NEW INSIGHTS IN FOOD RESEARCH AND ENGINEERING:

The Trend for World Sustainable Food Production

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New insights in food research and engineering: the trend for world sustainable food production

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Preface

The increase of chronic degenerative diseases in the population is mainly due to poor nutrition. The World Health Organization has established that a diet rich in plant foods and metabolites of animal origin can prevent the risk of acquiring such diseases. Increasingly, individuals are becoming aware of the need to consume healthy foods rich in antioxidants and other beneficial bioactive compounds to health because they can be preventive and even therapeutic. In recent years, some food industries in developed countries have been concerned about including sustainable technological processes to extract compounds of pharmaceutical interest or obtain foods that minimize the loss of healthy phytochemicals nutrients, which still preserve the quality and safety when consumed. However, the challenge remains high in underdeveloped countries since a large amount of food continues to be wasted, and food residues or wastes are not used. In this context, scientific research on innovation, science, biotechnology, and food technology continues to be carried out to encourage the food industry to improve its production processes and the use of materials to obtain active ingredients of high biological value.

This book consists of 15 chapters and is intended for scientists and technologists interested in food science and biotechnology research. The book covers topics on Emerging Technologies and Food Packaging, Food Enzymology and Molecular Biology, Food Fermentations, Food Processing, Food Safety, Food Microbiology, Functional Foods, Legislation and Quality Control, and Sensory Evaluation.

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Physiological changes in genetically modified tomatoes by application of hot water treatment

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Abstract

Tomato is the most widely produced and consumed horticultural crop worldwide and Mexico is the leading exporter. In this work, we evaluated the application of hot water treatment (HWT) on wild type tomatoes and genetically modified tomatoes as it is a treatment that has proven to be effective against cold damage and that the use of genetic technology can reinforce the effectiveness of the treatment. We aimed to preserve the physicochemical attributes in the fruit. *TomLoxB* Silenced Mutants var TA234 were used for this experiment. Genetically modified (GM) and wild type (WT) tomatoes were immersed in water at 40 ºC for 10, 20, or 30 s to evaluate changes in appearance, weight loss, external firmness, total soluble solids, and titratable acidity. GM tomatoes treated for 30 s showed reduced weight loss, delayed red color development, increased firmness, decreased total soluble solids and titratable acidity, in contrast to control and fruits exposed for 20 s, 10 s, as well as control and treated wild-type tomatoes. Hot water treatment for 30 s prolonged postharvest life in transgenic tomatoes up to 18 days, while 10 s delayed it by 12 days, and 20 s delayed it by 15 days, compared to WT tomato with a 9-day postharvest life.

Keywords: *Hot water treatment, solanum lycopersicum, postharvest life.*

1. Introduction

Tomato is the most widely produced and consumed horticultural crop worldwide, and Mexico is the leading exporter. An approach to increase its postharvest life has been the genetic modification of tomato (*Solanum lycopersicum*) variety TA234 by inserting the *TomloxB* gene in anti-sense. This procedure proved to be capable of delaying the onset of senescence and improving fruit color, flavor, and texture (Leon-Garcia *et al.*, 2017). In the hot water treatment (HWT) fruits are immersed for times ranging from seconds to hours in water with temperatures from 40 to 60 ºC. Its mechanism of action is based on enzymes inactivation, delay of metabolic processes of the fruit and inducing the fusion of the epicuticular wax, thus covering the cracks in the fruit epidermis, that prevents the attack by pathogens (Fallik, 2004; Mahajan *et al.*,2014), In addition, the application of this post-harvest treatment has been shown to counteract chilling injury, an effect that is evident when horticultural products are kept under refrigeration for prolonged periods of time. However, there are few studies related to the post-harvest performance of genetically modified fruits. The aim of this work was to assess the physiological changes in genetically modified tomatoes subjected to HWT.

2. Materials and methods

2.1 Plant material

The fruits (*Solanum lycopersicum*) variety TA234 *TomLoxB* Silenced Mutants were used for this experiment. Genetically modified (GM) and wild type (WT) were grown and collected at the UNIDA greenhouse in Veracruz, Mexico. The tomatoes were selected in a break stage ensuring that they were free of physical damage.

2.2 Hot water treatment

Wild type and genetically modified tomatoes were washed and disinfected with a 50 µL/L sodium hypochlorite solution, then were immersed in a water bath (Fisher Scientific Isotemp Model 28L-M, Pittsburgh, PA) filled with distilled water at 40 ºC for 10, 20 and 30 s. Control and treated tomatoes were stored at 25 ºC for 18 days.

2.3 External physical changes

The fruit during storage were evaluated by taking photographs of both GM and WT tomatoes every three days until the end of storage time using a Canon PowerShot mod. SX500IS camera.

2.4 Weight loss

The weight loss was assessed using the methodology of Cruz-Álvarez *et al*. (2012), weighing the tomatoes every 3 days during storage time, using an electronic balance (Sartorius model BL 2100, Germany).

2.5 Firmness

Firmness was determined in tomato pericarp by the method of Zapata *et al*. (2007), in three equidistant points of the equatorial region using a Fruit Texture Analyzer (model GS25, Quebec, Canada), using a stainless-steel needle probe 8 mm long by 2.5 mm wide.

2.6 Total soluble solids (TSS)

TSS were determined by the AOAC method (1990), taking 1 mL of the juice extracted from the tomato pulp, then measured in a refractometer (Leica Abbe Mark II, Buffalo, NY).

2.7 Titratable acidity

It parameter was determined with the method established by the AOAC (1990), taking an aliquot of 1 g of tomato pulp juice, two drops of 1% phenolphthalein were added as an indicator and titrated with a 0.1 N NaOH solution (Golden Bell®).

2.8 Statistical analysis

Results were analyzed using a one-way ANOVA to test for significant differences between the treatments. The comparison between means was made by Tukey's range test ($P<0.05$) with the statistics software Minitab® version 18.

3. Results and Discussion

3.1 Changes in appearance

WT tomatoes without HWT showed a light red coloration on day 3, while GM tomatoes showed the same degree of coloration until day 6 (Figure 1), exhibiting a delay in ripening induced by the *TomloxB* gene inserted in antisense (Leon-Garcia *et al.*, 2017). GM tomatoes with HWT for 30 s maintained its physical integrity throughout storage. Results suggest that HWT the effect of HWT for 30 s on the tomatoes GM, by decreasing enzymatic activity in metabolic processes, prevented the evaporation of water from the tissue, preserving its turgidity, unlike the rest of the treated fruits and the control (Cruz-Álvarez *et al.*, 2012; Kalantari *et al.*, 2015).

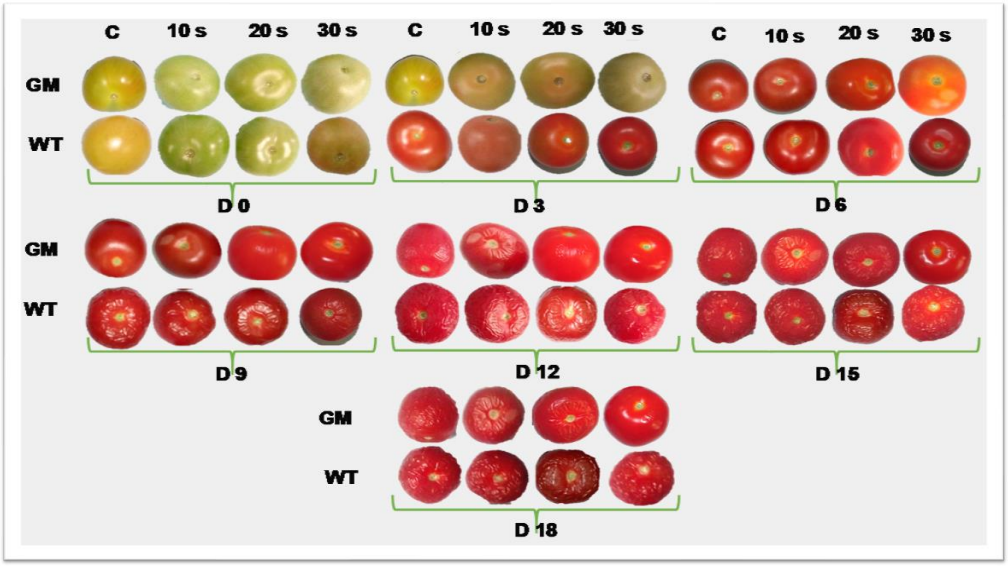

Figure 1. Changes in appearance in tomatoes with HWT 40 ºC for 10, 20, and 30 s storage for 18 days. (WT) wild type, (GM) genetically modified.

3.2 Weight loss

GM tomatoes with HWT for 30 s, showed a significantly lower weight loss of 13.91 % ($P < 0.05$) during 18 days of storage compared to control GM tomato with 22.93 %, 10 s treatment with 25.11%, and 20 s treatment with 21.01%. On the other hand, Wild-type tomatoes with HWT for 30 s showed a significantly lower weight loss of 18.79 % ($P < 0.05$) compared to control tomatoes with 31.07%, 10 s treatment with 27.24%, and 20 s treatment with 21.91 % (Figure 2). This fact can be attributed to the decrease in fruit respiration rate and transpiration caused by HWT (Cheng *et al.*, 1988), which delays fruit softening by interrupting protein synthesis and enzyme inactivation, thus avoiding excessive softening that contributes to a shorter post-harvest life (Paull and Chen, 2000; Bouzayen *et al.*, 2010). On the other hand, GM tomatoes with HWT for 10 s revealed significantly greater weight loss ($P<0.05$) during storage compared to the other treatments.

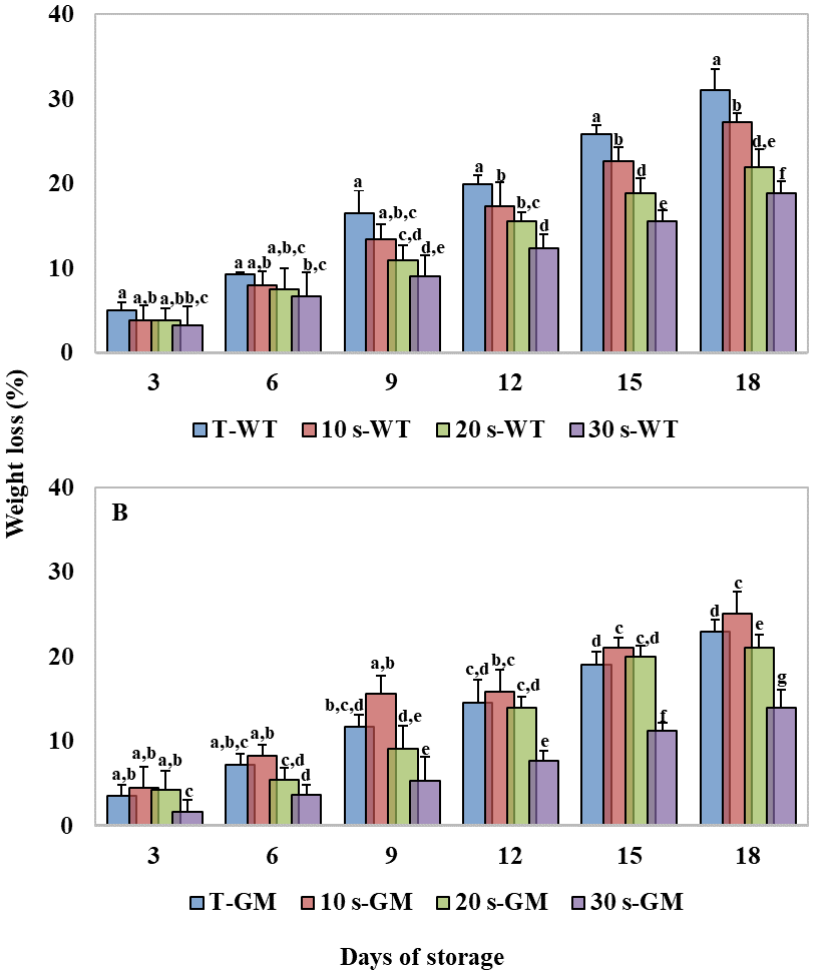

Figure 2. Weight loss in tomatoes with HWT 40 ºC for 10, 20, and 30 s storage for 18 days. (A) wild type (WT), (B) genetically modified (GM).

3.3 Firmness

GM tomatoes with HWT for 30 s showed a significantly higher firmness of 3.681 N ($P<0.05$) compared to control GM tomatoes with 2.931 N, with the 10 s treatment it was 2.46 N, and in the 20 s treatment it was 2.684 N at the end of storage time. Wild-type tomatoes with HWT for 30 s showed a significantly higher firmness of 2.822 N ($P<0.05$) compared to the 10 s with 2.722 N, and 20 s with 2.815 N treatments (Figure 3), even a slow decrease in firmness was observed. This is since effectively supplied heat can induce resistance and stabilization mechanisms in the membrane, as well as an inactivation of hydrolytic enzymes in the cell wall (Paull and Chen, 2000; Mama *et al.*, 2016).

However, GM tomatoes with HWT for 10 s showed a significantly greater decline than the rest of the treatments.

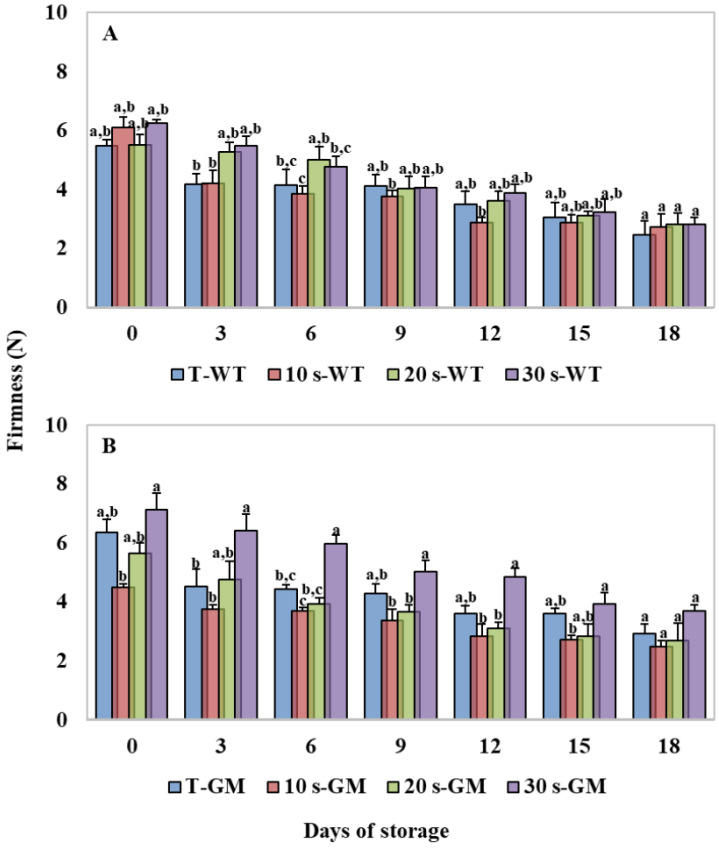

Figure 3. Firmness in tomatoes with HWT 40 ºC for 10, 20, and 30 s storage for 18 days. (A) wild type (WT), (B) genetically modified (GM).

