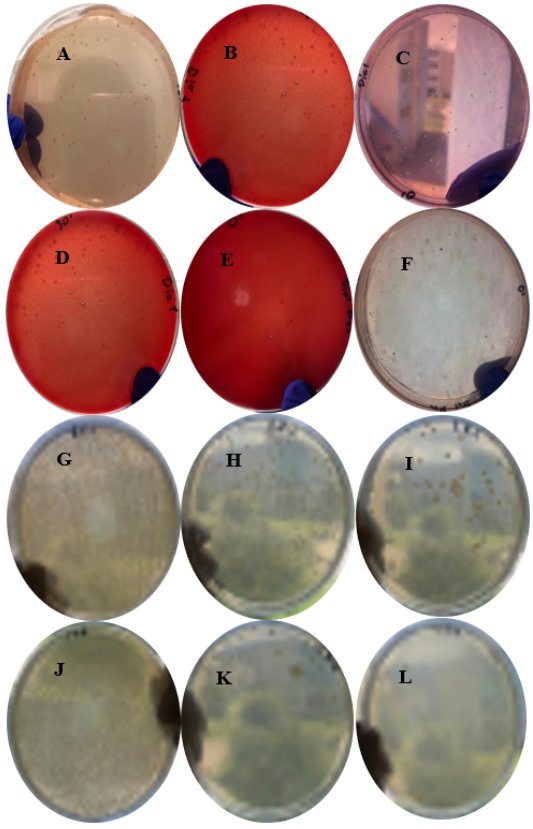

Figure 2. When using filter 2 (Palmira), of 10 cm, **A)** growth was of 95 CFU/100ml with respect to *E. coli* over a period of 30 minutes (low filtration). **B)** The growth of *S. aureus* was 74 CFU/100ml at 3 hours when using the 15 cm filter number 2, **C)** the growth of *E. coli* was 74 CFU/100ml in 0 hours. **D)** *S. aureus* growth was 108 CFU/100ml at 30 minutes. The use of filter 2 with 20 cm in length yielded the following results: **E)** *S. aureus* growth of 24 CFU/100ml at 0 hours. **F)** *E. coli* growth of 125 CFU/100ml at 0 hours. **G, H, I)** The pattern of consecutive dilutions for *E. coli* is observed until a dilution of 10^{-13}. **J, K, L)** Consecutive dilutions of *S. aureus* to a concentration of 10^{-11}.

Table 1. Comparison between diatom fiters from Guayaquil and Palmira

Measure from the filtering bed		0 hours		30 minutes		3 hours		10 hours		24 hours		
		CFU/100ml										
		S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*Pm*
Filter 1	10 cm	15	24	94	88	49	57	86	80	84	99	67.6
Filter 2		65	82	95	95	74	40	92	85	65	87	78.0
Filter 1	15 cm	77	82	80	90	77	53	27	44	27	40	59.7
Filter 2		80	74	88	92	94	96	67	71	85	89	83.6
Filter 1	20 cm	85	54	58	49	99	43	74	21	80	37	60.0
Filter 2		24	86	32	81	83	96	59	61	65	83	67.0

CFU/100ml: Forming units of colonies present in 100 milliliters of solution. Pm: Average growth per filter and measure. Filter 1: From Guayaquil. Filter 2: From Palmira.

Table 2 shows the results of the water filtration using Guayaquil diatoms as a filter bed. It may be noted that diatom growth varies at different degrees. When using 10 cm of diatoms, growth was 94 CFU/100ml at 30 minutes, then the growth decreased to 86 CFU/100ml at 10 hours and then increased again to 99 CFU/100ml at 24 hours. The growth was similar with 15 cm of diatoms, being 80 CFU/100ml at 30 minutes, 77 CFU/100ml at 3 hours and then it decreased to 27 CFU/100ml at 24 hours. On the other hand, the growth was lower with 20 cm of diatoms; 49 CFU/100ml at 30 minutes, 21 CFU/100ml at 10 hours and 37 CFU/100ml at 24 hours.

Table 3 shows the results of the water filtration using Palmira diatoms as a filter bed. When using 10 cm of diatoms, growth was 95 CFU/100ml at 30 minutes while that growth was reduced at 85 CFU/100ml at 10 hours and 87 CFU/100ml at 24 hours. With 15 cm of diatoms the growth was 88 CFU/100ml at 30 minutes, 96 CFU/100ml at 3 hours and the growth decreased to 85 CFU/100ml at 24 hours. On the other hand, there was a lower growth with 20 cm of diatoms, 32 CFU/100ml at 30 minutes, 59 CFU/100ml at 10 hours and 53 CFU/100ml at 24 hours.

4 Discussion

The filters reduce the contamination present in water, whether these contaminants are macroscopic elements or microorganisms such as bacteria (Apella and Araujo, 2000). Materials that have been widely used in the manufacturing industries have varied as new techniques and research emerge, favoring the purification of water, such as dust, coal, and others. As a result, the increase in the use of diatoms for multiple purposes related to water filtration has been observed in recent years (Colín-García et al., 2013). In a research carried out by Valencia (2014), it is mentioned that diatoms have a wide range of uses, among which seawater filtration and removal of the saline load. For this reason, it is considered that the purifying capacity of diatom is used when creating water filters, which facilitate the retention of contaminants such as dust and reduce the presence of bacteria and parasites.

Diatoms have multiple benefits when it comes to innovating and researching. Colín-García et al. (2013), mention the use of diatoms as a source of silica (SiO_2), which has many technological applications such as "drug release, photovoltaic cells, and high-performance ceramic materials". Likewise, Nájera-Arce et al. (2018), refer to the antimicrobial activity of diatoms in such a way that extracts obtained from species belonging to Bacillariophyceae s.l., have bactericidal qualities against common Enterobacteria such as *E. coli, S. aureus, Pseudomona.sp*, among others; therefore, diatoms, when applied to the water filtration for human consumption have a filter and an antimicrobial action.

By aggregating the results and contrasting diatoms from Guayaquil and Palmira, it was possible to show at first sight that the results obtained by filtration with diatoms from Guayaquil showed a higher filtering capacity (on average 77.03 CFU/100ml) unlike the diatoms from Palmira (on average 114.7

CFU/100ml) in *S. aureus* strain and *E. coli* strain. The filtration and retention effectiveness of bacteria increased as the number of diatoms used in the filter bed increased, with purification being higher when using a length of 20 centimeters of diatoms, followed by 15 centimeters and finally the lowest filter capacity when using 10 centimeters of diatoms.

Table 2. Water filtering with filter bed using diatoms from Guayaquil

FILTER 1	**0 hours**		**30 minutes**		**3 hours**		**10 hours**		**24 hours**	
	CFU/100ml									
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
10 cm	15	24	94	88	49	57	86	80	84	99
15 cm	77	82	80	90	77	53	27	44	27	40
20 cm	85	54	58	49	99	43	74	21	80	37

CFU/100ml: Forming units of colonies present in 100 milliliters of solution.

Table 3. Water filtering with filter bed using diatoms from Palmira

FILTER 2	**0 hours**		**30 minutes**		**3 hours**		**10 hours**		**24 hours**	
	CFU/100ml									
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
10 cm	65	82	95	95	74	40	92	85	65	87
15 cm	80	74	88	92	94	96	67	71	85	89
20 cm	24	86	32	81	83	96	59	61	65	83

CFU/100ml: Forming units of colonies present in 100 milliliters of solution.

These results differ from those obtained by Caballero and Zuni (2017), who in their research on the effectiveness of water filters using diatoms and chitosan obtained effectiveness with diatoms from the Andean region located at approximately 3000 meters. Using the filter they developed in their research, it was possible to reduce the bacterial load of an *E. coli* strain from 48 to 5 CFU/100ml, while in this research a significant improvement in filtration was obtained using diatoms from the coastal region (low geographic altitude), reducing the presence of the strain used from 100 CFU/100ml to 77.03 CFU/100ml. The data obtained show that diatoms alone have the ability to retain bacteria and decrease the bacterial load present in contaminated water (Caballero and Zuni, 2017). According to the study carried out by Pereira et al. (2017), the activated charcoal obtained from rice husks acts satisfactorily in the purification of water. However, the elimination of microorganisms present is not guaranteed. Therefore, it is necessary to study the combination of diatoms with activated carbon in the same filter in order to evaluate their behavior.

Pérez-Vidal et al. (2016), mention that the use of commercial filters, whose composition is better and more complex, is more effective in terms of water filtration, because the different materials arranged along the filter bed meet a percentage of water filtration, so if a layer of diatoms were added, bacterial filtration would increase and a more favorable result would be obtained (Calizaya-Anco et al., 2013).

5 Conclusions

When comparing diatoms from Guayaquil and Palmira as filter beds, it can be verified that diatoms from Guayaquil are the ones that yielded the best data.

S. aureus is used to obtain varying concentrations to contaminate water and pass it through the filter for filter bed verification. However, there is a

growth of no less than 15 CFU/100ml at hour 0 in filter 1 with 10 cm with diatom from Guayaquil.

Water contamination with *E. coli* bacilli is performed to obtain the varying concentrations to contaminate water and pass it through the filter for filter bed verification. There is a growth of no less than 21 CFU/100ml at 10 hours in filter 1 with 20 cm of diatom from Guayaquil.

The filtration time is not too important on the purification of water because contamination remained the same even with the variation of time.

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Environmental Pollution

Contrasting effects of air pollution asessment in two areas of the Quito Metropolitan District, Ecuador

Efectos contrastivos de la evaluación de la contaminación ambiental en dos zonas del Distrito Metropolitano de Quito, Ecuador

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Abstract

One of the main factors of air contamination within urban areas is burning gasoline by cars, light trucks and motorcycles. Gasoline burning liberates pollution gases such as Nitrogen dioxide, Sulfur dioxide, Carbon monoxide, benzene, besides others. Among these, Nitrogen dioxide (NO_x) is the one that stands out for the people's awareness of such contamination. We used the Hedonic Price Method (HPM) in order to determine people's perception of air quality as a proxy and estimate the effect of gases on the housing market. We selected two areas of Quito Metropolitan District in order to evaluate contamination effects on properties. One with a higher concentration of gases located within the historic district of Quito. The other with better air quality called the Bellavista district. The results of three different models indicated that a reduction of 1 ($\mu g/m^3$) of NO_x would increase an average of 4.54% of the housing market value, which it represents 2,032,326.24 USD in value for sample properties. A decrease of 5 $\mu g/m^3$ will increase in 22.7% of properties value.

Keywords: Greenhouse gases, hedonic price, housing value, air contamination, Quito.

Resumen

Uno de los principales factores de contaminación del aire dentro de las zonas urbanas es la quema de gasolina por parte de autos, camiones ligeros y motocicletas. La quema de gasolina libera gases contaminantes como el dióxido de

nitrógeno, el dióxido de azufre, el monóxido de carbono y el benceno, entre otros. Entre ellos, el dióxido de nitrógeno (NO_x) es el que destaca por la concienciación de la gente sobre dicha contaminación. En esta investigación se utilizó el Método de los Precios Hedónicos (MPH) para determinar la percepción de la gente sobre la calidad del aire como indicador y estimar el efecto de los gases en el mercado inmobiliario. Se seleccionaron dos zonas del Distrito Metropolitano de Quito para evaluar los efectos de la contaminación en los inmuebles. Una con mayor concentración de gases ubicada dentro del casco histórico de Quito, y la otra con mejor calidad de aire conocida como el sector Bellavista. Los resultados de los tres modelos diferentes indicaron que una reducción de 1 ($\mu g/m^3$) de NO_x aumentaría un promedio de 4,54% el valor del mercado inmobiliario, lo que representa un valor de USD 2.032.326,24 para las propiedades de la muestra. Una disminución de 5 ($\mu g/m^3$) aumentaría en un 22,7% el valor de las propiedades.