3.4 Total soluble solids (TSS)

Wild-type tomatoes with HWT for 30 s showed no significant difference ($P<0.05$) with respect to the rest of the treatments. The results obtained after storage time were as follows: control 10.6 %, and in the 10 s 10.8 %, 20 s 10.8%, and 30 s 9.3% treatments. On the other hand, GM tomatoes with HWT 30 s showed significantly lower TSS of 7.5 % ($P<0.05$) compared to the control 9.1 %, 10 s 11 %, and 20 s 10.5 % treatments during storage (Figure 4). However, GM tomatoes with HWT for 10

seconds displayed an increase in TSS; similar results were reported by Pinheiro *et al.* (2014), who attributed this result as a response to the HWT, by activation of sucrose synthase.

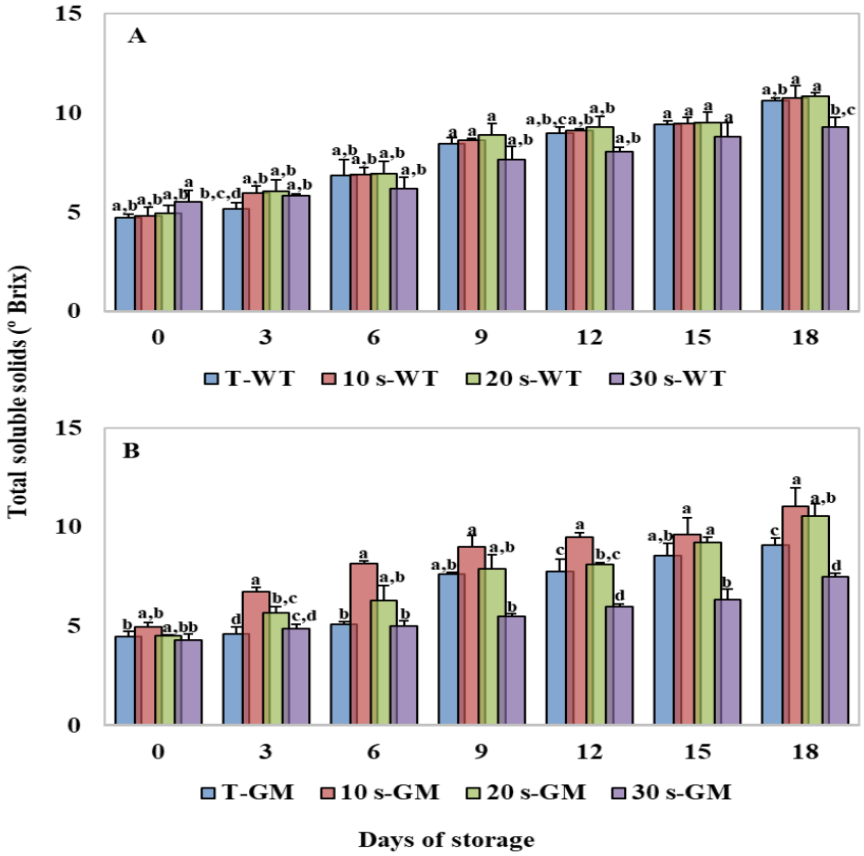

Figure 4. Total soluble solids in tomatoes with HWT 40 ºC for 10, 20, and 30 s storage for 18 days. (A) wild type (WT), (B) genetically modified (GM).

3.5 Titratable acidity

Wild-type tomatoes showed no significant differences in percent acidity ($P<0.05$) between the treated fruit (10 s with 0.35 %, 20 s with 0.36 %, and 30 s with 0.39 %) and the control with 0.34 %. GM tomatoes with 30 s HWT showed a significantly higher acidity percentage of 0.62 ($P<0.05$) compared to the rest of the treatments (control 0.52 %, 10 s 0.36 %, and 20 s 0.52 %) (Figure 5). The data suggest that application of HWT on GM tomatoes decreased the respiration rate of the fruit, which was consistent with other reports (Famiani *et al.*, 2015; Tadesse and Abtew, 2016).

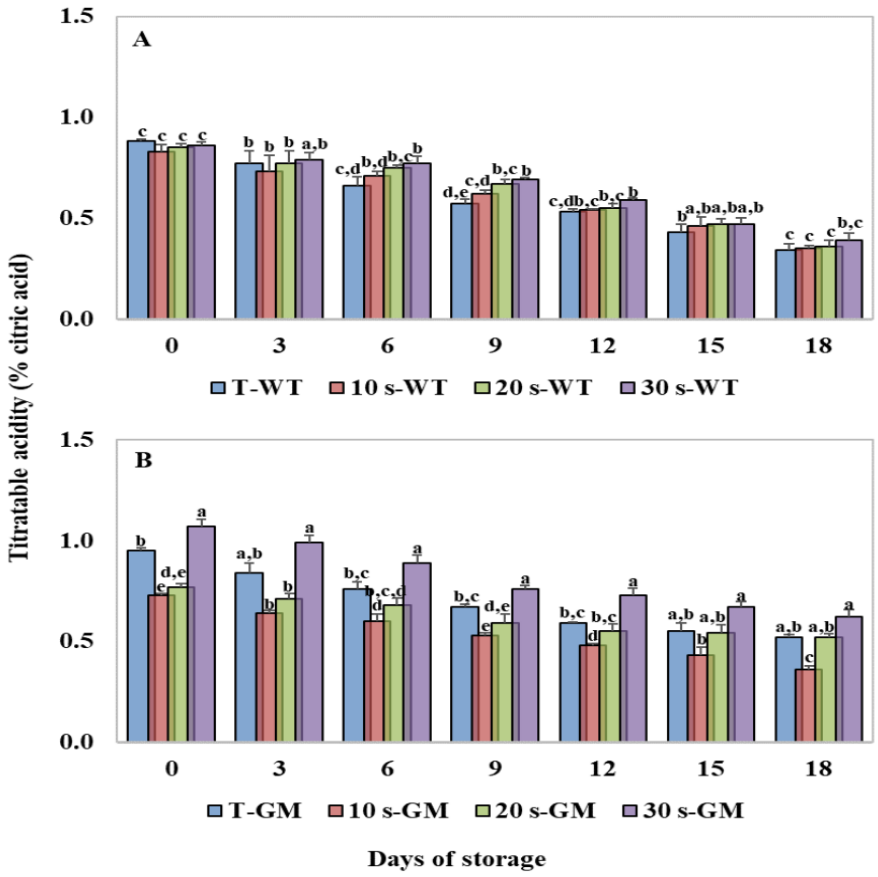

Figure 5. Titratable acidity in tomatoes with HWT 40 ºC for 10, 20, and 30 s storage for 18 days. (A) wild type (WT), (B) genetically modified (GM).

4. Conclusions

HWT for 30 s extended postharvest life in the genetically modified tomatoes to 18 days, while 20 s did it for 15 days, and 10 s for 12 days, compared to the wild type tomatoes that lasted 9 days. HWT for 30 s applied to the genetically modified tomatoes delayed the physiological changes of ripening, however at short exposure times it caused physiological irregularities.

5. Acknowledgments

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Volatile organic compounds produced by *Meyerozyma caribbica*: A potential mechanism of action against *Colletotrichum gloeosporioides*

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Abstract

Volatile organic compounds (VOCs) are compounds of low molecular weight (< 300 Da) whose production is strongly influenced by the microbial species and the growth phase. VOCs produced by biocontrol organisms such as *Meyerozyma caribbica* can be considered as an alternative to control fungal decay in the postharvest stage. Therefore, this study aimed to investigate the production of VOCs by *M. caribbica* alone and in the presence of *Colletotrichum gloeosporioides* under different conditions and its effect on fungus growth. The antifungal activity of VOCs was *in vitro* determined in Petri dishes and the identification of VOCs produced by *M. caribbica* was performed using headspace-solid phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC–MS). The VOCs produced by *M. caribbica* can inhibit the growth of *C. gloeosporioides* until 55 ± 4.5% at 25 °C after 7 days of incubation and 100% after 10 days of incubation at 6 °C. The main VOCs identified were alcohols such as 3-methyl-1-butanol and 1-phenylethanol, and esters such as ethyl and isopentyl acetate. *M. caribbica* produces the same VOCs in monoculture or co-culture with the fungus. However, the production of compounds such as ethyl acetate and isopentyl acetate by the yeast was detected at 6 °C. Otherwise, *C. gloeosporioides* was able to produce β-phellandrene. The production of VOCs by *M. caribbica* plays a vital role in its antagonistic activity against *C. gloeosporioides*; nevertheless, it is strongly influenced by the environmental conditions.

Keywords: *Biocontrol, postharvest, yeast, phytopathogenic fungi.*

1. Introduction

Fruit and vegetable losses caused by microorganisms, mainly by fungi and bacteria, along the food supply chain can reach more than 25% of the total production in industrialized countries, and up to 50% in low- and medium-income countries if storage conditions are not optimal (Sapper and Chiralt, 2018). Anthracnose is one of the most frequent postharvest diseases, characterized by the appearance of sunken necrotic dark brown or black lesions along with the formation of orange conidial mass, resulting in tissue rot. Anthracnose is mainly caused by *Colletotrichum* species being *Colletotrichum gloeosporioides* the main fungus associated with this disease. In avocado, anthracnose is controlled mostly by the application of synthetic fungicides such as prochloraz, carbendazim, and thiabendazole. However, the increment in the resistance of the phytopathogenic fungi to synthetic fungicides, the harmful to humans and the environment, as well as the growing consumer demand for healthy foods are leading to the development of alternative strategies to control postharvest diseases (Mari *et al.*, 2016). Several techniques have been studied to preserve fruits and vegetables including refrigeration, controlled atmosphere storage, and sterilization by UV and gamma radiation (Sapper and Chiralt, 2018).

In line with this, two of the most promising and popular green technologies for reducing postharvest losses are the use of edible coatings based on natural materials and biological control agents (BCAs). The BCAs have different mechanisms to achieve the inhibition of other microbial species. For example, *Meyerozyma caribbica*, a yeast, can inhibit phytopathogenic fungus through the competition for space and nutrients, parasitism, biofilm development, production of hydrolytic enzymes (Bautista-Rosales *et al.*, 2013) and synthesize volatile organic compounds (VOCs) (Iñiguez-Moreno *et al.*,

2020a). These compounds are produced by yeasts, molds, and bacteria during their primary and secondary metabolism. VOCs are low molecular weight (< 300 Da) products, characterized by their low polarity and high vapor pressure, whose synthesis is dependent on the kind of microbial species, growth phase, and environmental conditions (Korpi *et al.*, 2009). Moreover, VOCs are considered ideal antimicrobials because they do not require contact with the target microorganism or food matrix to achieve their activity (Contarino *et al.*, 2019). These compounds are widely used in the food and pharmaceutical industries for the modification of aroma and flavor (Valsami *et al.*, 2020). Otherwise, the application of VOCs in fresh and minimally processed foods is realized through their encapsulation and incorporation into edible coatings or modifying the packaging atmosphere. Recently, it has been reported that *M. caribbica* can produce VOCs at 6 and 25 °C in the presence of *C. gloeosporioides* (Iñiguez-Moreno *et al.*, 2020a). However, it was not determined if their profile changed along the incubation time. Henceforth, this study was aimed to i) determine the growth inhibition of *C. gloeosporioides* by the VOCs produced by *M. caribbica* and ii) identify the VOCs produced by this yeast alone and in the presence of *C. gloeosporioides* at 6 °C after 10 and 20 days of incubation and after 3 and 7 days at 25 °C.

2. Materials and methods

2.1 Microorganisms

C. gloeosporioides Pa14 (GenBank ID: MN477464) previously isolated from decayed avocados, was inoculated onto Potato Dextrose Agar (PDA, Oxoid, Basingstoke, UK) and incubated at 28 °C/10 days. The conidial suspension was prepared according to Iñiguez-Moreno *et al.* (2020b). *M. caribbica* (GenBank ID: JQ398674) was cultivated in yeast extract-peptone-dextrose (YPD) broth (Oxoid, Basingstoke, UK) as described by Bautista-Rosales *et al.* (2013).

2.2 Effect of VOCs on the growth of *C. gloeosporioides*

The antifungal activity of VOCs was determined *in vitro*. Briefly, each Petri dish with PDA was inoculated in the center with 50 µL of spore suspension (1×10^5 spores/mL). At the same time, 100 µL of the yeast suspension (1×10^9 cells/mL) were streaked on a new Petri dish containing PDA and were let dry for 1 h at 25 °C (Hernandez-Montiel *et al.*, 2018). Inoculated plates were placed mouth-to-mouth, sealed with three laps of parafilm, then were incubated for 3 and 7 days at 25 °C or 10 and 20 days at 6 °C. Petri dishes with spores' suspension in the absence of yeast were included as a positive control for *C. gloeosporioides* growth, Petri dishes without microorganisms were used for the determination of contamination. The percentage of inhibition of mycelial growth was calculated according to Eq.1.

$$Inhibition\ (\%) = \frac{dc-dt}{dc}100 \qquad \text{(Eq. 1)}$$

where dc (cm) is the mean of colony diameter for the control and dt (cm) is the mean of colony diameter for the treatment. Three replicates were used for each treatment and the experiment was repeated once.

2.3 Identification of VOCs produced by *M. caribbica*

Yeast VOCs were isolated and identified by headspace-solid phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC–MS). The cultures were obtained under the same conditions as above. At the end of the incubation period, the sealed Petri dishes were kept for 15 min at 30 °C to reach the equilibrium of VOCs in the headspace. The HS-SPME-GC–MS analysis was carried out according to Iñiguez-Moreno *et al.* (2020a). To identify the VOCs the GC retention index and mass spectra were cross-referenced with the National Institute of Standards and Technology (NIST 11 MS Library). Controls consisted of i) Petri dishes with PDA and ii) Petri dishes containing only *C. gloeosporioides*. Controls were performed under the same conditions as the sample

to exclude interfering substances. All measurements were made with three replicates, each replicate representing the analysis of a different Petri dish.

2.4 Statistical analysis

Data were processed by one-way analysis of variance (ANOVA). Before the ANOVA, percentages were arcsine-square-root transformed. The statistical data analysis was performed using the software Statgraphics Centurion XVI.I (Statpoint Technologies, Inc., Warrenton, USA), the post-hoc least significant difference (LSD) Fisher test ($p \leq 0.05$) was used for means comparison. The averages per treatment were reported as their untransformed values.