Palabras clave: Gases de efecto invernadero, precio hedónico, valor de la vivienda, contaminación del aire, Quito.

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1 Introduction

Clean air is considered as one of the most basic requirements of human health and well-being (World Health Organization, 2016). Its relative purity or contrarily its pollution may determine different social and economic problems (Panayotou, 2016; Gulia et al., 2015; Victor, 2017). An estimation of physical damages can be done by estimation visibility impacts, acid rain impacts, crop and other vegetation damages, property value impacts, corrosion and soiling impacts, recreation effects, and health effects. Poor air quality is associated with the incidence of short and long-term diseases, particularly neurologic and of the the respiratory system, as mentioned by several authors like Zivin and Shrader (2016); Calderón-Garcidueñas et al. (2016); Perera (2017).

Organization (WHO) estimated that in 2014, 82 % of the population in developing countries may be subject to levels that tend to exceed minimum quality standards as stipulated in their guidelines on air-quality (World Health Organization, 2006, 2016). This issue has a greater incidence in large cities on all continents (Smith and Huang, 1993; Bayer et al., 2009; Kennedy et al., 2007). Nevertheless, the impact on health of air pollution is difficult to assess. Several studies have used the value of statistical life (VSL) as a method to estimate marginal reduction in the risk of premature death (Sanchez Martinez et al., 2018; World Bank, 2016; Franchini and Mannucci, 2015; World Health Organization, 2016). However, these studies required a complete data of premature deaths to estimate its potential cost. In this particular study, we did not consider the effects of pollution on health because we did not have access to premature deaths information due to such information is restricted to health centers and health governmental offices that do not facilitate it.

Instead, we estimate the impact of physical damages on the property value. Several studies have demonstrated that it also allows to affect the price of homes, impacting on the value of the private patrimony of citizens (Bajari et al., 2012; Cebula, 2009; Neupane and Gustavson, 2008; Égert and Mihaljek, 2007; Chau et al., 2006; Kiel, 2006; Jackson, 2001; Harrison Jr. and Rubinfeld, 1978; Rodriguez et al., 2017). These investigations have been based on the classic study of Rosen (1974), who developed the theoretical basis of the hedonic model, and later on the studies of Freeman III (1974) as well as Harrison Jr. and Rubinfeld (1978) when performing empirical studies of the theory. These pioneering works determined the demand and benefits for clean air in urban areas. Air pollution is a problem originated of anthropogenic activities and has been directly related to the different particular and economic activities of citizens (Toulkeridis et al., 2020). This concern arises within the open urban spaces according to the intensity of the burning of fossil fuels either by industrial activity or by the use of vehicles (World Health Organization, 2016). Therefore, the reduction of pollution automatically allows citizens of any given city to breathe less contaminated or strictly better air (World Bank, 2016; World Health Organization, 2016), or improve the value of a property. This becomes an environmental service, whose monetary valuation enhances fundamentally as a guide to appropriate policy measures that protect health as well as the economic situation at a heritage level. Hereby, in a complementary way to the health benefits, the relative quality of the air affects the (de-) valorization of real estate due to the contamination levels of its location (Del Giudice et al., 2017; Saaty and De Paola, 2017).

In this sense, pollution represents a negative externality and the Hedonic Pricing method suits as a technique that allows the economic valuation of air quality as an environmental service. Consequently, it supports to infer the willingness to pay agents in order to receive better air quality from its impact on the real estate market. The underlying central assumption pursues individuals will pay a higher price for a house, apartment or office, which may be located in an area with less air pollution, compared to a property with similar characteristics in an area with higher contamination (Harrison Jr. and Rubinfeld, 1978; Echegaray-Aveiga et al., 2020; Robayo et al., 2020; Poma et al., 2021). With the support of the aforementioned methodology, the current study may estimate the economic value of the air quality in the city of Quito in central Ecuador, by contrasting the effects of air pollution in two exemplary areas such as Bellavista and the Historic District. This purpose will be based on their geographic location, geomorphological content, vehicle access, and house distribution, which may indicate different levels of average pollution (Toulkeridis et al.,

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2016; Nugra et al., 2016; Guanochanga et al., 2018, 2019; Fuertes et al., 2019; Borja-Urbano et al., 2021).

1.1 The case study of the city of Quito

The Metropolitan District of Quito (DMQ), in central Ecuador (Figure 1) is a large city at a considerable altitude, where an evaluation of the particular air quality situation has not been conducted yet. Nowadays, the city of Quito has approximately three million inhabitants, being the most populated in the country. Despite this, it is the city with the highest general industrial activity in the country with a Gross Value Added (GVA) of 23.7 billion USD according to the Central Bank of Ecuador Regional Accounts (BCE, 2015). Simultaneously, the vehicle fleet has grown by 57% since 2012, placing the city above the national average (INEC, 2016), increasing the air contamination in the DMQ.

According to the Air Quality Report of the Secretary of Environment of Quito (SAQ) in the DMQ (SAQ, 2017), the main source of air pollution of the DMQ is the transport that uses diesel fuel and gasoline, worsen by vehicular congestion caused by private vehicles. Additionally, there is a severe impact of the use of bunker fuel and fuel oil by thermoelectric plants and other industries in specific areas of the DMQ. Such activities are responsible for the concentration of pollutant particles in the air, such as Nitrogen Oxides (NO_x), Carbon Monoxide (CO), Sulfur Dioxide (SO_2) Particulate Matter (PM_{10}), $PM_{2,5}$, Ammonia (NH_3) or Benzene among others of lower amounts based on the Ecuadorian Standard of Air Quality (NECA). According to Baca (2014) main source of pollution is mobile sources such as private vehicles and public transportation, area sources of pollution such as waste disposal facilities are the second source of air pollution in the DMQ and in less degree stationary sources.

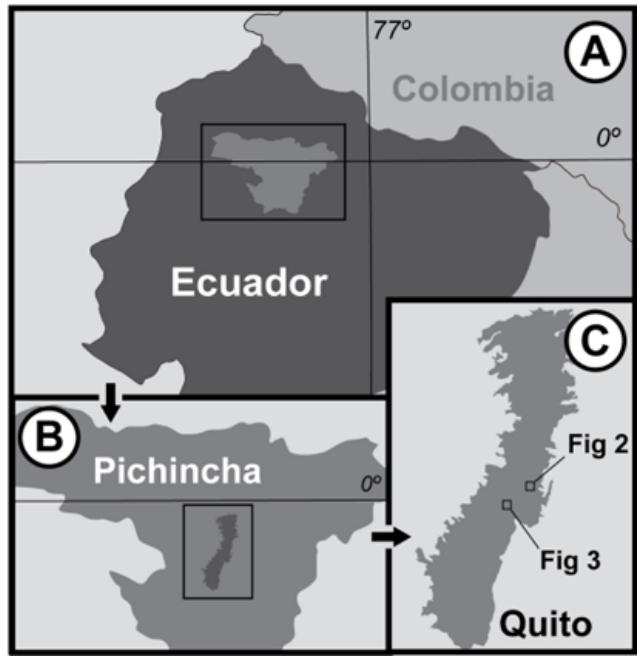

Figure 1. Study area.

Air quality is considered as an environmental service by The Economics of Ecosystems and Biodiversity Initiative (The Economics of Ecosystems and Biodiversity, 2013), and its economic or monetary valuation results, fundamental as guidance for policymakers in order to improve the health of citizens. Reasonably and as complementary to health benefits, the relative air quality impact on real estate property value or devaluation of a property due to air contamination levels as a negative externality may demonstrate air quality economic value. The hedonic price method allowed to value

the impact of this externality estimating buyers' willingness to pay for a better air quality through the housing market. It is assumed that buyers would pay an additional value for properties located in areas with less contamination (Harrison Jr. and Rubinfeld, 1978).

The current study tries to estimate the air quality economic value of Quito by contrasting the impact of contamination in two areas, Bellavista and the Historic District, which represent two different levels of contamination. The choice of these two sectors seeks to capture the effect of air purification by nearby forest ecosystems. Bellavista's neighborhood borders the Metropolitan Park of Quito, which is a forested recreational park, where pollution degrees certainly are relatively low. The Historic District of Quito represents some spatial characteristics such as narrow streets, constant traffic flow and traffic jams, public transport flow close to industrial establishment areas besides other air contaminating issues that allow an understanding that the environmental pollution estimates exceed the city average for this area.

Due to Quito's Metropolitan Atmospheric Monitoring Network (REMMAQ), which determines the air quality of the city, we have been able to quantify the air quality of both focused areas. The RENMAQ appraised air quality since 2003 with some 39 stations throughout the urban area, surrounding valleys as well as regional and street level (SAQ, 2017). It has been expected to allow transparency about the economic fees assumed by the citizens of Quito on the price of their homes. Therefore, we consider that the results of the current study may serve to present the benefits that they may represent for the city and, specifically to the heritage of the people of Quito. Additionally, the adoption of policy measures may improve air quality and protect the ecosystem that supports its purification.

Air purification is an environmental service provided by ecosystems. Within the classification of the Economics of Ecosystems and Biodiversity Initiative TEEB, which divides environmental goods and services by categories (supply, regulation, life support, and culture), air quality has been conceived as an ecosystem regulation service (The Economics of Ecosystems and Biodiversity, 2013). Meanwhile, in Ecuador, the NECA establishes polluted air as the presence of substances in the atmosphere, resulting from human activities or natural processes, present in sufficient concentration, for a sufficient time and under circumstances such as to interfere with comfort, the health or well-being of human beings or the environment (Ministerio del Ambiente del Ecuador, 2011). For NECA and the SAQ, the most representative substances in the atmosphere are mainly SO_2, CO, NO_2, O_3 and benzene and in lesser quantity M_{10}, $PM_{2,5}$, Cadmium and Inorganic Mercury. The review of preliminary studies on the assessment of air quality indicates a particular interest in the NO_2 variable as a proxy for contamination (Smith and Huang, 1993), while others suggest that the pollution variables are strongly correlated. If this is the case then it is recommended to use NO_2 as a proxy, as it is linked to the most visual pollution, being the product of combustion processes (Harrison Jr. and Rubinfeld, 1978).

1.2 Context of the Hedonic Pricing Method

The Hedonic Pricing Method (HPM) identifies the environmental service flow as characteristics that describe in a particular way a market good, typically being real estate (Hanley and Barbier, 2009). The theoretical validity of the method has been based on the Theory of Value from the proposed approach (Rosen, 1974; Lancaster, 1979), which indicates that a unit of good or service (hi) within the group of goods and services of the same market has been to be described by a vector of its characteristics, being named as Z (Hanley and Barbier, 2009). In this sense, the price of this unit of good or service ($p(h_i)$) may be seen as a function of the characteristics. Formally, the relationship is defined as follows:

$$p(h_i) = f(Z_i) \tag{1}$$

Already in practice, based on the proposed definition of the HPM, it has been established that the value of a house belonging to the set of houses of the universe of interest depends on two main conditions. The first is a vector of characteristics (number of rooms, square footage, neighborhood, criminality, etc.), while the second is a vector of environmental variables such as proximity to parks. By differentiating the price for the quantity of any characteristic, the implicit price is obtained in relative terms. The implicit price documents the average willingness to pay given a certain quantity or quality of one of its characteristics (Hanley and Barbier,

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2009).

If one seeks to adequately identify the magnitude of the effect of environmental good and service on the market price of the real estate, the theory allows the characteristics to be disaggregated and all those with the exception of the environmental variable to be kept constant; i.e., where the *ceteris paribus* condition applies (Schiffer, 1991; Boumans and Morgan, 2001). The logic underlying the concept of implicit price establishes that agents look forward to maximizing their utility, so they adapt their purchases to the condition of rationality and their perception of good qualities, leading to the point when the marginal rate of substitution (MRS) equals the implicit price of a certain characteristic (Hanley and Barbier, 2009).