3. Results and discussion

3.1 Effect of VOCs on the growth of *C. gloeosporioides*

The inhibitory effect of VOCs on *C. gloeosporioides* was up to 55 and 83% at 25 and 6 °C, respectively ($p \leq 0.05$, Table 1). These results are similar to those obtained with VOCs produced by *Aureobasidium subglaciale* against *Botrytis cinerea* (65.4%) at 25 °C after 5 days of incubation (Di Francesco *et al.*, 2020). In this study, the inhibitory effect of VOCs decreased as the time incubation increased ($p \leq 0.05$); probably because VOCs profile and concentration can change along the incubation period (Di-Francesco *et al.*, 2020). Besides, the fungus could adapt to environmental conditions and slowly grow in the presence of VOCs.

Table 1. Inhibition of *C. gloeosporioides* by VOCs.

Temperature of incubation (°C)	Inhibition (%) Incubation time	
6	10 days	20 days
	100.0 ± 0.00 [a]	83.9 ± 0.90 [b]
25	3 days	7 days
	79.53 ± 1.76 [a]	55.0 ± 4.50 [b]

Values are expressed as means ± standard deviation (n = 6). Values in the same row followed by different lower-case letters are significantly different according to Fisher's LSD test at $p \leq 0.05$.

3.2 Identification of VOCs produced by *M. caribbica*

The bioactivity of several VOCs produced by BCAs have been assessed in recent years (Contarino *et al.*, 2019; Di Francesco *et al.*, 2020). However, few studies report the VOCs synthesized by *M. caribbica* (Zhang *et al.*, 2014; Iñiguez-Moreno *et al.*, 2020a). In this research, the VOCs production was analyzed by HS-SPME-GC–MS at 6 and 25 °C at two different times of incubation. Under both tested temperatures the main identified compounds were alcohols and esters (Table 2). Alcohols and esters have been related to the antagonistic activity of *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae* (Contarino *et al.*, 2019), *Aureobasidium pullulans*, *Aureobasidium melanogenum*, and *A. subglaciale* (Di Francesco *et al.*, 2020). The mechanism of action proposed for alcohols is through the damage of the plasmatic membrane and protein denaturation, with consequent metabolism interference and cell lysis. Lipophilic compounds such as 2-methyl-1-butanol and 3-methyl-1-butanol have a high affinity for the plasmatic membrane and higher toxicity than ethanol (Weber and de Bont, 1996).

Table 2. Volatile organic compounds produced by *Meyerozyma caribbica.*

Peak number	Possible compound	Molecular formula	Retention Index (RI)	Retention time (min)
1	Ethanol	C_2H_6O	537	1.55
2	Ethyl acetate [a]	$C_4H_8O_2$	612	2.22
3	2-Methyl-1-propanol	$C_4H_{10}O$	625	2.35
4	3-Methyl-1-butanol	$C_5H_{12}O$	741	3.94
5	2-Methyl-1-butanol	$C_5H_{12}O$	744	4.02
6	Isopentyl acetate [a]	$C_7H_{14}O_2$	881	6.84
7	1-Phenylethanol	$C_8H_{10}O$	1063	11.88

[a]Detected only in samples incubated at 6 °C.

Otherwise, ethyl acetate and isopentyl acetate were only detected in samples incubated at 6 °C (Table 2). Some yeasts can produce ethyl acetate by esterification of acyl-CoA and ethanol by the catalysis of endogenous alcohol acyltransferase. However, ethyl acetate production through this pathway is particularly low. Because the acyl-CoA flows to the tricarboxylic acid cycle, but if the cycle is damaged or slowed down, it could be accumulated to form ethyl acetate (Zhao *et al.*, 2020). Otherwise, Plata *et al.* (2003) reported the formation of isopentyl acetate by wine yeasts such as *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* after 10 days of incubation in samples with low esterase activity. Low temperatures could decrease the activity of this enzyme, explaining the presence of isopentyl acetate in samples incubated at 6 °C. The mechanism of action of this compound on fungi is unknown. However, in *Escherichia coli* volatile isopentyl acetate enters the periplasm and is hydrolyzed to acetic acid and isopentyl alcohol by bacterial esterase. These compounds damage the cell membrane and impair the electron transport system, leading to loss of respiration (Ando *et al.*, 2015). Besides, the biocontrol through VOCs could be the result of a synergic effect of VOCs and carbon dioxide in the environment (Contarino *et al.*, 2019). The VOCs profiles obtained under both conditions were consistent, different conditions should be assessed to improve the production of VOCs of higher interest such as isopentyl acetate.

The VOCs produced by *C. gloeosporioides* Pa14 were included as controls in each assessed condition. After 7 and 20 days of incubation, β-phellandrene ($C_{10}H_{16}$, retention index 1026) was the main VOC synthesized by the fungus (Figure 2). This compound is a secondary metabolite (Yan *et al.*, 2018); for this reason, its production was lower or not detectable after 3 and 10 days of incubation at 25 and 6 °C, respectively (Figure 1). β-phellandrene is a monoterpene present in many plant essential oils, including pine, eucalyptus, and parsley. It has great commercial importance because of its applications in pharmaceuticals, flavor, fragrance, cosmetics, personal-care products, and domestic and industrial supplies. Also, β-phellandrene has been proposed as an advanced biofuel (Valsami *et al.*, 2020). Therefore, studies about the optimization conditions, extraction, and quantification of β-phellandrene produced by this fungus are required. Then, the production of this monoterpene by a fungus such as *C. gloeosporioides* can be considered as an alternative to the overexpression of the involved genes in its synthesis in microorganisms such as cyanobacteria.

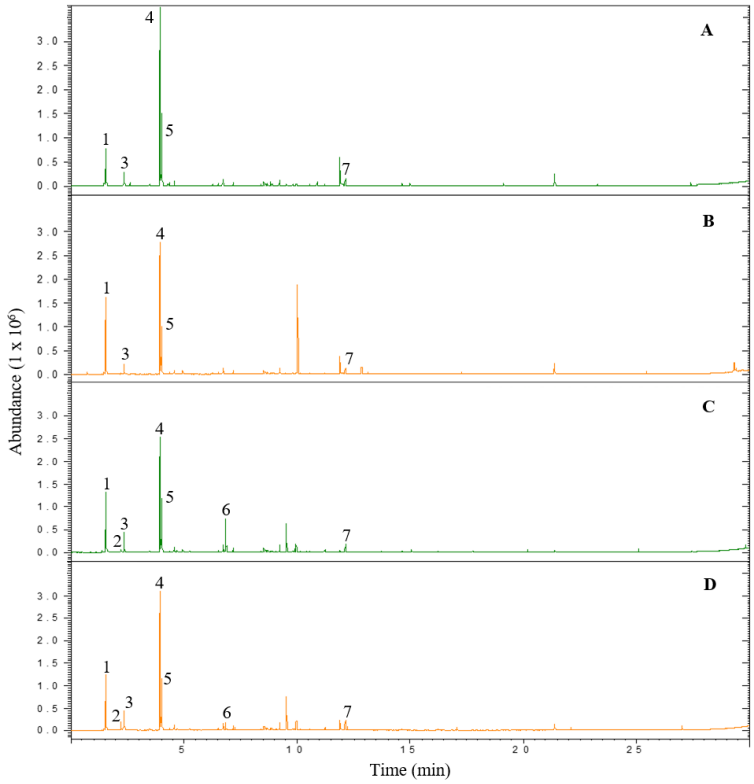

Figure 1. Volatile organic compounds produced by *Meyerozyma caribbica* at 25 °C after 7 days of incubation A) in the absence and B) in the presence of *Colletotrichum gloeosporioides*, and at 6 °C after 20 days of incubation C) in the absence and D) in the presence of *Colletotrichum gloeosporioides*. Not numbered peaks were also or just identified in the chromatograms of the fungus.

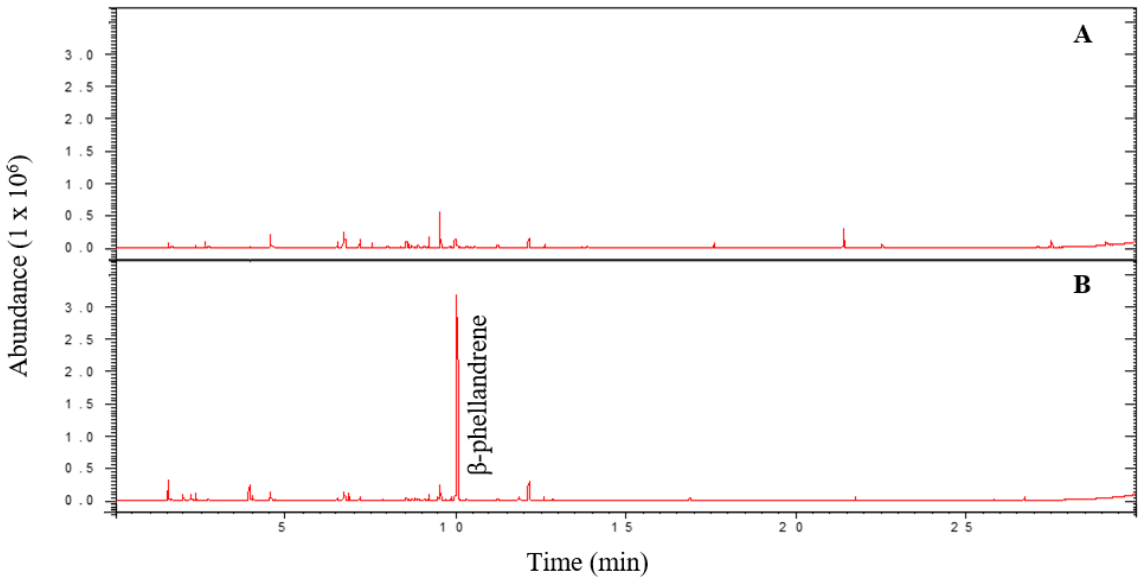

Figure 2. Production of β-phellandrene by *Colletotrichum gloeosporioides* incubated at 25 °C after A) 3 and B) 7 days.

4. Conclusions

The production of VOCs by *M. caribbica* plays a vital role in its antagonistic activity against *C. gloeosporioides*. Nevertheless, the production of important compounds such as isopentyl acetate is strongly influenced by environmental conditions, being favored at low temperatures. In the future, the extraction, purification, and microencapsulation of VOCs produced by this yeast can be considered as a biocontrol alternative to anthracnose control in tropical fruits. Besides, more studies must be carried out with the VOCs identified to determine their effect *in vivo* conditions. Because of the interactions between the VOCs, the assessed fruit (microbiome and volatilome), and the environmental conditions can affect the activity of VOCs. Finally, *C. gloeosporioides* Pa14 could be an alternative to the production of β-phellandrene.

5. Acknowledgements

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Isolation and techno-functional characterization of coconut (*Cocos nucifera* L.) storage proteins from the state of Guerrero, Mexico

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Abstract

Coconut cake is a by-product that results from extractions of coconut milk and oil, which has been reported to have around 20% protein. The state of Guerrero is positioned as the leading coconut producer in Mexico; however, there is a lack of diversification of coconut-based products. Therefore, the present work focused on the isolation and characterization of the storage proteins of the coconut cake. The methodology used was the following: The endosperm of *C. nucifera* L. was cut into cubes, ground, and strained to later dehydrated (65±3 °C) and finally defatted. Proteins were fractionated, and soluble protein was quantified by the Bradford method. SDS-PAGE monitored the protein profile. The protein fractions were analyzed for techno-functional characteristics as surface hydrophobicity, emulsifying activity index, and emulsifying stability index. The results exhibited that glutelins were the predominant proteins (68.65%), followed by globulins (28.42%). In the protein profile, cocosin was identified, which is the major protein of the coconut endosperm. The highest and lowest solubility of coconut protein was found at pH 8 to 12 and pH 4 to 6, respectively. Globulins showed greater surface hydrophobicity (304,574 ± 33,778). For the emulsion stability, no statistical differences were observed. Albumin and glutelins showed the highest emulsifying activity (11.52 ± 0.42 and 13.33 ± 1.57 m^2/g, respectively). In conclusion, the reserve proteins presented techno-functional properties.

Keywords*: Coconut cake, storage proteins, solubility, surface hydrophobicity, emulsifying properties.*

1. Introduction

The coconut (*Cocos nucifera* L.) is known as the tree of life due to its many uses (Foale *et al.*, 2020). It is a crop of economic importance; it is cultivated in more than 80 countries with a world production of 61,865,423 tons per year. Mexico ranks eighth as a producer with a contribution of 1´158,471 tons per year (FAOSTAT, 2018). The state of Guerrero is the leading producer of coconut with an annual production of 178,200 tons (SAGARPA, 2017). However, in Mexico, industrial exploitation is not oriented towards the diversification of value-added products. The main objective of the coconut industry is the production of oil (Persley, 1992). Coconut flour comes from coconut cake, which is the residue from oil and coconut milk extraction. It has been reported to be a good source of protein (up to 20% of the total protein) (Afoakwah *et al*., 2019; Mihirani *et al*., 2019; Stoin, 2016; Trinidad *et al.*, 2006; Sánchez and Ríos, 2002). Its use has been suggested to replace semolina (Sykut-Domańska *et al.*, 2020) (Figure 1 shows where the coconut cake comes from coconut milk and coconut oil extraction residue). The proteins are considered essential for the food industry due to their functional properties for food processing since they determine the texture, hardness, viscosity, absorption of water, and fat (Lad and Murthy, 2012). Therefore, the need arises to add value to the coconut cake proteins of the State of Guerrero, Mexico.

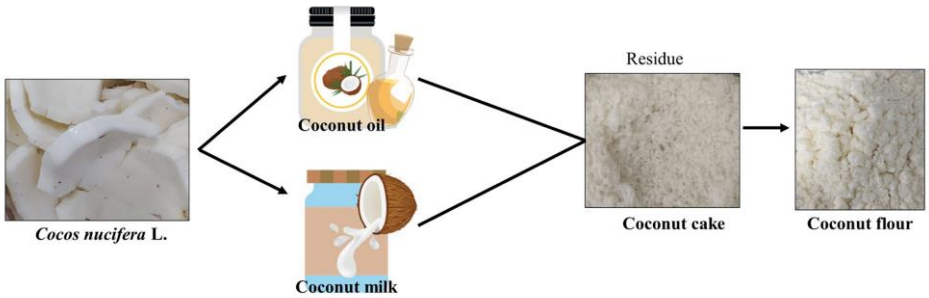

Figure 1. Shows where the coconut cake comes from (coconut milk and coconut oil extraction residue).

2. Materials and methods

2.1 Raw material

Coconut (*C. nucifera* L.) from 10 to 11 months old were collected from tall palm trees from Petatlan, Guerrero, Mexico.