This argument is important as it validates two fundamental assumptions: 1) the agents are in the ability to reflect and consider factors such as air pollution and other characteristics of a house when having to choose; 2) It is allowed to establish the principle that agents make an offer "on the margin". This implies that the money they are willing to pay is equal to the benefit generated by the set of characteristics of the house. In fact, this is a necessary condition for the existence of exchange. In this context, it is intuitive to think that the price of a house reflects, in part, the marginal willingness to pay for better air quality as a desirable feature.

1.3 Hedonic price function for the case of air quality

Here we intend to define the theoretical functional form between the price of the real estate and the environmental variable of interest, specifically the quality of the air. In addition, the relevant explanatory variables are proposed. A hedonic price function may be estimated by establishing the relationships between the price of the property and its particular characteristics:

$$p_i = f(CS_i, CN_i, CE_i + \varepsilon_i) \quad (2)$$

Where, following the categorization of Égert and Mihaljek (2007) and Hanley and Barbier (2009):

$p_i =$ property price i.
$CS_i =$ characteristics of the site (number of rooms, no bathrooms, surface, no garages, antiquity and green areas).
$CN_i =$ characteristics of the neighborhood (crime and distance to the nearest park).
$CE_i =$ environmental characteristics (in this case, contamination in the location zone is of interest).
$\varepsilon_i =$ error term.

The proposed hedonic price function allows obtaining the implicit price. Hereby, the marginal change in the price of the property associated with the marginal change of any of the proposed characteristics may be obtained. Regarding the relationship of interest between price p_i and air pollution, it is expected that it does not follow a linear pattern. Following the argument of Rosen (1974) air quality is desirable as it is directly related to the price of housing but at a decreasing rate. Therefore, it is expected that $\frac{\partial p_i}{\partial CE_i} > 0$, but $\frac{\partial^2 p_i}{\partial CE_i^2} < 0$: the marginal cost of air quality, its implicit price, falls while the level of air quality increases. This appreciation is intuitive. When there are low levels of contamination, people are expected to value proportionally less such attribute compared to a scenario with severe contamination problems.

2 Methodological Procedure

The current study considers two zones whose levels of contamination differ substantively. The first zone is the Historic District of Quito (HDQ) (Figure 2) and the second zone is Bellavista in the North Center of the city. It is fundamental to consider that a criterion of choice of the Bellavista zone is its location that circuits the extreme south-west of the Metropolitan Park of Quito (Figure 3), which allows continuous processes of air purification. Based on the limits of Figure 2, the following geographical map has been constructed according to the information of the Quito Open Government (GAQ). It is interesting to compare the geographical map of the HDQ (Figure 3) with that presented with the Bellavista zone (Figure 2). It results that there is no greater presence of park-like green areas in the Historic District. In addition, there is a higher density of housing construction. Based on these results according to a geographical division of the chosen zones, the following information matrix of the sample may be defined according to the information of the amount (N) of houses (Table 1).

Figure 2. Geographical map of the Bellavista area.

Table 1. Matrix of the number of housing for the sample.

Zone	No. Properties (N=)	Confidence level (%)	Error range (%)	Sample size
Bellavista	3.056	95%	5%	342
Historic Center	15.456	95%	5%	375

The results of the sample size are obtained based on the following calculation method:

$$n = \frac{\frac{z^2 * \sigma^2}{e^2}}{1 + \left(\frac{z^2 * \sigma^2}{e^2 N}\right)} \quad (3)$$

Where,
e = error range (= 5%)
z = score (at a 95% level of significance)
N = population size

In the current study, a focal sample has been taken for reasons of information availability. The total sample has a total of n = 149 observations, of which 80 correspond to the Historical District and 69 to the Bellavista area. The sample needs to include information on the value of the real estate and air pollution in the area where it is located. Additionally, it requires to collect data on other determinants of the price of the property that have been considered significant as control variables. Table 2 presents and defines the variables related to pollution levels in the HDQ or Bellavista, and those with additional control characteristics that potentially affect the price. Additionally, the information source for each variable is listed.

2.1 The Hedonic Pricing Method

2.1.1 Hedonic Price Model (HPM)

The hedonic price model (HPM) has been defined further below. Two basic HPM's are presented: I and II, considering the theoretical reflections presented in the previous sections. The HPM I demonstrates a linear evolution between the evolution of environmental variables and the price of housing. The difference between model II and I is the application of the assumption of the relationship to decreasing rates between the price of a property and environmental quality in the real estate market. In this sense, an exponential correction factor is applied. The transformation of the variable rooms, surface, and age are also considered, elevating them to the square in both zones for the general model II. Models are obtained with or without the application of the

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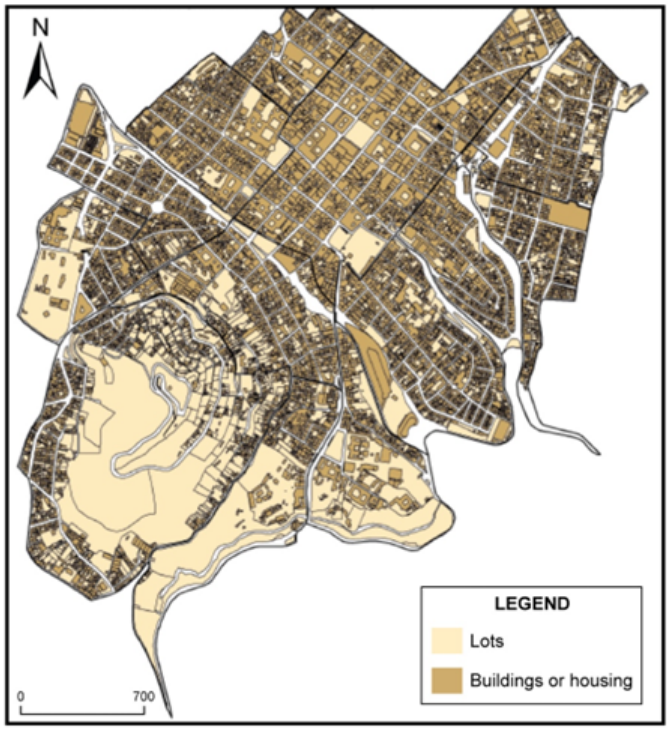

Figure 3. Geographical map of the Historical Center.

Table 2. Matrix of variables of the hedonic model. Sources: (a) Plusvalia.com, (b) Vive1.com, (c) OLX.com, (d) Secretaria de Ambiente del DMQ, (e) Fiscalia General del Estado

Concept	Variable	Definition	Unit	Source
Price	Market price	Market price of real estate (at the study date)	USD	a, b, c
Price m^2	Price m^2	Price m^2 of the real estate (at the study date	USD/m^2	a, b, c
Contamination	SO_2	Average annual concentration (= year 2016) of zoned sulfur dioxide	µm/m^3	d
Contamination	CO	Average annual concentration (= year 2016) of Zoned Carbon Monoxide	mg/m^3	d
Contamination	NO_2	Annual average concentration (= year 2016) of zoned Nitrogen Dioxide	µm/m^3	d
Contamination	O_3	Average annual concentration (= year 2006) of zoned ozone	µm/m^3	d
Contamination	Benzene	Average annual concentration (= year 2016) of zoned Benzene	µm/m^3	d
Rooms	No. of rooms	Number of rooms available	room	a, b, c
Bathroom	No. of bathrooms	Number of bathrooms available	bathrooms	a, b, c
Surface	Surface m^2	Surface extension	m^2	a, b, c
Garage	No. of garages	Number of own garages available	posts	a, b, c
Antiquity	Years of antiguaty	Number of years since the construction of the property	years	a, b, c
Green areas	Green areas	Availability of green areas such as gardens, green terraces, etc	0=no 1=yes	a, b, c
Crime	Criminality	Zoned sum of complaints of crimes against people and private property year 2016	complain	e

natural logarithm in the dependent variable. In this sense, Log-Log (elasticities) and Semi-Log (semi-elasticity) models are obtained, which also provides information of interest in this study. In general, the models are defined as follows:

Definition HPM I:

$$\ln(p_i) = \beta_0 + \beta_j \sum_{j=1} \ln\left(contamination_i^j\right) + \beta_k \sum_{k=5} \ln\left(characteristics_i^k\right) + \varepsilon_i \quad (4)$$

Definición del MPH II:

$$\ln(p_i) = \beta_0 + \beta_j \sum_{j=1} \ln\left(contamination_i^j\right) + \beta_k \sum_{k=5} \ln\left(characteristics_i^k\right) + \beta_l \sum_{l=13} \ln\left[\left(contamination_i^l\right)^{0,5}\right] + \beta_{19} rooms_i^2 + \varepsilon_i \quad (5)$$

Where,

$\ln(p_i)$ = property price i
$\ln\left(contamination_i^j\right)$ = natural logarithm of the variable $j = 1 \ldots 5$, of the set of pollution variables: { SO_2,CO_2,NO_2,O_3 and Benzene }
$\ln\left(contamination_i^l\right)^{0,5}$ = natural logarithm of the variable $l = 6 \ldots 10$, of the set of variables of the square root of contamination: {$SO_2^{0,5}$,$CO_2^{0,5}$,$NO_2^{0,5}$,$O_3^{0,5}$ and Benzene^{0,5} }
$\ln\left(características_i^k\right)$ = natural logarithm of the variable $k = 11 \ldots 18$, of the set of characteristic variables of the property: {rooms,ban,sup,garag,antiq,green areas and crime }
$rooms_i^2$ = number of rooms squared
ε_i = error term

3 Results and discussion

Air pollution between the Historic District of Quito and Bellavista differs when considering pollution variables resulting from the anthropogenic activities. Below are the results of the measurements of the concentration SO_2, CO, NO_2, O_3 and Benzene (Figure 4).

The results indicate that for all pollutants, the HDQ maintains lower levels of air quality compared to Bellavista. This corroborates the assertion about the existence of higher levels of pollution related to the result mainly of combustion processes (CO, NO_2, SO_2 and Benzene), and, indirectly, reflected in higher levels of O_3 between both zones. On the side of housing prices and price per square meter, the following information is obtained in the housing focus group.

The results of other house features were as anticipated. Variables such as construction area, parcel size, number of bedrooms, number of bathrooms, house's age, and presence of a garden were significant at 99%. Among neighborhood characteristics, only garage was significant at 95%, while green areas were insignificant. We selected all significant variables with the environmental variables and ran again the final regression model.

The average price of the Bellavista area exceeds that of the Historic District in USD 47,267 (Figure 5). The cases of properties on the average in the Historical District are reduced even though in this zone the most expensive property of the sample is offered with a price of USD 1,480,000, being almost USD 900,000 higher than the most expensive property in Bellavista. When this information is complemented with respect to the minimum prices obtained, it is concluded that there is greater variability in the price sample in the case of the Historic Center versus Bellavista. A further way to have a more accurate understanding of the real estate market in both sectors is by evaluating its price per square meter.

Figure 6 illustrates that the real estate market in the Bellavista area is more expensive compared to the Historic Center. Hereby, the average square meter is almost three times larger. This occurs even considering the maximum or minimum price per square meter. In general, the Bellavista area is more attractive from the point of the valuation that is yielded to the real estate offered there. This turns out to be a positive result based on the objectives of the current study, as there are clear differences between the Historic District and Bellavista regarding per square meter housing price.

In order to obtain the results of the empirical models of the hedonic price function, the average annual concentration of milligrams per cubic meter of NO_2 has been taken into consideration, since the pollution variables are highly correlated, avoiding multicollinearity issues. An intuitive reason for this problem is the close relationship among contamination sources, such as combustion processes of CO, NO_2, SO_2 and Benzene. A potential solution might be seen in the extension to further study areas, giving a greater variability to pollution measures, as stated as a particular case of this study. However, we found the most optimal functional form that allows the goodness of fit and greater possible significance for what different modeling proposals are composed of.

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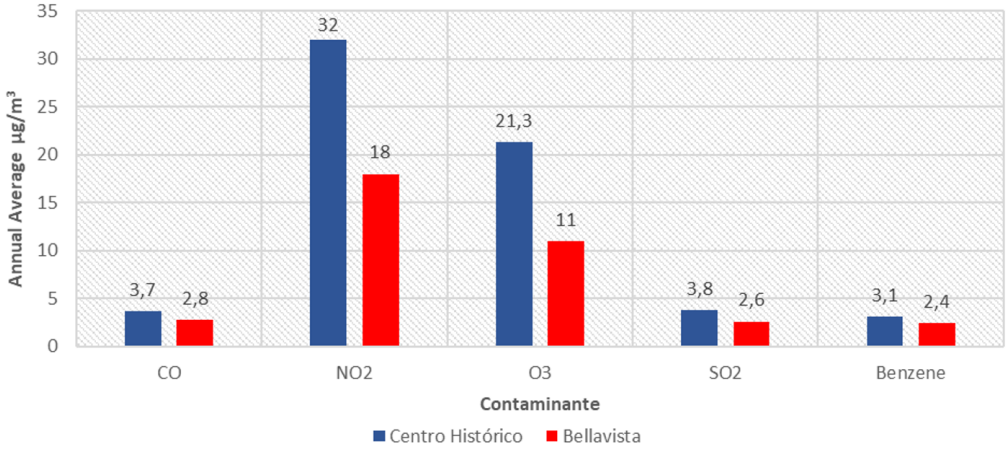

Figure 4. Comparison of pollutants by study area.

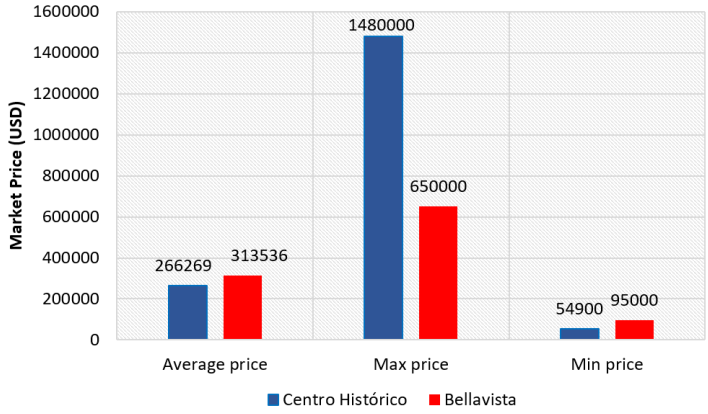

Figure 5. Comparison of housing market price according to study area. Adapted from Real estate websites.

In this sense, the results of the models in the semi-log version fit these specifications in a better way. The models that considered the relationship at decreasing rates between NO_2 and housing prices indicated a lack of improvement in the specification of the model. The estimation of adequate exponential value is more appropriate when studying a greater number of zones. Therefore, this may explain not giving a significant contribution to the explanation of the model despite having a theoretical validation. The models are corrected by heteroscedasticity. Particularly some variables such as the price per square meter (m^2) or housing age, for example, documented this issue. The results of the modeling in Table 3 are presented below.

We obtained high levels of R^2 values (91%), which have been similar to those yielded in Cebula (2009) of about $R^2 = 87\%$, Chau et al. (2006) $R^2 = 87\%$, and Jackson (2001) with $R^2 = 81\%$, indicating that the variables in the equation manage to explain to a considerable extent the variation of the housing price in the sample research. The variable NO2 has a negative sign and turns out to be highly significant, even at a 99% confidence level in the three proposed models. Additionally, considering Model III, it decreases by 1 μgm^{-3} in the average annual concentration of NO_2 increase with the average price of homes by 4.54%. With such data, we have been able to assess the quality of the air as documented in Table 4, while considering the results of Model III.

Table 3. Results of Models I, II and III. Statistics t are in parentheses; *Exceeds 95 % confidence level (t>1,96); **Exceeds 99 % confidence level (t>2,58).

Variable	Model I	Model II	Model III
Dependent	Log (pm)	Log (pm)	Log (pm)
Constant	8.99	6.30	1.217
	(9.72)	(12.02)	(0.44)
Log (Price (m2))	0.5288	0.8389	2.44
	(5.81)**	(15.26)**	(2.84)**
Log (Price (m2))2			-0.1227
			(-1.87)
Room	0.0852	0.0694	0.0677
	(3.86)**	(4.05)**	(4.00)**
Room2	-0.0029	-0.0022	-0.00213
	(-5.17)**	(-4.64)**	(-4.54)**
Bathrooms	0.0530	-0.0106	-0.011
	(3.28)**	(-0.88)	(-0.96)
Garage	0.0394	0.0106	0.008
	(2.25)*	(0.85)	(0.67)
Surface	0.0012	0.0039	0.0039
	(7.71)**	(11.87)**	(11.86)**
Surface2		-1.35e-06	-1.32e-06
		(-6.05)**	(-6.04)**
Antiquity	0.0045	0.0076	0.0063
	(2.76)**	(3.45)**	(2.87)**
Antiquity2		-0.00005	-0.00004
		(-2.58)**	(-2.38)*
Green areas	0.9188	0.0338	0.0336
	(1.40)	(0.71)	(0.44)
NO2	-0.0546	-0.0430	-0.0454
	(-3.79)**	(-4.99)**	(-5.08)**
R2	0.77	0.90	0.91

The estimates demonstrated that a decrease in the annual average of one milligram per cubic meter of NO_2 increases the price of housing by 4.5 %. Likewise, a decrease of five milligrams per cubic meter of NO_2 increases by 22.7 % the price of the homes of the research sample. In the first case, there is an increase in equity of 2,032,326.24 USD considering the 148 homes in the study area. In the second case, the equity increases, in aggregate terms, by 10,161,632.22 USD. The average price of the 148 homes increases between 13,731.93 and 68,659.67 USD, as air pollution decreases between one and five milligrams per cubic meter of NO_2. Obviously, a decrease of more than five (μgm^{-3}) of NO_2 would result in greater economic benefits. Thus, according to the reduction in the average annual levels of milligrams per cubic meter of NO_2, owners will benefit from an improved value of their properties, and therefore, their assets. Ultimately, their economic situation improves due to the relative quality of the air in the area where the real estate is located.

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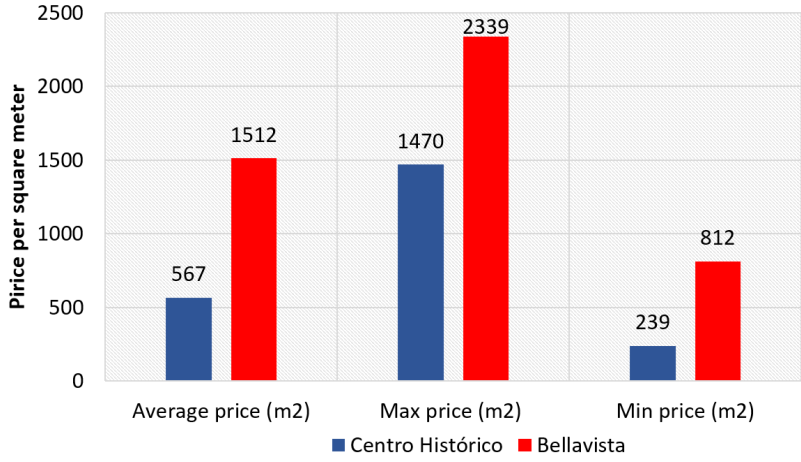

Figure 6. Price comparison m^2 of homes according to study area. Source: Real estate websites.

Table 4. Air Quality Assessment.

Variations		Focal sample				
Δ^- (NO_2) (μm/m3)	Δ^+ (Price) (%)	Average price (USD)	Amount households (n)	Δ Heritage (USD)	Δ Average price (USD)	Difference of average prices (USD)
1	4.5%	302 465.51	148	2'032 326.24	316 197.44	13 731.93
2	9.1%	302 465.51	148	4'064 652.49	329 929.38	27 463.87
3	13.6%	302 465.51	148	6'096 978.73	343 661.31	41 195.80
4	18.2%	302 465.51	148	8'129 304.98	357 393.24	54 927.74
5	22.7%	302 465.51	148	10'161 631.22	371 125.18	68 659.67

4 Conclusions

The two different studied zones of the city of Quito, are directly affected (being understood as a negative externality) by different economic activities that generate pollution. Our results demonstrate that NO_2 (32 μgm^{-3}) has the highest value among city pollutants. There is a significant difference between the Historic District and Bellavista, being 32 μgm^{-3}, and 23 μgm^{-3}, respectively. These levels of contamination have a direct effect on real estate price as it has been indicated. Differences in housing prices have been partially due to the varying levels of contamination between both zones.

Econometric models stated that a decrease in the annual average of one milligram per m^3 of NO_2 increases the price of housing by 4.5%, meaning an increase in equity of 2,032,326.24 USD. The average price of the 148 homes increases of about 13,731.93 USD if air pollution declines in one milligram per cubic meter of NO_2. Obviously, a higher pollutant reduction will result in higher economic benefits.

These results may help to establish policies that intend to improve air quality. Policies such as promoting green areas in sites with high levels of pollution may correct the unfair penalties in owners' properties. Additionally, the obtained results indicate the importance of maintaining forested areas that play an important role in purifying the air, allowing people to benefit directly from qualitatively adequate air.

The current study considers a focal sample and this leads to limitations when inferring the results at population levels. However, the R^2 (0.91) is very high, allowing to generalize our results and justify more extensive research in the DMQ regarding air

pollution and its effects on property. In addition, the study concentrated only on the NO_2 pollutant, thus it is necessary to measure the other 3 pollutants.

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Scientific paper/ Artículo científico

Veterinary Sciences

Effect of embryotrophic factors at different oxygen tension for *IN VITRO* culture on the embryonic development of alpacas up to the blastocyst stage

Efecto de factores embriotróficos a diferentes tensiones de oxígeno en cultivo *IN VITRO* sobre el desarrollo embrionario de alpacas hasta la etapa de blastocisto

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Abstract

The alpaca is the most important South American domestic camelid for Peru, a country that regionally has 87% of these animals. Since traditional forms of reproduction do not guarantee its genetic quality, *in vitro* reproduction is an alternative for its improvement. This study evaluated the influence of embryotrophic factors of epidermal development (EGF) and insulin-like growth factor (IGF-1) and oxygen tension on the *in vitro* development of alpaca oocytes up to the blastocyst stage. From ovaries of sacrificed animals, oocytes were obtained and placed in TCM-199 medium, supplemented with sodium pyruvate, glutamine, estradiol (E2), follicle stimulating hormone (FSH), luteinizing hormone (LH), embryotrophic factor (EGF or IGF-1), 5% fetal bovine serum and gentamicin (10μL/mL) for 32 hours, at 38.5 °C, with 5% CO_2 and relative humidity greater than 95%, with O_2 tensions between 6 and 20%. Subsequently, the oocytes were fertilized with fresh semen and cultured in KSOMaa medium for 48 hours. Cultures were differentiated by growth factors (EGF and IGF-1) and O_2 tensions (6% and 20%), plus the control group without EGF or IGF-I supplementation, to assess oocyte and blastocyst division rates from oocytes. By cluster analysis, significant differences were established between treatments with $\alpha = 0.05$ for each response variable, with the highest rate of oocyte divisions (24.8%) with EGF at 6% O_2 and the highest blastocyst/oocyte production (18.4%) with IGF-1 at 6% O_2. It is concluded that the addition of embryotrophic factors and low O_2 tension are favorable for *in vitro* embryo development in alpacas.