2.2 Sample preparation

The coconut endosperm was cut into cubes, and it was blended with drinking water (35 ±2 °C). The coconut milk was obtained, and the coconut cake was recovered. The recovered coconut cake was placed on trays for dehydration (65 ±3 °C) for approximately 4 hours (until dry to the touch).

2.3 Defatting of coconut cake

The San Pablo-Osorio *et al.* (2019) method was followed, where a 1:10 (w/v) ratio of flour/hexane was used, stirred for 4 h at 4 °C and filtered. Finally, the dried coconut meal was stored at 4 °C until use.

2.4 Protein identification analysis

2.4.1 Protein fractionation

The proteins of the coconut cake were extracted and fractionated according to the method of Osborne (1924), based on their solubility: albumins, globulins, prolamins, and glutelins. The extraction was carried out from the sieved flour. The suspensions (1:10 w/v) were shaken for 1 h at 4 °C and centrifuged at 11,000 rpm for 20 min at 4 °C. The first suspension was in distilled water and the supernatant corresponded to the albumin fraction. Next, the pellet obtained was resuspended in 0.4 N NaCl, the supernatant was recovered (globulins). Next, the pellet was resuspended in 70% ethanol, from which the prolamins fraction was recovered. Finally, the pellet was resuspended in 0.1 N NaOH, and the supernatant represented the glutelin fraction. The fractions obtained were stored at 4 °C for later use.

2.4.2 Protein quantification

According to Bradford (1976), protein content was determined using bovine serum albumin as standard. Briefly, 100 µL of sample and 3 mL of the Bradford reagent were placed in test tubes. Then, it was gently vortexed and allowed to react for 10 min. Subsequently, 250 µL of the sample was placed on a microplate and read at 595 nm.

2.4.3 Electrophoresis in polyacrylamide gels under denaturing conditions (SDS-PAGE)

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) method. Proteins were prepared in reducing conditions with *β*-mercaptoethanol (+*β* ME) and nonreducing conditions without *β*-mercaptoethanol *(-β* ME). A total of 5 µg of protein was applied. The molecular weight of the proteins was determined by Precision Plus Protein^{TM} All Blue Protein Standards.

2.5 Techno-functional characterization of proteins

2.5.1 Protein solubility

The solubility of the proteins present in coconut cake was carried out according to Salcedo-Chávez *et al.* (2002), with modifications. First, 1:10 coconut flour was used with 0.1 N NaOH and stirred for 1 hour at room temperature. Subsequently, it was centrifuged for 10 min at 17,000 g (25 °C). Finally, the pH (3-12) was adjusted with 2 N HCl, and soluble protein was quantified by the method of Bradford (1976) method.

2.5.2 Surface hydrophobicity

The surface hydrophobicity (H_0) analysis was determined by fluorescence using 1-anilino-8-naphthalene-sulfonate (ANS) as a molecular probe according to the method of Kato and Nakai (1980) with modifications of Resendiz-Vazquez *et al.* (2017). The starting point was a protein concentration of 0.6 mg/mL.

2.5.3 Emulsifying properties

The emulsifying activity index (EAI) and the emulsifying stability index (ESI) were made by the method of Pearce and Kinsella (1978) with modifications of Rodsamran and Sothornvit (2018), using 0.5 mg/mL of the protein fractions.

2.6 Statistical analysis

Analysis of variance (ANOVA) was carried out using Statgraphics Centurion software. Significant differences were established between the means ($P<0.05$) using the LSD test. Different letters indicate significant differences. Data are the average of three independent determinations.

3. Results and Discussion

3.1 Protein identification analysis

3.1.1 Protein content

Table 1. Protein fractions of coconut cake

Protein Fraction	Protein content (%)
Albumins	2.79 ± 0.60^{c}
Globulins	28.42 ± 1.80^{b}
Prolamins	0.14 ± 0.20^{d}
Glutelins	68.65 ± 2.60^{a}

Glutelins was the majority fraction (68.65 ± 2.60%), followed by globulins (28.42 ± 1.80%) and to lesser extent albumin (2.79 ± 0.60%), while the content of prolamins was scarce (0.14 ± 0.20%) (Table 1 shows the storage protein content of the coconut cake). Studies by Kwon *et al.* (1996) reported globulins as the majority fraction (40.1%) in coconut flour from the Philippines, in the same way. Patil and Benjakul (2017) said globulins as the majority fraction in coconut endosperm from Thailand. Rodsamran and Sothornvit (2018) reported glutelins (52.06%) as the primary protein

fraction in coconut flour. The variation in protein content can be affected by the origin of the coconut and the extraction conditions used (Kwon *et al.*, 1996; Li *et al.*, 2018).

3.1.2 Protein profile

Four main polypeptides were observed in albumins (10, 15, 25, and 50 kDa), both under reducing and nonreducing conditions (Figure 2 shows the electrophoretic profile of the albumin, globulin, and glutelin fractions under reducing (a) and non-reducing (b) conditions). Li *et al.* (2018) reported three main polypeptides under non reducing and reducing conditions in coconut cake. A band with a molecular weight of 50 kDa was observed in the globulin fraction, corresponding to the majority protein, 11S globulin, or cocosin (Garcia *et al.*, 2005). Cocosin has been reported to play a prominent role in the stability of coconut milk (Tangsuphoom and Coupland, 2008). Kwon *et al*. (1996) analyzed cocosin, which under non reducing conditions, its molecular weight (MW) was approximately 50 kDa, while under reducing conditions, the acid subunit of 50 kDa and the basic subunit of 20 kDa of 11S globulins were observed. Also, proteins with MW of 16 and 22 kDa were observed, which correspond to 7S globulin. Thus, four polypeptides (50, 34, 20, and 15 kDa) were observed for the glutelin fraction.

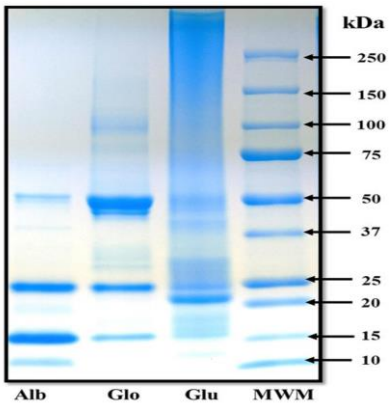

Figure 2. Electrophoretic profile of the albumin, globulin, and glutelin fractions under reducing (a) and non-reducing (b) conditions. MWS: Molecular weight standard, Alb (+), albumin (+β-ME); Glo (+), globulin (+β-ME), Glu (+); glutelin (+β-ME); Alb (–), albumin (–β-ME); Glo (–), globulin (–β-ME); Glu (–), glutelin (–β-ME). (+β-ME), with β-mercaptoethanol, (–β ME) without β-mercaptoethanol.

3.2 Techno-functional characterization of proteins

3.2.1 Protein solubility

The maximum solubility of coconut flour proteins was observed from pH 8 to 12 (between 90.81 ± 4.84 and 97.58 ± 2.87%), while the lowest solubility point was between pH 4 to 6 (1.40 ± 0.61 to 3.16 ± 1.23%). Similar results have been reported by Samson *et al.* (1971), indicating that coconut flour proteins precipitate at pH 4 and their maximum solubility at pH 12. Thus, the solubility of proteins provides us with valuable information to define the food matrix that could be incorporated.

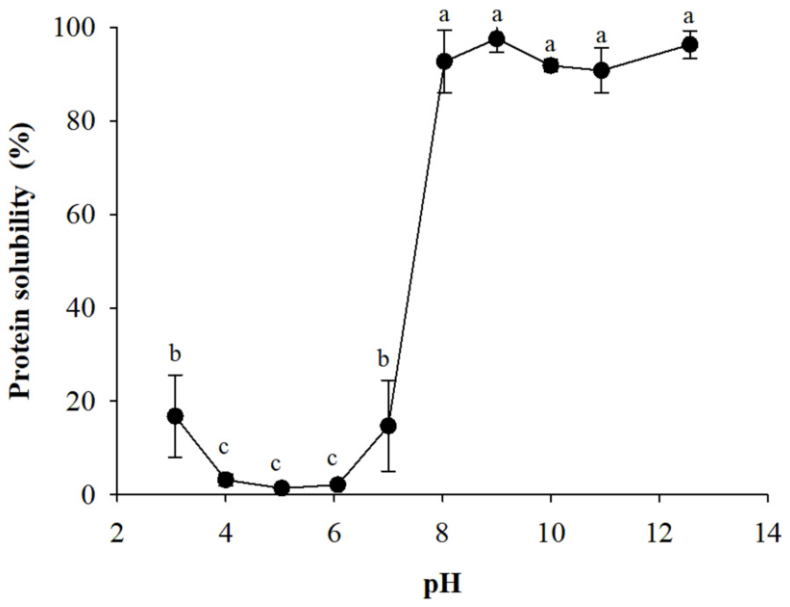

Figure 3. Solubility of coconut cake.

3.2.2 Surface hydrophobicity

Figure 4 shows the surface hydrophobicity for globulins and glutelins. Globulins showed higher H_0-ANS (304,574.33 ± 33,778.46) compared to glutelins (110,191.00 ± 9,320.21), and in the case of albumins, no H_0-ANS was detected. The property is closely associated with the functional properties

of proteins and has been related to the initial anchoring of the molecule in the oil/water interfaces (Castel, 2010).

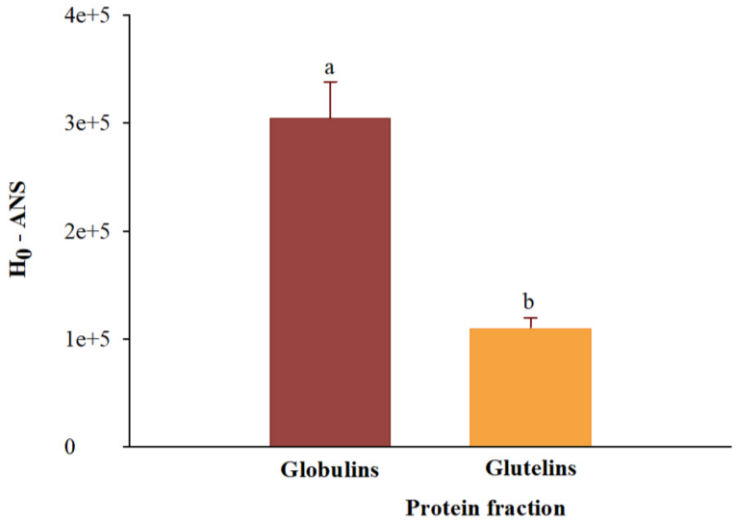

Figure 4. Surface hydrophobicity of globulins and glutelins

3.2.3 Emulsifying properties

Regarding the emulsifying properties, Figure 5 shows the emulsifying activity index, and Figure 6 shows the emulsion stability index. The fractions with the highest emulsifying activity were albumins and glutelins (11.52 ± 0.42 and 13.33 ± 1.57 m^2/g, respectively), while globulins showed the lowest activity (5.07. ± 0.37 m^2/g).

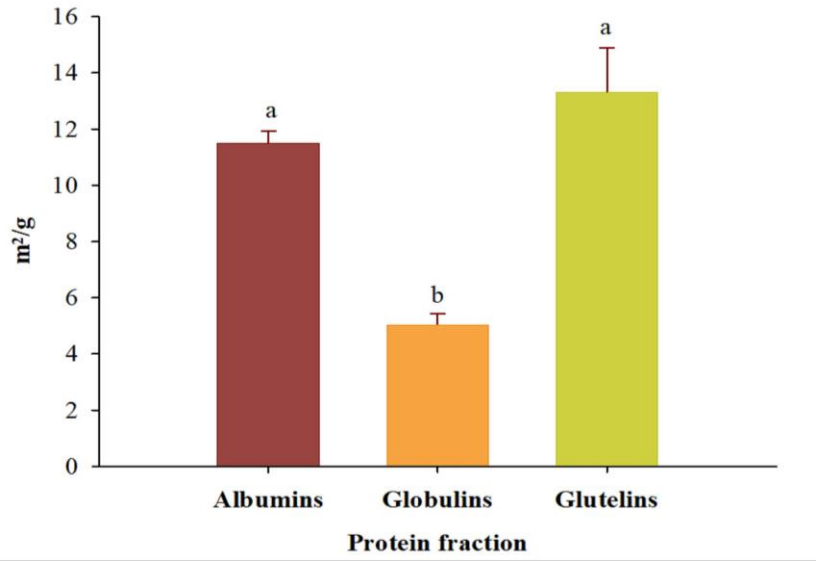

Figure 5. Shows the emulsifying activity index

However, for the emulsion stability index, no statistically significant differences to albumins, globulins, and glutelins (23.18 ± 2.79, 27.04 ± 5.44, and 21.68 ± 4.49 minutes, respectively) ($P < 0.05$). These results were like those reported by Rodsamran and Sothornvit (2018). Emulsifying properties correlate with protein solubility, surface charge, surface hydrophobicity, and molecular flexibility. Therefore, denatured proteins expose their hydrophobic groups by dissociation and partial unfolding, which increases oil-water adsorption (Soria and Villamiel 2010).

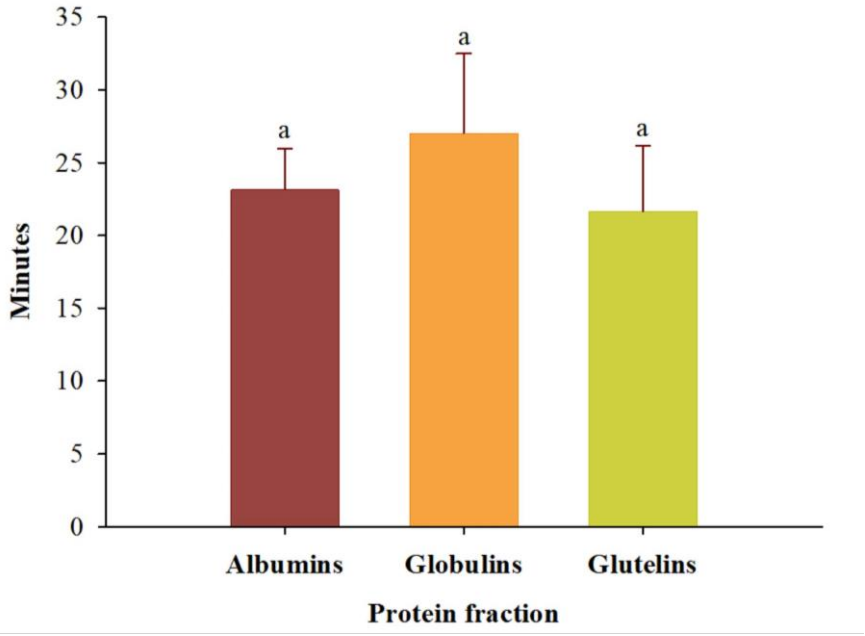

Figure 6. Shows the emulsion stability index

4. Conclusions

The main storage protein fractions of coconut cake were isolated, which presented techno-functional properties; therefore, they can be considered as an alternative to be incorporated into food matrices.