Keywords: Alpaca, camelids, cleavage, fertilization, oocytes, reproduction.

Resumen

La alpaca es el camélido sudamericano doméstico de mayor importancia para el Perú, país que a nivel regional cuenta con el 87% de estos animales. Ya que las formas tradicionales de reproducción no garantizan su calidad genética, la reproducción *in vitro* es una alternativa para su mejoramiento. Este estudio evaluó la influencia de los factores embriotróficos de desarrollo epidérmico (EGF) y de crecimiento insulínico (IGF-1) y tensiones de oxígeno en el desarrollo *in vitro* de ovocitos de alpaca hasta la etapa de blastocistos. A partir de ovarios de animales sacrificados, se obtuvieron ovocitos que se colocaron en medio TCM-199, suplementado con piruvato de sodio, glutamina, estradiol (E2), hormona folículo estimulante (FSH), hormona luteinizante (LH), factor embriotrófico (EGF o IGF-1), 5% de suero fetal bovino y gentamicina (10μL/mL) durante 32 horas, a 38,5 °C, con 5% de CO_2 y humedad relativa mayor de 95%, con tensiones O_2 entre 6 y 20%. Posteriormente, los ovocitos fueron fecundados con semen fresco y cultivados en medio KSOMaa durante 48 horas. Los cultivos fueron diferenciados por factores de crecimiento (EGF e IGF-1) y tensiones de O_2 (6% y 20%), más el grupo control sin suplementar con EGF o IGF-I, para valorar las tasas de división de ovocitos y blastocistos a partir de ovocitos. Mediante análisis de conglomerados, se establecieron diferencias significativas entre los tratamientos con $\alpha = 0{,}05$ para cada variable de respuesta, observándose la mayor tasa de divisiones de ovocitos (24,8%) con EGF a 6% de O_2 y la mayor producción de blastocistos/ovocito (18,4%) con IGF-1 a 6% de O_2 . Se concluye que la adición de factores embriotróficos y una baja tensión de O_2 son favorables para el desarrollo embrionario *in vitro* en alpacas.

Palabras clave: Alpaca, camélidos, clivaje, fertilización, ovocitos, reproducción.

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1 Introduction

The breeding of domestic camelids such as llamas (*Lama glama*) and alpacas (*Vicugna pacos*) in South America is part of an ancestral culture, especially in Peru and Bolivia, which account for 98.89% of the total alpaca population (87.9% Peru and 10.9% Bolivia) and 93.4% of total llamas (60.8% Bolivia and 32.5% Peru), according to figures reported by the Ministry of Agriculture and Irrigation of Peru (MINAGRI, 2015). Peru reports a population of 3 685 516 alpacas and 1 257 000 llamas, hence genetic and reproductive studies of these species are paramount for the country, due to their great productive and commercial potential, especially alpaca based on the production of fiber, meat, skin and manure as organic manure, as well as its use as an animal for recreation and production of therapeutic solution (MINAGRI, 2019). According to the last agricultural census of Peru carried out in 2012 by the National Institute of Statistics and Information Technology (INEI), the regions with the highest density of alpacas are Puno with 39.6%, Cusco with 14.8% and Arequipa with 12.7%; the Huacaya variety represents 80.4% of the total population (INEI, 2013).

Despite the importance of alpacas in Peru, their reproduction mainly occurs by natural mating, without considering genetic variability and improvement of the species for commercial purposes, which has led to a decrease in genetic quality (Huanca, 2012). *In vitro* reproduction is shown as a viable alternative for genetic improvement and increased productivity of alpaca, since this technique is highly developed in buffalo, cattle, sheep and pigs (Liang et al., 2020; Javvaji et al., 2020; Dubeibe et al., 2019; Gonella Diaza et al., 2013; Rodrigues et al., 2013). The reproduction feasibility of camelids by artificial insemination, embryo transfer and *in vitro* fertilization has also been reported (Ruiz, 2018; Pérez et al., 2017; Pacheco et al., 2016), being necessary to study the conditions that allow the successful development of the reproduction of alpacas by *in vitro* techniques in Peru since there are no specific protocols for it.

Since *in vitro* production of embryos is limited due to the reduced survival in both embryonic and fetal and the high frequency of fetal, placental or neonatal abnormalities, alternatives are sought to improve the production of embryos with this technique, focused on growth factors that regulate the processes of cellular mitogenesis, differentiation, and apoptosis under *in vivo* conditions (Block, 2007; Kane et al., 1997).

The growth factors or embryotrophic factors more used in experimental studies to increase the maturation efficacy of oocytes and *in vitro* production of embryos are the insulin growth factor (insulin growth factor IGF), because of their effects on growth, follicular development and maturation induced by gonadotrophins (Lenz et al., 2007) and contribution to the pre-implantation and development of the embryo in cattle (Lima et al., 2006; Stefanello et al., 2006; Block, 2007), the epidermal growth factor (EGF), which stimulates cell proliferation and differentiation (Adams, 1999), and which is related to oocyte maturation (Harper and Brackett, 1993), the transforming growth factor (TGF), and the growth factor derived from platelets (Platelet-derived growth factor, PDGF) (Block, 2007).

On the other hand, the best yields of embryonic cultures are obtained with O_2 concentrations lower than atmospheric, mainly due to the reduction of the generation of O_2-free radicals, reducing their deleterious effect (Legge and Sellens, 1991; Noda et al., 1991; Umaoka et al., 1992). In addition, the reduced intrauterine O_2 tension reported in vivo studies would imply protection for the preimplantation of blastocyst (Clark et al., 2006). Thus, a better development of matured bovine oocytes in 5% of O_2 has been reported (Hashimoto et al., 2000; Van Blerkom et al., 1997).

Therefore, the aim of the research was to establish the ideal conditions for alpaca embryonic development by evaluating the influence of the embryotrophic factors EGF and IGF-1 and the oxygen tension at 6% and 20% on the division rate of oocytes after the first embryonic culture at 48 h in KSOMaa medium, and the blast rate from oocytes after seven days of second embryonic culture in SOFaa.

2 Materials and Methods

2.1 Study area

The research was carried out at the Quimsachata Research and Production Center (CIP), of the agri-

cultural experimental station ILLPA-Puno, National Institute of Agricultural Innovation-INIA -Peru, located between Santa Lucia and Cabanillas, provinces of Lampa and San Roman in Puno, 15° 44′ 00″ South Latitude and 70° 41′ 00″ West Longitude, in the agro-ecological area known as a dry puna, with an average altitude of 4300 masl, and a temperature that fluctuates between 2°*C* (May to July) and 15°*C* (September to December) (Díaz, 2013).

2.2 Collection of ovary simples

The collection of ovaries was made from animals at the slaughtering livestock in Nunoa, with random sampling that did not consider the reproductive status of alpacas. The ovaries collected were placed and transported in a thermos between 35 and 37°*C*, immersed in 0.9% saline and supplemented with gentamicin (10μL/mL).

2.3 Processing of oocytes

Using the modified Slicing method (Lorenzo et al., 2015), 1051 oocytes were collected, selected in categories I and II. For their maturation, oocytes were placed in TCM-199 medium, supplemented with sodium pyruvate, glutamine, estradiol (E2), follicle stimulating hormone (FSH), luteinizing hormone (LH), embryotrophic factor (EGF or IGF-1) at concentrations between 10 and 50 ng/ml, 5% fetal bovine serum and gentamicin (10h/ml) for 32 hours at 38,5°*C* with 5% CO_2, and relative humidity higher than 95%, with O_2 of 6% or 20%. Table 1 shows the distribution of oocytes based on inclusion of embryotrophic factors and O_2.

Table 1. Distribution of alpacas oocytes.

Embryotrophic Factor	Oxygen tension %	Number of Oocytes
EGF	6	205
	20	219
IGF-I	6	206
	20	210
Control	6	211
	20	211

After maturation, the oocytes were transferred to a fertilization medium (FER-TALP supplemented with 0.25 mM of sodium pyruvate, 6mg/mL of BSA, and 50μg/mL of gentamicin), in which they were washed three times. At the same time, sperm preparation was done by washing it in Sperm-TALP supplemented with 1.0 mm of sodium pyruvate, 3 mg/ml of BSA fraction V and 50μg/ml of gentamicin with 4 μL of heparin and 30 μL of PHE/ (penicillamine, hypotaurine and epinephrine) and centrifuged at 1500 rpm/10 min. The pellet formed was resuspended in 1ml of the FERT-TALP medium. *In vitro* fertilization was done with sperm from a fertile male, which after being prepared were transferred to a 80 μl drop from the fertilization medium and placed in an incubator for 10 hours.

2.4 Embryo development

At the end of the fertilization period, the possible zygotes were removed from the fertilization drops and introduced into multiwell plates with 500 μl KSOM-AA culture medium, where EGF or IGF-1 (10-50 10 and 50 ng/ml) were added at 38,5°*C*. Maximum relative humidity $> 95\%$, CO_2 voltage 5% and 6 or 20% O_2 voltage. The zygote division rate was evaluated 48 hours after fertilization and then transferred to the SOFaa culture medium, adding EGF or IGF-1 under the above conditions. On day seven after fertilization, blastocyst stages were observed.

2.5 Statistical data treatment

The experiment combined EGF IGF-1 embryotrophic factors and a control group with two O_2 levels (6% and 20%). Euclidean distance cluster analysis was used for close neighbors with a 95% confidence level to establish differences and similarities in the joint application of treatments on oocyte and blast cell division rates obtained from oocytes (blasts/oocytes). This technique allows the grouping in clusters of variables according to their distances, where there is no significant difference according to what is established in the analysis, since they are exclusive with respect to factors that do not belong to the group, not establishing hierarchies, but statistically differentiated groups (Cuadras, 2020). The InfoStat version 2018 statistical package was used for all statistical analyzes.

3 Results and Discussion

3.1 Oocyte division rate

Once the experiment was conducted, it was observed that the lower O_2 (6%) produced the highest rate of oocyte division and blastocyst formation per oocyte, compared with the control group and 20% of O_2 (Table 2).

Table 2. Oocyte and blastocyst division rates per oocyte after application of EGF and IGF-I with two O_2 tensions.

Embryotrophic Factor	O_2 tension (%)	Oocyte Divisions (%)	Blastocysts/ Oocytes (%)
EGF	6	51(24.9)	29(14.1)
	20	13 (5.9)	12 (5.5)
IGF-I	6	42(20.4)	38(18.4)
	20	24(11.4)	14(6.7)
Control	6	33(15.6)	6 (2.8)
	20	33(15.6)	6 (2.8)

Similarly, the percentage of oocyte divisions (24.9%) was higher with the embryotrophic EGF factor with 6% of O_2, lower than the reported by Benavides et al. (2015), who, when analyzing the influence of oxygen tension on bovine embryonic development, obtained 69.7% of oocyte divisions with 5% of O_2. However, these authors do not assess the effect of EGF; while Ahumada (2011), when adding EGF, obtained 74.15% of cleavage in bovine oocytes grown at 5% of O_2.

In Figure 1, the dendrogram for oocyte divisions presents three clusters as groups that differ significantly when the cut is taken according to the result of the cophenetic distance calculated in 0.72. A cluster with EGF and IGF-1 treatments with 6% of O_2 stands out, which, when separated from the control group with different O_2 would indicate that EGF and IGF-I significantly increase oocyte division, although there were no differences between embryotrophic factors with this O_2. On the other hand, that IGF-I with 20% of O_2 form a cluster with the control group, and with both O_2 it would suggest that IGF-I, under these conditions, does not affect the division of oocytes. Meanwhile, EGF treatment with 20% of O_2 would have an inhibitory effect on this variable.