5. Acknowledgments

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Molecular characterization of bacteriophages of lactic-acid bacteria (LAB) associated with sausages spoilage

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Abstract

Lactic acid bacteria (LAB) have ecological and metabolic characteristics of economic and technological importance in food; however, LABs are also implicated in the spoilage of foods, causing unpleasant tastes, odors, acidity, and turbidity. In sausages, LABs generate economic losses by decreasing their shelf life. An alternative to treat this problem is the use of bacteriophages (bacterial viruses) since they are harmless and can contribute significantly to reducing the deterioration of these foods. The goal of this study was to characterize lytic bacteriophages associated with LAB: *Lactobacillus plantarum*, *L. paraplantarum* and *Leuconosctoc mesenteroides*.

Keywords: *LAB, sausages spoilage, bacteriophages, biological control.*

1. Introduction

Lactic acid bacteria (LAB) are a heterogenous group of Gram-positive bacteria and catalase negative. They are classified according to their morphology, fermentative pathway, growth temperature, configuration of lactic acid produced, and tolerance to high salt concentrations, and wide range of pH. The LAB´s use food carbohydrates and produce lactic acid as the main product of fermentation (Bintsis, 2018). These metabolic characteristics play an important role in a variety of fermentation processes. Currently, the genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* are considered the core group. Additionally, in recent years have proposed several new genera and the remaining group now includes the following: A*erococcus*, *Alloiococcus*, *Carnobacterium*,

Dolosigranulum, *Enterococcus*, *Globicatella*, *Lactococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Khalid, 2011; Bintsis, 2018). Currently, the main use of BAL is as a starter culture in a wide variety of fermented foods such as yogurt, cheese, vegetables, and some cereals. However, LAB as well are implicated in the spoilage of fresh meat products, causing aftertaste, off-odors, acidity, slime and turbidity, a few days following production. Generating economic losses by rejection of the product and reducing its shelf life. Food manufacturers must ensure food safety and quality by controlling microbial contamination through good hygiene practices and by limiting microbial growth (Ngoc-Du *et al.*, 2020). Moreover, additional preservation barriers have been implemented, such as: heat treatment, vacuum packaging and use of chemical preservatives. However, one common disadvantage of these techniques is that the product is also affected in its organoleptic characteristics. Therefore, in recent years, strategy has focused on the use of new tools to establish additional barriers, safe for humans and at the same time does not affect the characteristics of the finished product, and that can be used alone or in combination with the techniques described above. One such technique is the use of lytic bacteriophages to attack specific bacteria. This approach is called "bacteriophage biocontrol " or "phage biocontrol" (Moye *et al.*, 2018).

Bacteriophages or simply phages, are viruses that kill bacteria by intracellular lysis and are the most abundant organisms on the planet. They were first identified during the early part of the 20th century by Frederick Twort and Felix d'Herelle. Since they were discovered, phages have been considered an inexpensive and safe tool for bacterial control. Although therapeutic applications of phages were largely forgotten in the west after the widespread acceptance of antibiotics during the 1940s and 1950 and their use was limited to laboratories (Anderson *et al.*, 2011). Later, in the 1990s, with the increase of antibiotic resistant bacteria, phages have experienced a renewed interest. Currently, phages are considered a safe tool with multiple advantages that can be used as ecological alternatives in the

biocontrol of bacteria in medical, human, veterinary, and industrial areas (Sulakvelidze *et al.*, 2001). In a food context, use of phages as biological control offers a unique opportunity to attack pathogenic bacteria in food, without altering normal microflora and organoleptic properties (Moye *et al.*, 2018). Therefore, the main goal of our study was characterizing specific LAB-phages.

2. Methods

2.1. Bacterial strains

Strains of lactic acid bacteria belonging to the laboratory of quality and food safety of the Food Technology Unit at the CIATEJ, were used in the present study. The strains *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Leuconosctoc mesenteroides*, were previously isolated from spoilage sausage packages, identified by biochemical tests and Gram stain (Prescott *et al.*, 2002), and confirmed by matrix-assisted laser desorption/ionization- time of flight / mass spectrometry (MALDI-TOF/MS) (Martinez-Garcia, 2020).

2.2. Isolation and purification of phages

Phages were isolated from a sausage production plant, samples were collected from surfaces, water, ingredients, and finished product. Additionally, a water sample was collected from a wastewater treatment plant, both sampling sites located in Guadalajara, Jalisco, México. All samples were stored at 4 °C and processed the same day.

First, solid samples were mixed with 100 mL of sterile water. Next, 50 mL of each sample was centrifuged at 16000×*g* (F15-6x100y rotor, Thermo Scientific, Pittsburgh, PA, USA), and the supernatant was filtered through a 0.22 μm membrane (PVDF; Millipore, Bedford, MA, USA) to eliminate bacteria and/or diverse residues. The phages in the supernatants were isolated using *Lactobacillus plantarum, Lactobacillus paraplantarum*, *Leuconosctoc mesenteroides*, as receptor strains in double-layer plaque assays with MRS soft agar (De Man, Rogosa and Sharpe agar, 10 g/L peptone, 10 g/L meat extract, 5 g/L yeast extract, 20 g/L glucose, 1 mL sorbitan monoleate, 2 g/L

dipotassium phosphate, 5 g/L sodium acetate, 2 g/ ammonium citrate, 0.2 g/L magnesium sulfate, 0.05 g/L manganese sulfate, 7 g/L agar). In brief, 1 mL of each sample and 100 µL of the receptor strain in the exponential growth phase were mixed, and 4 mL of melted soft MRS agar was added. The mixture was poured onto Petri dishes with nutrient agar (15 g/L agar) and incubated at 32 °C for 48 h.

Lytic plaques were selected and incubated in 50 mL of MRS broth (supplemented with 10 mM $CaCl_2$ and inoculated with ~4×10^8 colony-forming units (CFU/mL) of each receptor strain) at 32 °C for 48 h. The cellular debris was eliminated by centrifuging the samples at 10000×g/10 min/4 °C (24-Pl Rotor, Thermo Scientific, MA, USA), and filtered through a 0.22 µm membrane (Millipore PVDF, Bedford, MA, USA).

2.3. Phage host range

The lytic activity of the purified phages was analyzed by a dot plaque assay employing 3 strains of *Leuconostoc mesenteroides*, 3 strains of *Lactobacillus paraplantarum* and 2 of *Lactobacillus plantarum* identified as previously mentioned. To assess phage activity, these strains were cultured on MRS agar until log phase and incubated with 10 µL of a phage suspension (10^8 plaque forming units [PFU]/mL) at 32 °C for 48 h (Powell *et al.*, 1986).

2.4. Genomic DNA extraction

DNA was extracted as follows: a 500 µL aliquot of each phage suspension (1×10^8/mL PFU) was treated with 100 U of DNase I (Invitrogen, Carlsbad, CA, USA), 2 µL of proteinase K (20 mg/mL) (Vivantis, Oceanside, CA, USA), and 50 µL of SDS (10%) at 56 °C for 1 h. The purified DNA was treated with phenol-chloroform-isoamyl alcohol (25:24:1 ratio), precipitated with 3 M sodium acetate and isopropanol. Next, DNA pellets were washed twice with ethanol 70% and resuspended in DNase-free water.

2.5. Genome comparison by restriction endonuclease patterns

DNAs from isolated phages was characterized by restriction fragment length polymorphism (RFLP) technique, using *AvaII*, *BamHI*, *DpnI*, *EcoRI*, *PvuI*, *EcoRV*, *HaeII* and *HhaI* restriction enzymes according with manufacturer instructions. Later, restriction patterns generated were encoded in a binary matrix and analyzed by cluster analysis with average method. Additionally, the phylogenetic tree was constructed using STATGRAPHIC Centurion software (StatPoint Inc. 2005. StatGraphics v.15.02.06. Warrenton, Virginia).

3. Results

3.1. Isolation of bacteriophages

26 bacteriophages were isolated from ground turkey samples (10 isolates), surfaces (5 isolates) and wastewater (11 isolates). Likewise, of the 26 phages, 8 were isolated with *L. mesenteroides*, 12 with *L. plantarum* and 6 with *L. paraplantarum* (Table 1).

Table 1. Total lactic-acid bacteria specific lytic phages identified and their source.

Lactic-acid bacteria receptor	Bacteriophage isolation source		
	Sausage factory		Sewage water
	Ground turkey	Surfaces	
Leuconostoc mesenteroides	4	-	4
Lactobacillus plantarum	6	2	4
Lactobacillus paraplantarum	-	3	3

3.2. Host specificity of the identified phages

In addition, the 26 isolated phages were tested using 90 LAB strains, of which, 10 phages (38.46%) were able to infect 8 of the bacteria used (8.89%), producing both clear (54 LAB, 67.5%) and opaque

plaques (26 LAB, 32.5%) (Table 2). Interestingly, all phages produce both plaque types, clear and opaque, and the lytic activity observed shows clear plaques ~1 mm in diameter without halos. The phages with greatest lytic activity were LM803 (7/8), LM601, LM603 and LM801 (6/8 each), also, the LAB strains 55 (10/10) and 56 (9/10). Furthermore, phages LM601, LM603, LM801, LM802, LM803a, LM804, LP803 and LP804, reveal a broad host range, infecting ≥ 2 LAB species (Table 2). For the next experiments, only the phages from surfaces and sewage water were used, and unfortunately, the phage Lp801 was lost, and it was not possible to recover it.

Table 2. Host range of isolated lactic-acid bacteria phages analyzed by spot test assay.

Strain	Lactic-acid bacteria	Bacteriophage										Lytic phage sensitivity
		LM6 01	LM6 03	LM6 01	LM8 02	LM8 03	LM8 03a	LM8 04	LP8 01	LP8 03	LP8 04	
44	*Leuconostoc mesenteroides*	1	1	0	1	1	1	1	1	1	1	9
48	*Leuconostoc mesenteroides*	1	0	1	1	1	0	0	0	0	0	4
55	*Lactobacillus paraplantarum*	1	1	1	1	1	1	1	1	1	1	10
56	*Lactobacillus paraplantarum*	1	1	1	0	1	1	1	1	1	1	9
62	*Lactobacillus plantarum*	1	1	1	1	1	0	0	1	1	1	8
65	*Leuconostoc mesenteroides*	1	1	1	1	1	0	0	1	0	0	6
66	*Lactobacillus paraplantarum*	0	1	1	0	1	1	0	1	1	1	7
32	*Lactobacillus plantarum*	0	0	0	0	0	0	0	1	0	0	1
Bacterial strains infected lytic phages		6	6	6	5	7	4	3	7	5	5	

0= opaque plaques (lysogenic); 1= clear plaques (lytic).

3.3. Restriction Fragment Length Polymorphism (RFLP) analysis

The phylogenetic analysis was performed in 9 lytic phages (Table 2). The phages were tested with the *AvaII, BamHI, DpnI, EcoRI, PvuI, EcoRV, HaeII* and *HhaI* restriction enzymes. The results revealed 79 distinct restriction fragments, 24 with *EcoRV* and *HaeII* restriction enzymes, and 31 with *HhaI* (Figure 1). The phylogenetic tree grouped the 9 phages into three groups. First group consisted of the phages, LM 804 (P1), LM 603 (P8) and LM 803a (P10); the second group consisted by the phages, LM 601 (P3), LP 803 (P21) and LP 804 (P22); and the third group by the phages, LM 802 (P6), LM 801 (P9) and LM 803 (P13). Additionally, the phages LP803 (P21) and LP804 (P22), both isolated with *L. plantarum*, showed the highest similarity of all phages analyzed (Figure 2).

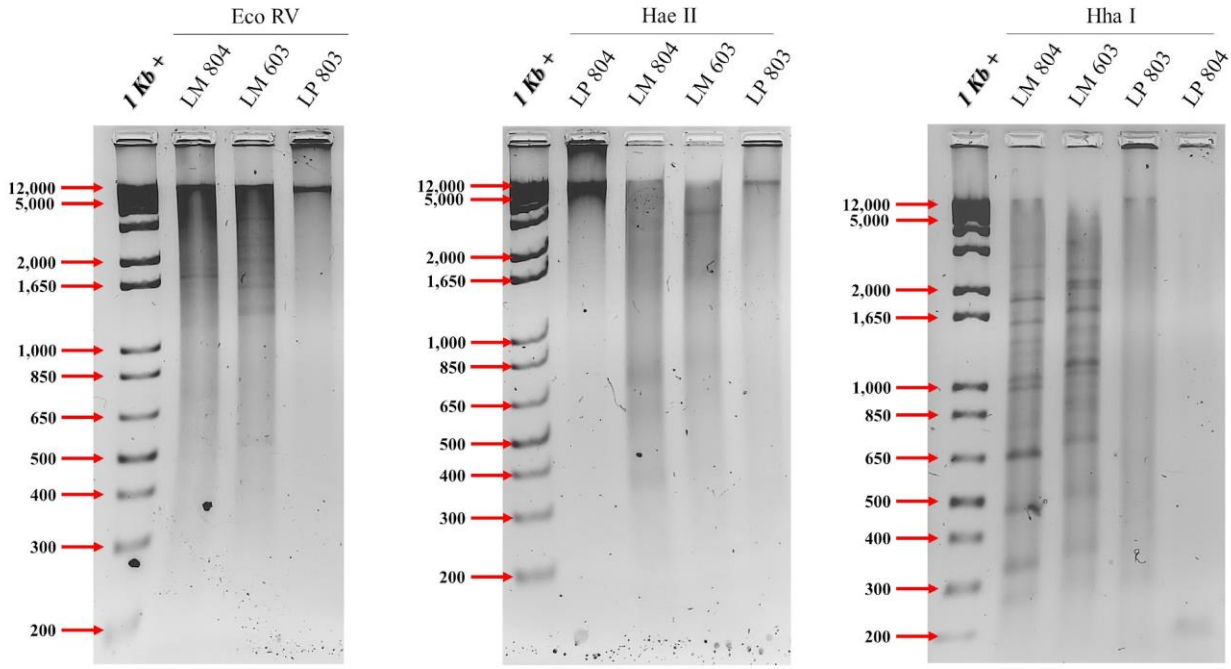

Figure 1. Diversity in the restriction fragment length polymorphism (RFLP) of viral genomes. Using restriction enzymes *EcoRV, HaeIII* and *HhaI* on DNA of the phages LM804, LM603, LP803, LP804.