These results are consistent with those reported by Delgado (2018), who observed a greater division of oocytes with 2% of O_2 and an improvement in the quality of bovine embryos than with 5% and 20% of O_2 in the culture. Likewise, Arias et al. (2007) reports similar results to current research applied to bovine embryos, under conditions of high (20%) and low (7%) O_2. In this regard, studies in sheep and swine have concluded that the absence of O_2 promotes the ability to activate, and improves the parthenogenesis of oocytes *in vitro* cultures (Iwamoto et al., 2005; Loren et al., 2016; Yao et al., 2019).

In contrast, He et al. (2020) reported that the excision rate of yak oocytes was significantly lower ($P < 0{,}05$) at 5% of O_2 concentration than at 10% and 20% concentrations, improving the maturation and competition of oocyte development. Rodrigues et al. (2013) found that the division of canine oocytes was not affected by O_2 of 5% or 20%. The differences between these results are probably explained by the characteristics of the embryonic development of these species.

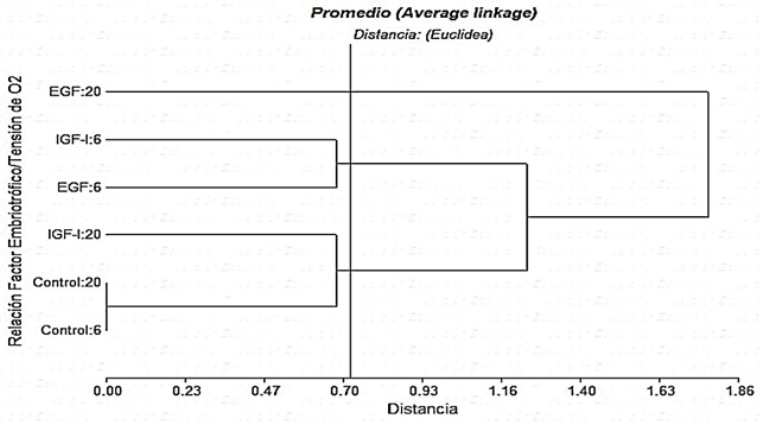

Figure 1. Conglomerate dendrogram for the response variable number of oocyte divisions.

3.2 Blastocyst rate from oocytes

Figure 2 shows the dendogram of cluster analysis with four well-defined clusters with the cut-off according to the cophenetic distance of 0.48, indicating that there are significant differences between the experimental treatments and the control group with both O_2, i.e., that the use of EGF and IGF-1 with 6 and 20% of O_2 increases the formation of blastocysts. On the other hand, the percentage of blastocysts/oocyte obtained is higher than the 14.0% reported by Soto-Martínez et al. (2019), also with bovine embryos evaluated in sequential synthetic oviductal liquid, avoiding the accumulation of embryotoxic substances at a maximum of 5% of O_2.

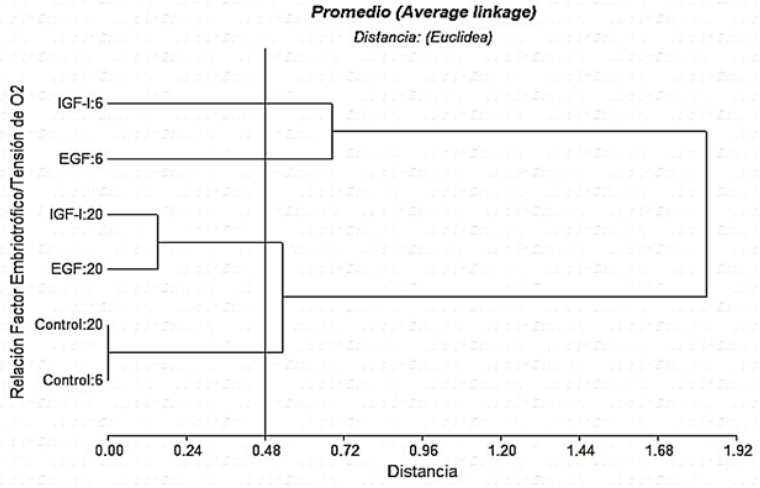

Figure 2. Cluster dendrogram for the variable response number of blastocysts per oocyte.

However, the number of blast cells per oocyte was not modified by EGF and IGF-I when using an O_2 of 20% so they were located in the same cluster. Whereas the 6% IGF-1 embryotropic factor of O_2 showed the highest number of blasts per oocyte (18.4%). This result is similar to that reported by Sirisathien and Brackett (2003), who obtained a higher number of blast cells per oocyte with IGF-1 than with EGF in cattle; i.e., similar proportions of parthenogenetically activated oocytes became blastocysts than inseminated oocytes (28.8%). Yong et al. (2017), highlighted the importance of growth

factor treatment for *in vitro* maturation of porcine oocytes, which is consistent with the results of this study.

Regarding the effect of IGF-1, Javvaji et al. (2020) report that the addition of this factor significantly improved the maturation of oocytes in ovine compared with untreated oocytes by regulating PI3K/Akt expression and apoptosis signaling, which are related to the activation of oocytes in ovines. Finally, the addition of epidermal growth factor (EGF) to the maturation medium stimulates oocyte maturation, but only EGF supplementation increases embryonic and blastocyst development. This evidence is consistent with Richani and Gilchrist (2018), who determined that the EGF also dominates the translation of maternal transcripts into the inactive oocyte, a phase that is necessary to the competition of the oocyte. In addition, it is similar with the study of Salgado et al. (2013), showing that there was a significant difference ($p < 0{,}05$) of the EGF over the proportion of oocytes, explaining the highest proportion of oocytes under treatment with 50 ng/mL.

4 Conclusions

In vitro division rate of alpaca oocytes grown in KSOM-AA medium and blastocyst formation in SOFaa medium, both with embryotrophic factors (EGF and IGF-1), was favored by low oxygen (6%), with significant results in the control groups, observing a higher percentage of oocyte divisions with EGF treatment and in oocyte blastocysts with IGF-1 treatment.

Although the use of the embryotrophic factors EGF and IGF-1 and the low O_2 resulted in an increase in the number of divisions and the number of blasts per oocyte, indicating that under the conditions of this study they can be used for an improvement *in vitro* embryonic development in alpacas, it is necessary to have additional information to clarify the mechanisms of action of embryotropic factors in order to optimize the procedure and achieve a viable *in vitro* alpaca reproduction that leads to the genetic improvement of the species.

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Scientific paper/ Artículo científico

Food Science

Risks of chemical contamination in milk and its derivatives

Riesgos de contaminación química en leche y sus derivados

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Abstract

Milk is a complete and balanced food that, along with its derivatives, represent important components of a healthy diet for the population, since they provide proteins, lipids, carbohydrates, vitamins, minerals and bioactive compounds. However, these foods are susceptible to contamination by a wide variety of chemical products, whose presence beyond certain established legal limits determines a chronic intake of small doses of these compounds. By accumulating in the body, and depending on their toxicity, they have the potential to cause serious affections in various organs and systems, constituting a major public health problem. This review seeks to describe the entry of chemical contaminants (aflatoxins, veterinary drug residues, dioxins, polychlorinated biphenyls, dioxin analogues, disinfectants and detergents) into the food chain, as well as the potential effects on consumer health, the Maximum Residue Limits of these contaminants established for bovine milk and the most frequent methods used for their detection. On this basis, measures are proposed to avoid this type of contamination in dairy products, whose quality is closely related to the conditions of the surrounding environment, associated with anthropogenic activities, agricultural practices, animal production and processing conditions.

Keywords: Foods, Bovine, Milk, Safety, Toxicity

Resumen

La leche es un alimento completo y equilibrado que, junto a sus derivados, son componentes importantes de una dieta saludable en amplios sectores de la población, pues suministran proteínas, lípidos, hidratos de carbono, vitaminas, minerales y compuestos bioactivos. Sin embargo, estos alimentos son susceptibles de contaminación a partir de una amplia variedad de productos químicos, cuya presencia más allá de ciertos límites legalmente establecidos, determina una ingesta crónica de pequeñas dosis de estos compuestos. Al acumularse en el organismo, y en función de su toxicidad, tienen el potencial de ocasionar severas afecciones en diversos órganos y sistemas, constituyendo un importante problema de salud pública. Esta revisión busca describir el ingreso de contaminantes químicos (aflatoxinas, residuos

de fármacos veterinarios, dioxinas, bifenilos policlorados, análogos a las dioxinas, desinfectantes y detergentes) a la cadena alimenticia, así como los potenciales efectos sobre la salud del consumidor, los Límites Máximos de Residuos de estos contaminantes establecidos para la leche bovina y los métodos más frecuentes utilizados para su detección. En base a esto, se plantean medidas tendentes a evitar este tipo de contaminación en productos lácteos, cuya calidad está estrechamente relacionada con las condiciones del medio circundante, que a su vez se asocia con actividades antropogénicas, prácticas agrícolas, de producción animal y condiciones de procesamiento.

Palabras clave: Alimentos, Bovino, Leche, Inocuidad, Toxicidad

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1 Introduction

Milk is a white liquid secreted by breasts of mammals to feed their offspring (RAE, 2019). With an estimated production of 522 million metric tons in 2019, bovine milk is the most consumed by the population (85%), although in some regions there is significant consumption of buffalo milk (11%) and other ruminants (3.9%) (Kalyankar et al., 2016; STATISTA, 2020). In all latitudes, dairy products are susceptible to chemical contamination (OMS, 2018; Dimitrieska et al., 2016), which occurs through soils, agricultural practices, dairy production practices and processing (Nguyen and Flint, 2020; Priyanka et al., 2017), becoming an important problem for public health due to 1) several of the chemical agents are highly stable, so are incorporated into the food chain even when their use has been banned for decades, 2) are not susceptible to decrease after the application of physical, chemical or biological treatments of milk, contaminating dairy products, 3) the lipophilic nature of several contaminants determines a cumulative effect on the animal organism and its presence in dairy fat 4) chronic exposure to these pollutants has the potential to severely impair the health of the population (Akhtar and Ahad, 2017; Ismail et al., 2019). Therefore, the set of contaminants in milk and its derivatives will be reviewed in relation to their structural characteristics, in aspects such as their entry into the animal organism, the mechanisms by which they exert harmful effects on health after chronic consumption of dairy products with concentrations of chemical agents at levels above the maximum residue limits (MRLs) allowed in bovine milk, and which are described in this review. Finally, the aim is also to mention the main methods used for its detection and propose strategies to prevent and control chemical contamination of dairy products.

2 Chemical contaminants

2.1 Aflatoxins

Aflatoxins (AF) are widely distributed mycotoxins, which after contaminating crops and entering the food chain affect animal health and productivity and food safety in the population by presenting toxic, mutagenic, teratogenic, carcinogenic and immunosuppressive effects (OMS, 2018; Dimitrieska et al., 2016; Ayar et al., 2007).

Although more than twenty types of AF have been reported (Nguyen and Flint, 2020), AFB1, AFB2, AFG1 and AFG2 are the main mycotoxins related to dairy contamination. AFB1 and AFG1 differ structurally from AFB2 and AFG2 with an additional dual link. On the other hand, AFG have a furan ring, while AFB have a lactone ring. Depending on their degree of toxicity and carcinogenicity, the following order is found: AFB1 > AFG1 > AFB2 > AFG2, whose structures are shown in Figure 1 (AECOSAN, 2015).

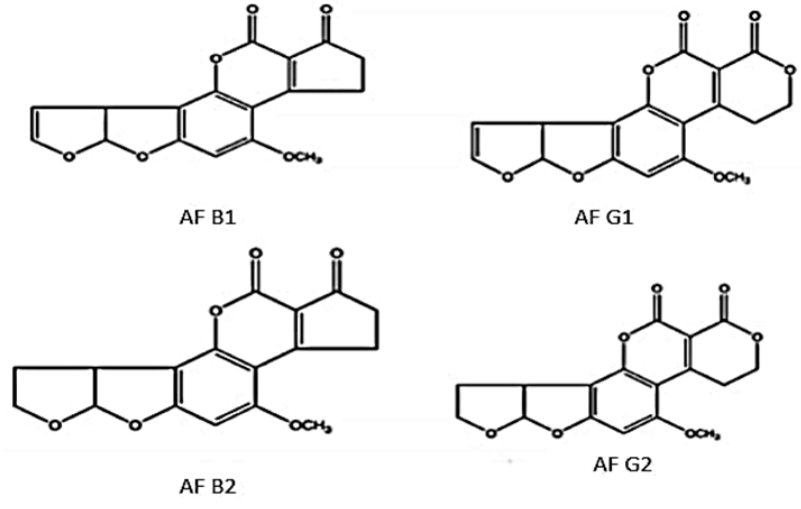

Figure 1. Molecular structure of B and G aflatoxins (AECOSAN, 2015)

Aflatoxin B1 (AFB1) is produced by fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus* (Van der Fels-Kler and Camenzuli, 2016; Duarte et al., 2013). *A. flavus* colonizes mainly the aerial parts of the plants, being found in stored foods such as peanuts, corn and cotton seeds. *A. parasiticus* is found mainly in the soil and has a similar distribution to *A. flavus*, but is rarely seen in maize (Van Asselt et al., 2016; IARC, 2002). Although temperature and humidity are the main factors affecting the presence of AF in food, international transport of agricultural products determines that no region of the planet is free from it (IARC, 2002).

In the liver tissue of ruminants, AFB1 is metabolized to aflatoxin M1 (AFM1), which is secreted into urine, feces and milk, mainly within 48 hours of contaminated food consumption, reducing to undetectable levels 96 hours later (Marchese et al., 2018; Nguyen and Flint, 2020). Transfer rates from AFB1 to AFM1 in milk have been estimated from 0.3% to 6.2% (Vaz et al., 2020), being maize and other contaminated concentrated foods the main sources of AFM1 in raw milk (Fink, 2008), which in turn is the main introducer of this aflatoxin in the human diet (Dimitrieska et al., 2016; Duarte et al., 2013; Vaz et al., 2020). AFM1 is hardly affected by pasteurization (Neagu et al., 2009), found in pasteurized milk (Van Asselt et al., 2016), ultra-pasteurized milk (UHT) (Duarte et al., 2013), cheese (Urbán et al., 2009) and yogurt (Rahimirad et al., 2014).

Although a tolerable daily intake (TDI) has not been specified for AFM1, the European Union has established its MRL at 0.05 μg/L for raw milk, pasteurized milk, and milk used in the manufacture of dairy products, and 0.025 μg/L for infant formula and diet foods; whereas in the United States, China and Brazil the MRL for AFM1 in milk is 0.5 μg/L. Although maximum AF levels are regulated in more than 80 countries, there is no international equality (Nguyen and Flint, 2020; Akbar et al., 2019; Vaz et al., 2020; Rahimirad et al., 2014).

Due to the stability, toxicity and MRL allowed for AF, particularly AFM1, their quantification is extremely important. In this sense, enzyme-linked immunosorbent assay (ELISA) is commonly used for screening purposes, while high-performance liquid chromatography (HPLC) with fluorescence detection or mass spectroscopy is frequently used for the identification and quantification of AF in food (Vaz et al., 2020; Rahimirad et al., 2014).

Crop rotation, prevention of infestations and grain deterioration, the use of resistant seed varieties, low moisture crops, the promotion of dehydration, proper storage and transport of grains and monitoring of FA in food have been considered to reduce fungal contamination and FA production in food (Van Asselt et al., 2016; IARC, 2002). Although it is impossible to completely prevent milk contamination (Ayar et al., 2007), limiting dairy cattle access to food with high concentrations of AFB1 helps prevent milk contamination (Dimitrieska et al., 2016; Ayar et al., 2007). On the other hand, the application of physical, chemical and biological methods as alternatives for reducing the milk content of AFM1 is questionable and poses an additional risk to food safety (Nguyen and Flint, 2020; Rahimirad et al., 2014).

2.2 Residues of veterinary drugs

Antibiotics

Antibiotics have been used as promoters of animal growth and disease prevention and treatment (Albright et al., 1961; Sachi et al., 2019). Non-compliance with milk withdrawal times, use of non-prescribed antibiotics, their use as food additives and a limited or non-existent monitoring system, among other factors, determine MRLs higher than legally established antibiotic residue (AR), which represents a serious threat to public health, especially of vulnerable age groups, and contributing to the emergence of microbial resistance (Priyanka et al., 2017; Albright et al., 1961; Kurjogi et al., 2019; Sachi et al., 2019). Screening and confirmatory methods are used for the detection of AR in milk, the former include bacterial growth inhibition assays, receptor binding enzyme assays and immunoassays (Navrátilová, 2008; Padol et al., 2015), while chromatographic tests are confirmatory, offering greater sensitivity, specificity and quantification of the analyte (Priyanka et al., 2017; Sachi et al., 2019).

Measures to reduce the concentration of RA in dairy products have been proposed, such as the education of the dairy farmer, strict compliance with the milk withdrawall time (Albright et al.,

1961), the use of adequate techniques for the detection of AR in dairy (Priyanka et al., 2017; Sachi et al., 2019), the suppression of antibiotics as growth promoters, the adoption of management and hygiene practices during milking and processing of milk, and the minimization of the use of antibiotics or their replacement by probiotics, immunomodulators, organic acids and food supplements (Priyanka et al., 2017; Sachi et al., 2019; Padol et al., 2015; Yang et al., 2019).

Anthelmintics

Anthelmintics are used to treat parasitosis of flat worms (tapeworms and trematodes) and round worms (nematodes). Depending on their chemical structure and mode of action, they are mainly classified in benzimidazoles (albendazole, fenbendazole, flubendazole, mebendazole, oxfendazole, thiabendazole, triclabendazole), tetrahydropyrimidines (levamisole, pyrantel, morantel), imidazoles (tetramisol, levamisole) and macrocyclic lactones (abamectin, doramectin, ivermectin, selamectin, moxidectin) (Romero-González et al., 2014).

Incorrect use of anthelmintics contributes to their entry into the food chain, with residues observed in milk (Romero-González et al., 2014; Cerqueira et al., 2014). In this food, the concentration of benzimidazoles is not affected by cooking, cold storage (−18 °C), baking or microwave action (Tsiboukis et al., 2013), while levamisole residues are stable during fermentation processes and heat treatment of serum, remaining in cheese (Whelan et al., 2010). In contrast to thermal treatment, macrocyclic lactones present in milk are partially degraded, which does not happen when they are present in the cream of milk (Avcı and Filazi, 2020).

In several species, benzimidazoles have been related with carcinogenic, genotoxic, embryotoxic and teratogenic effects (Romero-González et al., 2014; Tsiboukis et al., 2013; Santos et al., 2019), while ivermectin presents mutagenic and teratogenic effects in several mammals, in addition to an ecotoxic effect (Santos et al., 2019; Pérez-Cogollo et al., 2018). Thus, anthelmintic residues in food represent a public health risk (Padol et al., 2015; Romero-González et al., 2014; Avcı and Filazi, 2020). Table 1 shows the permitted MRLs of various anthelmintics and antibiotics in bovine milk. Furthermore, monitoring veterinary prescriptions, observing withdrawal periods in milk production, following the application of anthelmintics and controlling and monitoring antiparasitic residues contribute to the prevention of their contamination. Chromatographic methods are the most commonly used to identify and confirm the presence of anthelmintics in milk (Cerqueira et al., 2014; Santos et al., 2019).

Organochlorine and organophosphate pesticides

Pesticides are "chemical compounds intended to prevent, destroy, attract, fight or control any pest, including unwanted species of plants or animals during the production, storage, transport and process of food, agricultural products or animal food that can be administered to animals for the control of ectoparasites" (FAO/OMS, 2013).

Depending on their ability to enter the food chain, bioaccumulation and toxicity, organochlorine pesticides (OCPs) and organophosphorus pesticides (OPs) stand out as contaminants in milk and milk derivatives, which, despite their progressive prohibition, still represent a risk to public health.

OCPs or chlorinated hydrocarbons are broad-spectrum synthetic chemicals that include ethane derivatives such as dichlorodiphenyltrichloroethane (DDT), cyclodienes, including chlordane, aldrin, dieldrin heptachlor, endrin and toxaphene, and hexachlorocyclohexanes (HCH) as lindane (Zaragoza-Bastida et al., 2016).

The main routes of OCP contamination include inadequate management practices, such as the storage of pesticides next to food and fumigation of crops in areas close to dairy farms, causing pesticide deposition in water and food (Bedi et al., 2018), transcutaneous transmission, soil fertilization with residual sludge and the use of animal feed from countries where lindane and DDT are still used (Rusu et al., 2016; Fischer et al., 2016). High environmental stability and liposolubility determine the deposition of OCP in animal tissues especially rich in fat and milk (Zaragoza-Bastida et al., 2016; Rusu et al., 2016), being considered a persistent organic pollutant (POPs) (OMS, 2020). The mobilization of adipose tissue to maintain milk secretion, particularly in grazing-based livestock or in case of malnu-

trition of livestock, implies that the milk of these animals registers an increase in the concentration of these pesticides (Fischer et al., 2016).

Table 1. Maximum Residue Limit (MRLs) for antimicrobials and anthelmintics in bovine milk (FAO/OMS, 2008).

Antimicrobial	**MRLs (μg/l)**
Amoxicillin	4
Benzylpenicillin	4
Ceftiofur	100
Chlortetracycline/ oxytetracycline/ tetracycline	100
Dihydrostreptomycin/ streptomycin	200
Spiramycin	200
Gentamicin	200
Monensin	2
Neomycin	1500
Pyrithromycin	100
Sulphylendazole	25
Tylosin	100
Anhelmintics	**MRLs (μg/l)**
Albendazole	100
Doramectin	15
Eprinomectin	20
Febantel/ Fenbendazole/ Oxfendazole	100
Ivermectin	10
Thiobedazole	100

Despite reports of decreasing concentrations of OCP and its derivatives in relation to previous studies, probably because these compounds were banned decades ago, OCP contamination is still reported in different regions of the world, occasionally exceeding the MRLs established in raw milk, pasteurized milk, sour cream, cheese and butter. Thus, humans are exposed to these pollutants mainly through animal food (Akhtar and Ahad, 2017; Ishaq and Nawaz, 2018; Rusu et al., 2016), representing a threat to public health due to its mutagenic, teratogenic and deleterious effects on the endocrine, cardiovascular and respiratory systems. In addition, several organochlorine pesticides such as DDT, HCH, and hexachlorobenzene (HCB) are potential human carcinogens (Rusu et al., 2016).

On the other hand, organophosphate pesticides (POF) such as dichlorvos, diazinon, dimethoate, chlorpyrifos, malathion and methylparathion have replaced OCPs with less stability and persistence. Thus, they enter the animal organism through contaminated food and water, either by inhalation, through the use of insecticides in pastures, stables and dairy factories, or through the skin after the application of ectoparasites and subsequent violation of the established withdrawal period of time (Bedi et al., 2018; Fischer et al., 2016; Fernández et al., 2010). The lipophilic nature and its ability to covalently bind to proteins determine the presence of OPS in milk and its subsequent incorporation into the food chain, being detected in pasteurized milk, raw milk and cheese (Salas et al., 2003; Al-Julaifi et al., 2015; Pagliuca et al., 2006). OPS have potential effects on human and animal health, causing excessive stimulation of acetylcholine receptors and thus muscle

weakness or paralysis, excessive secretory activity, and changes in consciousness (Bedi et al., 2018; Fernández et al., 2010). Table 2 shows milk MRLs for various organochlorine and organophosphate pesticides.