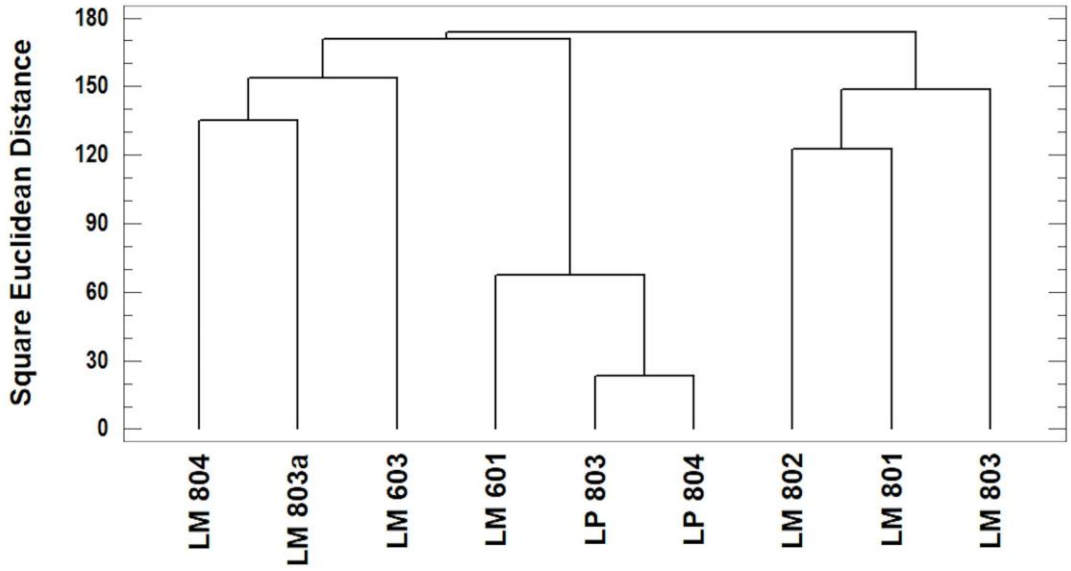

Figure 2. Cluster analysis of the 9 bacteriophages associated with lactic acid bacteria involved in the deterioration of sausages using the data of the restriction profiles of the enzymes *EcoRV*, *HaeII* and *HhaI* through the statistical procedure of the average method (unweighted pair group method with arithmetic mean, UPGMA).

4. Discussion

Bacterio(phages) as biological entities are abundant and widely distributed in the world and have great relevance in bacterial communities. In the food industry, infection of starter cultures of LAB by bacteriophage represents a serious economic problem of considerable importance worldwide. On the other hand, phages are an important tool as natural antibacterial to control food pathogens and spoilage organisms at the pre- and postharvest stages of production. However, despite important advances in biological and molecular areas (Jarvis, 1989; Endersen *et al.*, 2014), the potential use of phages for biological control of spoilage-associated LAB, has not been explored.

In the present work, nine LAB lytic phages were isolated from sewage water treatment plant and surfaces in sausage factory, moreover *L. mesenteroides* and *L. plantarum* were most sensitive to phage infection. Both strains are commonly associated with vacuum-packaged processed meat spoilage (Dykes *et al.*, 1994, 1995; Yost and Nattress, 2000; Chenoll *et al.*, 2007). To date, sixteen *Leuconostoc* phage genome sequences are available in GenBank, isolated principally from dairy

industry and sauerkraut (fermented cabbage), while the phages of *Leuconostoc mesenteroides* isolated in the present work were isolated from non-dairy related sources. Interestingly, the number of phages isolated from dairy sources is very low, and there are no reports of bacteriophage attack in dairy fermentations associated with *Leuconostoc* spp. (Endo, 2016).

Our results also reveal the presence of *L. plantarum* phages in sewage water treatment plants. Furthermore, *Lactobacillus plantarum* is a versatile LAB employed in the food industry, as starter and adjunct culture from dairy to wine fermentations (Kyrkou *et al.*, 2020). Furthermore, *L. plantarum* also have been reported as spoilage bacteria in meat products, causing defects such as souring, off-flavors, discoloration, gas production, slime production and decrease of pH (Chenoll *et al.*, 2007). Currently, more than 230 *Lactobacillus* phages have been identified, most of them belonging to the Caudovirales order (Villion and Moineau, 2009), of which 84 complete genome sequences are available in GenBank. While the first reference of *L. plantarum* phages dates to 1969, since then, several virulent and temperate phages of *L. plantarum* have been discovered, most of them belonged to the Siphoviridae family (Kyrkou *et al.*, 2019). In addition, *Lactobacillus* phages have been reported from various sources, from sewage water to vaginal environment (Villion and Moineau, 2009). Moreover, our results show the ability of these phages to tolerate extreme conditions as sewage water. In this regard, phages in this environment have been described, though little information about their population dynamics and their interaction with the microbial community has been extensively studied (Hantula *et al.*, 1991).

Interestingly, the 9 LAB phages reported here suggest that these phages have low host specificity. However, despite the high number of phages reported, the information about their host range is very poor, for example, since discovering the phages of *L. plantarum* only a few have a high level of strain specificity (Kyrkou *et al.*, 2019). In general, it is believed that most phages are only capable of infecting a narrow range of bacteria that are closely related. This is due to a combination of factors

as host binding proteins, biochemical interactions during infection, presence of related prophages or plasmids and bacteriophage-resistance mechanisms (Ross *et al.*, 2016). However, many of these reports are based on methods that may inadvertently select for narrow-host-range phages, consequently, these phages are consistently overlooked (Yu *et al.*, 2015). Thus, to confirm the host promiscuity of LAB phages, further studies are needed.

Additionally, the phylogenetic relationship of the 9 phages were explored by restriction fragment length polymorphism (RFLP) analysis. Three well defined groups were identified, the first group corresponds to *L. mesenteroides* phages LM801, LM802 y LM803, which come from the same sampling site. The second group corresponds to the *L. plantarum* phages LP803 and LP804 are genetically similar, moreover, both have the same host range, suggesting that they have a common ancestor. Surprisingly, both also have related with the *L. mesenteroides* phage LM60, which was isolated from the sausage factory. Commonly RFLPs are employed to check the small and specific variations in a sequence of double-stranded DNA. Nonetheless, various limitations should be considered, such as the amount of sample DNA, low levels of polymorphism in some species, few loci detected per assay, phage anti-restriction mechanisms, and DNA methylation. Therefore, to confirm our results, it is necessary to explore other techniques such as PFGE or PCR-RFLP.

5. Conclusion

Currently, various lactic acid bacteria phages have been described, most of them used as starter cultures in the dairy industry and in fermented products. In the present work, we report nine LAB phages isolated from sewage water and surfaces from sausage factories. Furthermore, most of the phages have the capability of infecting different LAB genera, and only LM 803 has a narrow host range of infection. Hence, broad-host-range phages are valuable resources that need to be explored in detail. Finally, the lytic feature of the phages presented here, may be employed as an alternative tool for biological control in control of spoilage-associated LAB.

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Production of biosurfactants by lactobacilli from agro-industrial waste

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Abstract

Biosurfactant (BS) production in the present study was carried out by means of four strains of lactobacilli using agro-industrial wastes as a carbon source, focusing on the formulation of a sustainable and low-cost culture media and its potential applications in the food industry. Biosurfactants produced in the formulated culture media were isolated and analyzed. The biosurfactant produced by *Lactobacillus pentosus* and extracted with phosphate buffered saline (PBS) reduced the surface tension (ST) from 70.0 mN/m to 35.0 mN/m, on the other hand the biosurfactant produced by *Lactobacillus paracasei* showed the best performance in emulsification index (EI) with 42.9%. The reduction in the cost of the culture media and the use of biodegradable raw materials opens the possibility of producing biosurfactants useful in industrial applications.

1. Introduction

One of the most used additives in the food industry are the emulsifiers, which aid to form a stable mixture of two immiscible substances (Zelman, 2017). There are two classes of surfactants, those obtained through chemical synthesis and those of biological origin called biosurfactants. The ability of biosurfactants to make emulsions must be close to the performance of the main surfactants used in the food industry, such as Tween 80, carboxymethyl cellulose, OSA starches and gums such as arab to achieve this purpose. Some of the products that contain them are mayonnaise, dressings, sweets, confectionery, bakery (Asia Pacific Industry, 2017).

The diversity of biosurfactants makes these groups of compounds attractive for a variety of industrial applications. These compounds are classified according to their structure as glycolipids, rhamnolipids, trehalolipids, sophorolipids, lipopeptides and lipoproteins, fatty acids, and phospholipids, as well as polymeric biosurfactants (Sharma *et al.*, 2016). In comparison with these, surfactants have disadvantages due to their characteristics, generally they have greater toxicity, less biodegradability, and a decrease in their performance at high temperatures and extreme pH values. In contrast, biosurfactants are easily biodegradable, their toxicity is very low, and they can be produced specifically for different applications by changing only the production conditions (Sharma and Saharan, 2014).

The main reason why these compounds are not used is due to the economic feasibility of the biotechnological process. Recently, a variety of strategies have been tested to make fermentation products more competitive, for example, using agro-industrial wastes, optimizing growing conditions and improving the recovery efficiency of the process.

This work describes the use of a culture media formulated from agro-industrial residues for the production of biosurfactants with different strains of lactobacilli, the analysis of the medium's cost, and explores future possibilities, such as the optimization of the medium already formulated by means of experimental designs, in order to further reduce costs and increase production, using lignocellulosic and dairy industry residues, improve the process of purification and recovery of the biosurfactant, as well as the characterization of its chemical structure and its application in food production. The aim of this work was to produce biosurfactants by means of lactic acid bacteria, using agro-industrial residues as a source of carbon.

2. Materials and Methods

2.1 Microorganisms

Lactobacillus casei NRRL B-1255, *Lactobacillus paracasei* NRRL B-1913, *Lactobacillus pentosus* NRRL B-227, *Lactobacillus plantarum*. The known structural composition of the biosurfactants produced by *L. casei* and *L. paracasei* is glycoprotein (Golek *et al.*, 2009) and glycolipids (Sharma and Saharan, 2014), corresponding to *L. pentosus* is from glycolipids (Vecino *et al.*, 2014) and glycolipopeptides (Vecino *et al.*, 2015) and that of *L. plantarum* is from glycolipid (Sauvageau *et al.*, 2012) and glycoprotein (Madhu and Prapulla, 2014).

2.2 Preparation of inocula and culture media

All lactobacillus strains are preserved in Petri dishes with Man, Rogosa and Sharpe Agar (MRS) medium, stored at 4 °C. Cultures in MRS Agar medium were used to inoculate test tubes with MRS broth, which were incubated at 37 °C for 12-18 h. MRS broth and EEFD media, formulated using agro-industrial residues and lactose, glucose, sodium acetate, NH_4Cl, NaCl, KH_2PO_4, $MnSO_4$, $MgSO_4 \cdot 7H_2O$, $FeSO_4$, peptone, yeast extract, etc. The EEFD media was sterilized by autoclaving at 120 °C for 15 min.

2.3 Growing conditions

Experiments were performed in 300 mL beakers, at 30 °C, 150 rpm and pH 7, all experiments were carried out in triplicate.

2.4 Extraction of the biosurfactant

The fermentation was centrifuged at 2800 rpm for 15 min. PBS (Phosphate-buffered saline, 1x, pH 7.4) was used for extraction. Required components to prepare 1 L of PBS: NaCl (8 g, 0.137 M), KCl (200 mg, 0.0027 M), Na_2HPO_4 (1.44 g, 0.01 M), KH_2PO_4 (245 mg, 0.0018 M) (ATT Bioquest, 2020).

2.5 Emulsification index

The emulsification index (EI) was obtained for the cell-free supernatant and the biomass pellet, adding 2 mL of the sample to be analyzed and 2 mL of soybean, canola and corn oil in a test tube that was homogenized in a vortex for 2 min. The mixtures were left in repose for 24 hours, then the total height, the height of the emulsion and the aqueous phase were measured. The index was calculated with equation 1 (Amodu *et al.*, 2014).

$$EI = \frac{Total\ height\ of\ the\ emulsion}{Total\ height\ of\ the\ emulsion + aqueous\ phase} \times 100 \quad \text{(Eq. 1)}$$

2.6 Emulsification index for *L. pentosus* y *L. plantarum*

To have a standard, an experiment was carried out to measure the emulsification index of a Tween 80 suspension with NaCl (M concentration), the MRS broth culture media and the EEFD, adding 2 mL of the sample and 2 mL of corn oil in a test tube which was homogenized in a vortex for 2 min. The mixture was left to stand for 24 h. The index was calculated with Equation 1.

2.7 Tensiometry

The surface tension of cell-free supernatant and the biosurfactant extracted with PBS was measured by the Du Noüy method with a tensiometer of Du Noüy and a platinum ring (1.9 cm) (Phywe Systeme GmbH, 1998). For the test, 1 mL of the sample was added to 39 mL of distilled water in a beaker (50 mL), the mixture was stirred manually for 2 min, then it was introduced into the Du Noüy tensiometer for the respective measurement.

3. Results and Discussion

3.1 Emulsification index

It can be observed that all three strains produced biosurfactants, with the highest values in the emulsification index for *L. pentosus* (42.2%) and *L. casei* (40.5%) strains in the tests with corn oil, *L. paracasei* has the highest performance with soybean oil (42.9%), while the lowest percentages of emulsification index were obtained with canola oil. It is worth mentioning that the emulsification index measurement for MRS broth and EEFD media was performed with corn oil, both producing emulsification of 37.2% and 32.6% respectively. The results obtained of the emulsification index tests are depicted in Figure 1.

An interesting point to note is that the percentage difference between the rate of emulsification of Tween 80 and the supernatant of *L. pentosus* in the medium EEFD, is 2.1%, a performance in its emulsifying activity very close, so you could expect an emulsification rate equal to or greater than that of Tween 80 for the purified biosurfactant.

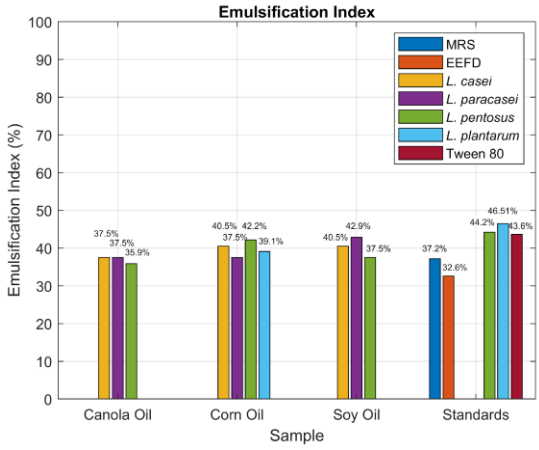

Figure 1. Production of biosurfactants by lactobacilli from agro-industrial waste.