It has been proposed that the control of environmental pollution in dairy farms (Bedi et al., 2018), the improvement of storage conditions for dairy food, farmer education programs, the control of pesticide, the application of organic agriculture, the implementation of integrated pasture management methods (Rusu et al., 2016), the identification of contamination sources, the monitoring of pesticide residues in animal food and dairy products by gas chromatography (GC), liquid chromatography/mass spectrometry (LC/MS), and liquid chromatography coupled to tandem mass spectrometers (LC/MS/MS) (Akhtar and Ahad, 2017) can help reduce the presence of these pesticides in milk and milk products.

2.3 Heavy metals

These are metallic and metalloid elements with a higher density compared to other metallic elements (5 g/cm^3 or an atomic weight between 63.5-200.6 g/mol), are widely distributed in the environment and induce systemic toxicity even at low exposure levels (Ismail et al., 2019; Mahmoudi et al., 2017; Jan et al., 2015). Although iron (Fe), zinc (Zn), nickel (Ni) and copper (Cu) have been considered in this group when found in food products beyond certain limits, the most toxic heavy metals found in food are generally mercury (Hg), arsenic (As), cadmium (Cd) and lead (Pb). According to the 2011 and 1999 editions of the Codex Alimentarius Commission, the maximum permitted level of Pb and Cd in milk has been established at 0.02 μg/ml and 0.01 μg/ml, respectively. The European Union has set the maximum permitted level of As at 0.1 μg/ml, while Indian legislation stipulates 1.0 μg/ml as the maximum permissible limit for mercury in milk and milk products (Ismail et al., 2019).

Anthropogenic activities such as urbanization, industrialization, irrigation with contaminated water, the application of fertilizers containing heavy metals and non-hygienic conditions in the processing and distribution of milk determine its entry into the body, reported in samples of raw, pasteurized and powdered milk (Ismail et al., 2019; Mahmoudi et al., 2017; Jan et al., 2015). Contamination of milk and milk products with heavy metals is important to public health because they cause diseases, especially in infants and the elderly, who are the main dairy consumers (Ismail et al., 2019; Mahmoudi et al., 2017). Thus, one of the main causes of saturnism is precisely the consumption of lead-contaminated milk, whose absorption rate in children is 40 % higher than in adults (Ismail et al., 2019; Harlia et al., 2018).From a pathophysiological point of view, metals stimulate the generation of reactive oxygen and nitrogen species, generating oxidative stress and impairing the cellular antioxidant system (Jan et al., 2015).

Methods used to detect heavy metals in dairy products include capillary electrophoresis, pulse voltammetry and spectrometric methods. To prevent these elements from entering the food chain, the idea is to reduce their concentrations in the water destined for dairy cows, using adsorbent agents such as smectite, palygorskite and zeolite, as well as food monitoring, the use of safe materials for dairy processing and packaging, periodic analysis of dairy products and monitoring of water used in milk and dairy processing (Mahmoudi et al., 2017). In addition, the supply of cumin (*Cuminum cyminum L.*), white turmeric (*Curcuma zedoaria Rosc.*) and mango ginger (*Curcuma mangga Val.*) in dairy cow food reduces the concentration of lead in milk and increases it in feces, probably by modulating the ruminal microbiota (Nurdin et al., 2013). In addition, a lower concentration of heavy metals has been observed in yogurt compared with raw milk, an effect attributed to fermentation processes as a result of bacterial activity (Enb et al., 2009).

2.4 Dioxins and polychlorinated biphenyls analogue to dioxins (PCB-AD)

These are a group of polychlorinated compounds, almost aromatic planar with similar physical and chemical structures, consisting of 75 polychlorinated dibenzo-p-dioxins (PCDD) and 135 polychlorinated dibenzofurans congeners (PCDF). Out of the dioxins (PCDD/Fs), 17 exhibit toxicological properties, and 12 show 209 polychlorinated biphenyls

Table 2. MRLs for various organochlorines and organophosphates (Ishaq and Nawaz, 2018; Pagliuca et al., 2006).

Organochlorines	**MRLs μg/kg**
DDT	40
DDE	40
Dieldrin	6
γ-HCH	1
α- endosulfan	100
β-endosulfan	100
Endosulfan sulphate	100
Organophosphates	**MRLs μg/kg**
Acephate	20
Chlorpyrifos	10
Chlorpyrifos- methyl	10
Diazzinon	10
Methamidophos	10
Metidation	20
Forate	20
Pyrimifos-methyl	50

(PCBs), and when adopting a dioxin-like planar structure they exhibit toxicological properties similar to dioxins (analogue to dioxin PCB-AD) (DO, 2011; AECOSAN, 2018).

These compounds are characterized by being highly stable in the environment, by their ubiquity, their toxicity and their ability to enter the organism through air, soil or sediments, inhalation, skin absorption and especially by contaminated food (AECOSAN, 2018; Gallego et al., 2005). In ruminants, the intake of PCDD/Fs and PCB-AD occurs mainly during grazing, by the consumption of contaminated grass and soil particles, as well as by the consumption of silage and fodder subjected to drying processes (Bogdal et al., 2017).

Once accumulated in the organism, mainly in animal adipose tissue, gestation and lactation will mean the mobilization of these compounds, increasing their concentration in milk which, along with their derivatives, becomes a potential source of dioxins and PCB-AD for the consumer (Gallego et al., 2005; Schulz et al., 2005; Piskorska-Pliszczynska et al., 2017). Dioxins have been detected in more than 90% of samples of infant formula, butter, and other dairy products (yogurt, frozen dairy desserts, baked products containing dairy) and various types of cheese (CFIA, 2019), being a serious threat to public health. PCDD/Fs and PBC-AD have carcinogenic, teratogenic, and mutagenic effects and are associated with dermal and hematic alterations, which act as an endocrine disruptor causing reproductive, immunological, and neurological disorders (Gallego et al., 2005).

In order to prevent food intended for milk-producing animals from being contaminated by dioxins and PCB-AD, it has been proposed the identification of agricultural areas with significant presence of these compounds, as well as the identification and monitoring of feed and feed ingredients coming from these areas, the monitoring of the concentration of dioxins and PCB-AD in sewage sludge and compost used in agriculture, and the identification and control of critical feed manufacturing processes (for example, artificial drying by direct

heating) (FAO/OMS, 2018). Ingestion of dioxin and PCB contaminated soil particles can also be prevented by reduciing animal density during grazing and increasing pasture availability. Since dioxins remain in the animal organism for 30 to 60 days before they are excreted through milk, it has been proposed to transfer animals to uncontaminated soil for three months to reduce dioxins in the milk. Since milk contamination with PCB-AD has been reported following the ingestion of painting remains in stables and grazing in areas near industrial centers, these should be considered as potential sources of contamination (Gallego et al., 2005; Bogdal et al., 2017; Schulz et al., 2005).

PCDD/Fs and PCB-ADs, along with organochlorine pesticides, are the most common persistent organic pollutants (POPs) (OMS, 2020), which are released into the environment as a result of various anthropogenic activities and enter the food chain due to their capacity for transport, toxicity and persistence (Figure 2).

Detection of PCDD/Fs and PCB-AD is based on gas chromatography along with high-resolution mass spectrometry (GC-HMRS). Gas chromatography-based methods are also used with tandem mass spectrometry (GC-MS/MS). Additionally, bioassay techniques have been developed as high-throughput screening methods (FAO/OMS, 2018).

2.5 Disinfectants and Detergents

Cleaning and disinfection are critical steps in primary production and later dairy processing, allowing the removal of milk residues and minimizing the level of bacterial contamination in milking facilities. Detergents including surfactants in their composition and disinfectants are used for this purpose with a wide range of products containing biocides such as chlorine, iodine, quaternary ammonium and chlorine dioxide (Van Asselt et al., 2016; Fischer et al., 2016; Kirsanov et al., 2020). If the washing and disinfection procedures in dairy cows, as well as the washing and rinsing of milking and storage equipment are performed incorrectly, the residues of detergents and disinfectants contaminate milk and dairies (Fischer et al., 2016; Merin et al., 1985; Šalomskienė et al., 2013; Siobhan et al., 2012). Thus, equipment and utensils used in milk processing must be cleaned, disinfected and rinsed with drinking water (unless the manufacturer's instructions indicate otherwise) and subsequently drained and dried (FAO, 2004).

Although chlorine residues in milk degrade rapidly, without presenting a health risk (Fischer et al., 2016; Šalomskienė et al., 2013), chlorine contact with organic matter results in milk containing residues of contaminants that are not intentionally added, such as trichloromethane (TCM) or chloroform (Siobhan et al., 2012). Other disinfectants such as chlorine dioxide generate by-products whose intake inhibits the absorption of iodine (Van Asselt et al., 2016), while quaternary ammonia is stable in milk, negatively affecting the health of consumers and inhibiting milk fermentation and cheese processing (Siobhan et al., 2012). The MRL for disinfectants and detergents in milk and other foods has been estimated at 0.1 mg/kg (DO, 2014). On the other hand, the high intake of iodine from its residues in milk can lead to disorders in thyroid function, mainly in children (Fischer et al., 2016).

Due to the wide variety of disinfecting products, several analytical techniques have been described for their detection in food, including simple colorimetric tests, potentiometer with ion selective electrodes, thin layer chromatography and liquid and gas chromatography. For the quantification and detection of chlorates, perchlorates, and quaternary ammonium in milk and dairy products, chromatography-tandem mass spectrometry is used (Fischer et al., 2016).

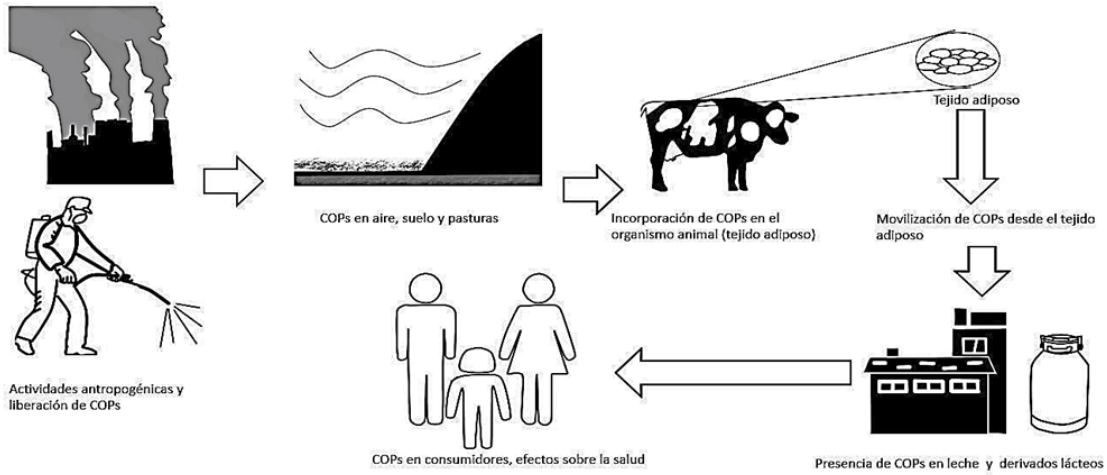

Figure 2. Entry of Persistent Organic Pollutants (POPs) into the food chain

3 Conclusions

Milk is a source of nutrients for a significant number of the population. However, anthropogenic activities aimed at improving animal productivity and dairy processing cause a risk of chemical contamination of these products. Due to the ubiquity of these pollutants and their effects after their chronic intake through milk and dairy products, maximum residual limits have been established for most of these compounds, and it is necessary to prevent, or at least minimize, and monitor the concentration of chemical contaminants in dairy products, using appropriate screening techniques and contributing to the food security of the population.

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