3.2 Tensiometry

The standard range for considering a biosurfactant to have good performance in reducing the surface tension of water is between 72.4-40.8 mN/m (Sharma and Saharan, 2016). Tween 80, one of the most used surfactants in the food industry, can reduce the water surface tension to 37.96 mN/m (Shrinivas *et al.*, 2007), for the tests with the cell-free supernatant a reduction of the surface tension was obtained in a range between 44-39 mN/m and for the biosurfactant extracted with PBS, values in a range between 37-35 mN/m, with the best performance for the biosurfactant extracted with PBS from the *L. pentosus* and *L. casei* strains, both with 35.0 mN/m, this value is already below the standard range, so we can consider that it has a higher performance than the standard e and very close to the value of Tween 80. It is important to observe that the performance of the pure biosurfactant has not been measured, so it is possible that its analysis shows a higher reduction of the surface tension, compared to the data obtained in this experiment. The results obtained of the tensiometry tests are depicted in Figure 2.

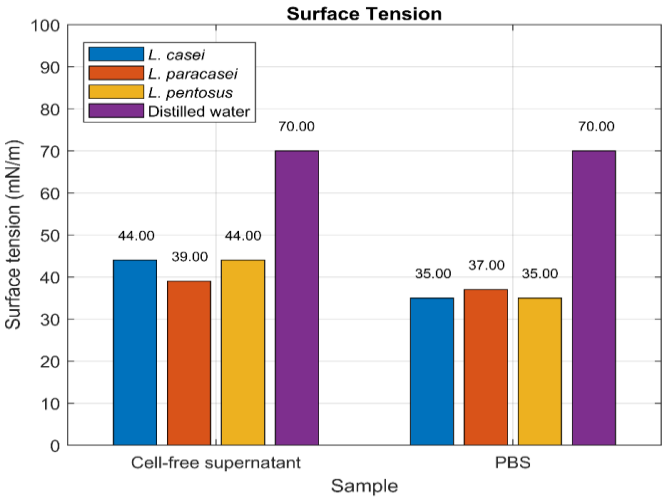

Figure 2. Surface tension.

3.3 Analysis of the cost of the culture media

The cost in Mexico of a MRS broth unit is $1,949.00 MXN (Merck, 2020); due to its high cost its use in the production of biosurfactants on an industrial scale is not feasible, therefore it is important to obtain alternative sources that provide the nutritional requirements of the producing microorganisms at a low cost. The medium formulated EEFD, satisfies the nutritional requirements of the studied microorganisms, with a cost of 12.14% (Table 2 and Table 3) with respect to the MRS broth, and allows the production of bioemulsifiers in a similar way to the medium of reference, reason why it is possible to think about its future industrial application.

Table 2. Cost per 100 L of MRS and EEFD media

MRS (MXN)	EEFD (MXN)
$22,218.60	$2,698.13

Table 3. Cost per component for 100 L of EEFD medium

Component	Cost (MXN)
Lactose	$377.11
Glucose	$6.38
CH_3COONa	$477.10
NH_4Cl	$18.46
NaCl	$5.63
KH_2PO_4	$282.40
$MnSO_4$	$0.11
$MgSO_4 \cdot 7H_2O$	$0.63
$FeSO_4$	$6.50
Peptone	$793.49
Meat extract	$694.59
Yeast Extract	$35.73

3.4 Future work

3.4.1 Use of different types of agro-industrial waste

The use of agro-industrial waste not only reduces production costs, but it also reduces the polluting emissions generated in the production of chemical surfactants, promotes the circular economy and sustainability in the processes of the food industry. Exploring the effect of different types of agro-industrial wastes on the growth of microorganisms and the production of biosurfactants would allow for cheaper culture media formulations, an optimization in the performance of biosurfactants and an economic impact on the agricultural and livestock industry that provides its wastes for the production chain. Some of the proposed wastes come from the dairy, tequila, and agricultural industries (Córdoba *et al*, 2013).

Perform an experimental design to obtain the best combination of nutrients to ensure the growth of the microorganism and the production of biosurfactants.

3.4.2 Biosurfactant purification and recovery

Some of the separation methods proposed for the extraction of biosurfactants are centrifugation-dialysis and extraction with different solvents such as ethyl acetate, etc. Another proposal for the purification process is the method of recovery of biosurfactants from corn liquor. The corn washing liquors are submitted to a dilution until their critical micellar concentration (CMC), once they are solubilized in water they are submitted to an extraction with organic solvents (ethyl acetate; trichloroethylene (TCE); tributyl phosphate (TBP); methyl butyl ether (MTBE); isoamyl alcohol; chloroform; dichloromethane; hexane; heptane; xylene), then the aqueous phase is separated from the organic phase, the aqueous phase does not have biosurfactants and can be used as a nutritional component in the fermentation process, the organic phase is subjected to distillation in order to evaporate the solvents and obtain the biosurfactant (Moldes *et al.*, 2014).

3.4.3 Characterization tests

Biosurfactants are complex molecules, described as multicomponent mixtures of proteins, polysaccharides, phosphate groups, among others, whose structure has not been completely elucidated. The main structural components of biosurfactants from *L. casei*, *L. paracasei*, *L. pentosus* and *L. plantarum* can be analyzed by a set of characterization techniques. The characterization tests for the fermentation products are FTIR (Fourier-Transform Infrared spectroscopy), RMN (Nuclear Magnetic Resonance spectroscopy), HPLC (High Performance Liquid Chromatography), CG-MS (Gas Chromatography-Mass Spectrometry). Their joint analysis would allow us to know their structure and even whether the microorganisms of interest produce one or several types of biosurfactants with the same substrate (Rosas-Galván, 2018).

3.4.4 Food applications

The purified biosurfactants will be tested on different types of foods such as dressing and sauces (mayonnaise-like products and dressings), ice cream and special emulsions (Norn, 2015).

4. Conclusions

The culture media formulated with glucose and lactose allows the satisfactory growth of the strains, observing in *L. pentosus* and *L. casei* the best results in reducing the surface tension, while the best performance in the emulsification index was for *L. pentosus* and *L. plantarum* with corn oil and *L. paracasei* with soybean oil.

As surface tension is reduced in the samples of the cell-free supernatant and the biomass pellet, we can infer the presence of biosurfactant attached to cells, in addition to that excreted by the lactobacilli, therefore, the extraction and concentration of the biosurfactant should be done for both phases.

The emulsification index in corn oil (42.2%) and the reduction of surface tension (35.0 mN/m) for the biosurfactant extracted with PBS from *L. pentosus* have values very close to the performance of

Tween 80 (EI: 43.6%; ST:37.9 mN/m), which makes it an excellent candidate for testing as a substitute in food products containing Tween 80 in its formulation (desserts, ice cream, sauces, dairy products, etc.).

The reduction in production costs provides areas of opportunity in industrial and research applications, allowing biosurfactants to be a competitive option in the emulsifier market and at the same time promoting the development of sustainable processes and the transition to a circular economy. We thank PhD Martín Porras Godinez from FES Cuautitlán for his support in the tensiometry analysis.

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Comparative study of physicochemical and nutraceutical properties of Huitlacoche (*Ustilago maydis*) organically grown vs. commercial product

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Abstract

Huitlacoche (*U. maydis*) is a fungus known since the pre-Columbian stage belonging to the group of basidiomycetes, this food has antiparasitic, immunostimulant, antioxidant, anti-tumor properties and contains essential nutrients such as Lysine and Omega 3. In this work a comparative analysis between commercial huitlacoche and organically grown product was carried out, studying value parameters such as: protein, ash, moisture, fiber, and fat content, as well as nutraceutical properties (amino acids and antioxidant power). The results show that the product obtained by cultivation with organic fertilization protocols achieved significant increases in the quality and nutritional parameters of the studied product, obtaining for protein a value of 14.75%, amount of ash 5.78%, 11.06% fiber and 1.78% fat. Regarding amino acids, we have hydrolysate factors corresponding to: Aspartic Acid (3.19 $mg_{Aspartic\ Acid}/g_{protein}$), Glutamic Acid (2.96 $mg_{Glutamic\ Acid}/g_{protein}$), Alanine (1.06 $mg_{Alanine}/g_{protein}$), Leucine (1.28 $mg_{Leucine}/g_{protein}$), Lysine (0.85 $mg_{Lysine}/g_{protein}$); respect to the

antioxidant capacity, a maximum value of 0.068 mM of Trolox was obtained. Similarly for the proper development of the product of interest it is necessary to have adequate humidity conditions. Likewise, the statistical analysis used (Tukey) gives certainty to the results obtained in this experimental work.

Keywords: *Huitlacoche, organically grown product, commercial product, physicochemical properties, nutraceutical properties.*

1. Introduction

The huitlacoche (*Ustilago maydis*) that occurs naturally, in the fields, is collected and sold in bulk in local markets; Due to the delicacy that this product acquires once cooked, it has come to be called the "Aztec caviar", this dish has peculiar properties of flavor, aroma and particular organoleptic characteristics (Ruiz-Herrera *et al.*, 1998; Ruiz-Herrera *et al.*, 2008). In this sense we can speak of the flavor of huitlacoche, in the same way that we refer to the flavor of chocolate, strawberry, vanilla, etc. that is, as a classic flavor (Lizárraga-Guerra *et al.*, 1997). Recently, studies have been carried out to know the bromatological composition of huitlacoche, which have shown that it has a higher protein content than corn and other foods, in addition to having antiparasitic, immunostimulant, antioxidant, antitumor properties, among others; the compounds that impart these properties are: fatty acids, terpenoids, phenolic compounds among others, for which it can be attributed a nutraceutical potential that gives it added value (Beas *et al.*, 2011). The pathogenicity of this species is strictly coupled to the sexual development of the fungus, in such a way that the presence of two haploid cells of a complementary mating type is required. Once these sexually compatible haploid cells are close enough, chemical messenger media recognition reactions take place, these being the pheromone and the pheromone receptor. This will cause the fusion of two strains of complementary sex, thus carrying out the mating reaction. Once the cells have successfully mated, a new form of growth known as hypha is generated and whose main characteristics are two: I) it is a dikaryotic cell

(presence of two unfused nuclei) and II) it is the infective form of the fungus (Brefort *et al.*, 2009).

Global food trends in recent years indicate greater consumer interest in certain foods that, in addition to their nutritional value, provide benefits to the physiological functions of the human body (Astiasarán and Martínez, 1999). The term Functional Food refers to those processed foods which contain ingredients that perform a specific function in the physiological functions of the human body, beyond their nutritional content (Arai, 1996). Some of the main functions are those related to optimal growth and development, the function of the cardiovascular system, antioxidants, xenobiotic metabolism, the gastrointestinal system, among others (Palou and Serra, 2000).

2. Materials and methods

2.1 Sample location

For this work, samples of huitlacoche collected in the Serrano and Márquez community, located in the north of the town of Irapuato, Guanajuato, were used. Such samples come from maize grown under the conditions shown (see Table 1).

Table 1. Data on conditions and origin of the samples collected for analysis.

	Crops	Condition	Seed type
1	M 1	Conventional	Common
2	M 2	Organic	Common
3	M3	Conventional	Improved
4	M4	Organic	Improved

2.2 Freeze drying of samples

The samples were identified and stored frozen at -20 ºC in the Microbiology laboratory of the Life Sciences Division, Campus Irapuato. The next day after the collection, work was done on separating the tumors from the ears, the tumors were weighed, the weight was recorded, poly-paper bags were stored, and they were again frozen. From the samples that were frozen at -80 ºC, 250 g of tumor

were taken, which were macerated in sterile pistil crucibles that at the time of maceration were kept frozen by means of liquid nitrogen to prevent the tumors from being beaten and thus achieve the objective of this technique, which was to obtain a fine flour from huitlacoche.

2.3 Determination of the chemical composition

The Kjeldahl method was used, acid digestion (sulfuric acid) of the sample and then an alkaline distillation (sodium hydroxide), using 6.25 as a conversion factor from nitrogen to protein, (Method 954.01).

To determine the ash content in the huitlacoche and corn samples, Method 923.03 was used, which is the inorganic residue resulting from incineration at 550 °C until the total loss of organic matter for 4 h.

The fiber and fat content were obtained by the acid-alkaline digestion method (method 962.09) and by the Soxhlet method, by means of an extraction of the total free fat with hexane, (Method 920.34), respectively.

The following protocol was carried out for the determination of amino acids: 1 g of lyophilized sample was taken, and 5 mL of deionized water were added, in a 50 mL falcon tube, it was shaken in vortex to later add 5 mL of 100% acetonitrile and vortex again. It was left to rest for 2 h in ice and subsequently it was centrifuged for 15 min at 3000 rpm, the supernatant was removed, frozen with liquid nitrogen and lyophilized; The lyophilized sample was resuspended in 300 µL of MeOH [100%], confining it in 3 tubes, 100 µL each, allowed to dry at 4 °C and derivatized with PITC (Phenyl isothiocyanate) to finally resuspend it in 25 mL of phosphate sample buffer (pH 7.5) and inject 4 µL into the HPLC (Agilent Technology 1200).

For the determination of the antioxidant capacity, the working ABTS substrate solution was prepared by adding 25 µL of 3% hydrogen peroxide solution (catalog number 323381) to 10 mL of ABTS substrate solution. 100 µg of tissue/0.5 mL of assay buffer were weighed and then centrifuged at

1200 rpm for 15 min at 4 ºC; 10 µL of a Trolox standard and 20 µL of working myoglobin solution were added; 10 µL of sample test and 20 µL of working myoglobin solution were added separately; 10 µL of test sample and 20 µL of working myoglobin solution were added separately, 150 µL of working ABTS substrate solution was added to each test, then it was incubated for 5 min at room temperature and 100 µL of stop solution (catalog number S3446) to each test; before using the stop solution, warm to room temperature and mix until homogenized. Finally, absorbance at 405 nm was recorded using a plate reader (Synergy 2 Biotek).

3. Results

3.1 Amount of protein (%)

Figure 1 shows the results obtained in terms of the amount of protein for the different samples studied, in this sense the samples are in an interval between 5% and 17%, with M3 as the best sample , which corresponds to the seed improved in conventional treatment. The results indicate that the samples M2, M3 and M4 are within the appropriate range in terms of protein quantity, which goes from 11.5% to 16.4% (Méndez-López *et al.*, 2019). This indicates that the best results are obtained when using improved seed for both conventional and organic agriculture.

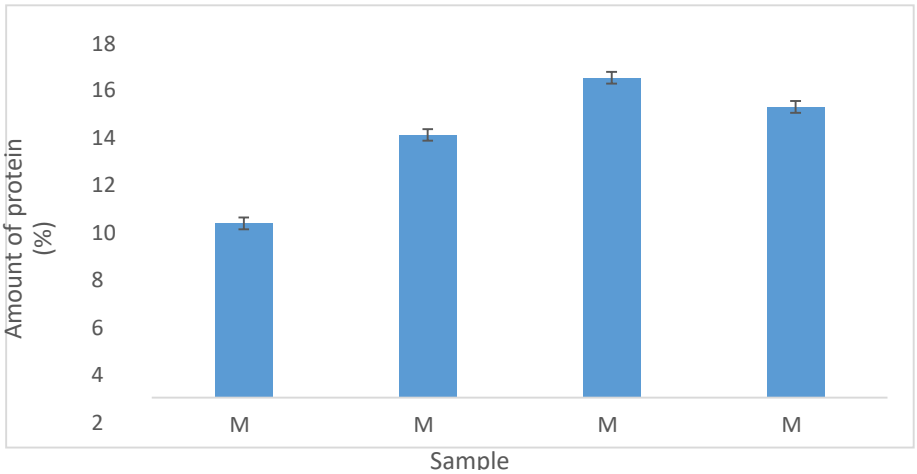

Figure 1. Amount of protein (%) in huitlacoche in various samples.

3.2 Ash amount (%)

Figure 2 shows the results obtained for ash for the different samples studied, in this sense the samples are in an interval between 3.7% and 5.8%, having as the best sample M3 and very close to this M2, which corresponds to improved seed in conventional treatment and creole seed with organic treatment. The results indicate that samples M2, M3 and M4 are above 5% ash, which is indicative that when carrying out any of the modifications (seed improvement or organic treatment) this parameter is improved.

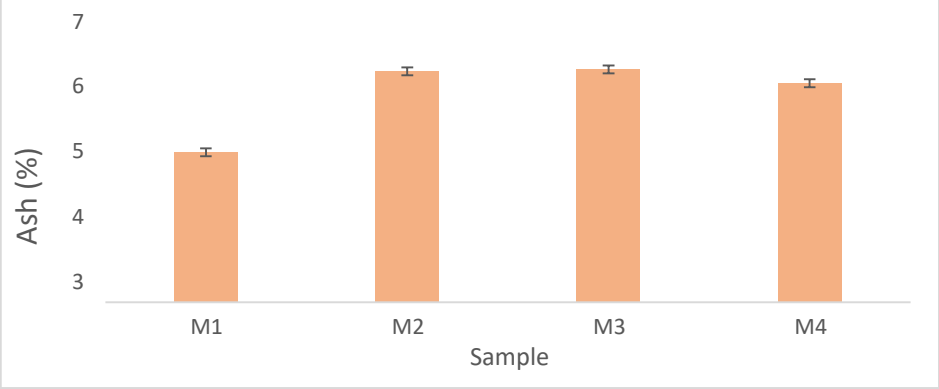

Figure 2. Amount of ash (%) in huitlacoche in various samples.

3.3 Fiber quantity (%)

Figure 3 shows the results obtained for fiber for the different samples studied, in this sense the samples are in an interval between 5% and 11.1%, having as the best sample M2 corresponding to Creole seed with organic treatment, followed by M3 and M4. The results indicate that the samples M2, M3 and M4 are within the appropriate range in terms of protein quantity, which goes from 8.6% to 15.5% (Beas *et al.*, 2011). This indicates that the best ones are obtained when applying organic treatment and / or using improved seed.

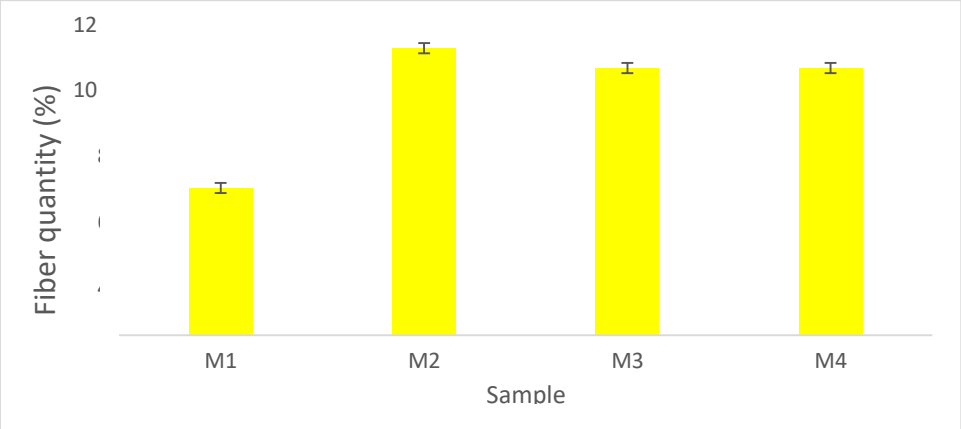

Figure 3. Amount of fiber in huitlacoche in various samples.

3.4 Amount of fat (%)

Figure 4 shows the results obtained corresponding to the amount of fat (%) for the different samples studied, in this sense the samples are in an interval between 1.3% and 1.8%, having M2 corresponding to the Creole seed with organic treatment as the sample with the highest content, followed by M3 and M4. The results indicate that the samples M2, M3 and M4 are above 1.4% of fat content, which is indicative that when carrying out any of the modifications (seed improvement or organic treatment) this parameter increases.

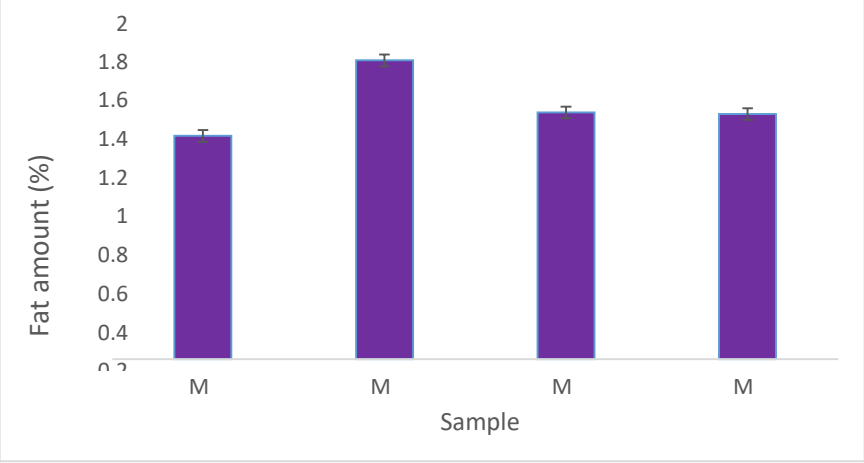

Figure 4. Amount of fat (%) in huitlacoche in various samples.

3.5 Amino acid content

Table 2 shows the amount of some amino acids for the sample that yielded the best value of these, these data indicate that the sample M3 obtained the best values in Aspartic Acid (3.19 $mg_{Aspartic\ Acid}/g_{protein}$), Glutamic Acid (2.96 $mg_{Glutamic\ Acid}/g_{protein}$), Leucine (1.28 $mg_{Leucine}/g_{protein}$) and Lysine (0.85 $mg_{Lysine}/g_{protein}$). Similarly, the M4 sample obtained the best Alanine value. (1.06 $mg_{Alanine}/g_{protein}$). In the same way as in the previous cases, the best results for this parameter were obtained in cases where there was some modification, in this case for seed improved with both conventional and organic treatment.

Table 2. Amino Acid Content in Huitlacoche samples

Sample	Amino acid
M3	Aspartic Acid (3.19 $mg_{Aspartic\ Acid}/g_{protein}$)
M3	Glutamic Acid (2.96 $mg_{Glutamic\ Acid}/g_{protein}$)
M3	Leucine (1.28 $mg_{Leucine}/g_{protein}$)
M3	Lysine (0.85 $mg_{Lysine}/g_{protein}$)
M4	Alanine (1.06 $mg_{Alanine}/g_{protein}$)

3.6 Antioxidant Capacity (mM of Trolox)

Figure 5 shows the values obtained for the antioxidant capacity (mM of Trolox) for the different samples studied, in this sense the samples are in an interval between 0.02 mM of Trolox and 0.07 mM of Trolox , having M2 corresponding to the Creole seed with organic treatment as the sample with the highest content followed by M4. The results indicate that when carrying out an organic treatment, the antioxidant capacity of huitlacoche is favored and therefore its nutritional benefits are increased.

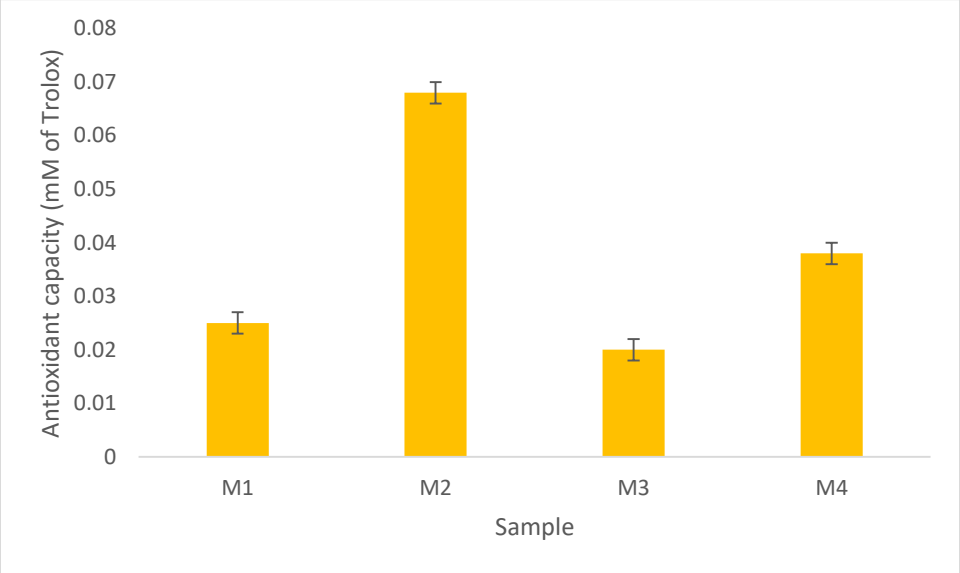

Figure 5. Antioxidant capacity (mM of Trolox) in various samples

4. Conclusions

This work presents the effect on nutritional and nutraceutical parameters of huitlacoche when produced conventionally and organically, in this sense the results show that the best results in the amount of protein, amount of ash and amino acids were obtained in the case were used improved seed grown conventionally (M3), likewise for fiber, the amount of fat and the antioxidant capacity the best values were obtained for the Creole seed grown organically (M2). In this sense, it can be observed that the evolution of nutritional parameters in Huitlacoche is carried out in a differentiated way, having in common that when using an improvement in seed or an organic crop these properties are favored, the above can be elucidated through the data shown, which indicate that the sample with lower values is the one that did not use improved seed and was cultivated conventionally. In support of the above, the statistical analysis showed a significant difference between the various parameters studied; These results are encouraging as it contributes to the study and development of sustainable alternatives for cultivation and improvement of this food product typical of Mexican cuisine.

5. Acknowledgments

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Sorghum (*Sorghum bicolor* L. Moench): chemical composition and its health benefits

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Abstract

Sorghum is one of the most widely produced cereals in the world after wheat, rice, maize, and barley. It is a high-yield, low-cost crop with high resistance to drought, which is why it is considered a fundamental ingredient in semi-arid and arid regions of the world, especially in a large population of Africa and Asia where it is considered a staple food. Sorghum is mainly made up of carbohydrates, followed by proteins, lipids, crude fiber, and ash; it also provides minerals such as phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn); vitamins A, D, E and B complex; In this regard, it should be said that it is also a rich source of dietary fiber and antioxidants such as phenolic compounds. According to its nutritional and functional characteristics, it is a product that can compete with the most consumed cereals, such as wheat and maize. However, only an average of 35% is destined for human consumption, the rest is destined for animal feed, industrial products, and ethanol production. Although its use has been limited, sorghum is a crop that is becoming increasingly important in the developed world. It has been sought to promote its consumption in foods such as bread, cakes, cookies, tortillas, pasta, beer; as well as the incorporation of sorghum to products made with flours from other cereals since it increases the nutritional profile of the final product.

Besides, as it is a gluten-free grain, it constitutes an option of products for patients with celiac disease who are intolerant to these proteins that are found in other cereals such as wheat, barley, and rye. For its nutritional profile, sorghum has important health benefits, such as a reduction in cardiovascular diseases and type II diabetes; decreased risk of thrombosis and plasma cholesterol; antioxidant activity, and anticancer properties. The analysis of this review focuses on the valorization of the sorghum grain as a source of nutritive and functional compounds that have a wide variety of health benefits and that can be considered as an alternative to the lack of food and malnutrition suffered in the world.

Keywords: *amino acids, lipids, fiber, vitamins, minerals, antioxidants*.

1. Introduction

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most important cereal in the world after wheat, rice, maize, and barley. It is considered as a potential alternative to produce food-oriented to human nutrition, due to its high resistance to drought in agro-ecological zones with scarce rainfall, where the production of other cereals is inadequate (López *et al.*, 2011). It is a fundamental ingredient in semiarid (Ebadi *et al.*, 2011) and arid regions of the world, especially in a large population in Africa, where it is considered a staple food. Likewise, sorghum is a cereal that is consumed by the poor masses of many other countries such as India (Iyabo, Ibiyinka, and Deola, 2018).

In the world hunger and malnutrition represent serious problems, in some countries the production of food is insufficient (García-Casal, 2007); a good alternative could be the cultivation of sorghum, which has been identified as an ideal substitute for maize, due to its adaptive agronomic characteristics that make it ideal for cultivation in different environmental conditions (Mabelebele *et al.*, 2015). There is a wide variety of colors in sorghum grains, ranging from white, lemon yellow, red, and black (Dykes *et al.*, 2011). It is a high-yield, low-cost cereal with potential health benefits,

such as slow starch digestibility, reduction of cardiovascular disease, antioxidant activity, and anticancer properties (Nguyen *et al.*, 2015). In this regard, regular consumption of sorghum grain can reduce the risk of cancer and type II diabetes (Xiong *et al.*, 2019). Despite its low cost and easy production, global consumption of sorghum remains low relative to wheat and maize. Currently, new opportunities have arisen for the use of sorghum as a raw material for human consumption, due to its environmental conservation and the growing interest in healthy and functional foods (Cayres *et al.*, 2020). Sorghum has higher energy compared to other grains such as wheat, rice, cassava, etc. and it is rich in protein, complex carbohydrates, and fat. Recently, the consumption of sorghum has increased due to its rich nutritional profile and that it is also a gluten-free grain, for this last quality has been considered very important as an alternative food for people suffering from celiac disease or wheat allergy (Sharanagat *et al.*, 2019).

The content of this review emphasizes the importance of the sorghum grain in terms of its chemical composition, several of these compounds have been shown to possess certain biological activity that contributes to various benefits on human health, qualities that make it an excellent functional ingredient in the food.

2. Sorghum uses and applications

Of the group of cereals, sorghum is one of the most cultivated in the world. Used especially for animal feed, industrial products, and biomass, approximately 35% is destined for human consumption. Its particular use as a staple food is in parts of East and South Africa, Latin America, China, and India (Alfieri *et al.*, 2017). Sorghum is very resistant to drought compared to other cereals, it has the advantage of growing under difficult conditions such as infertile soils and excessive heat, circumstances that are not suitable for maize or wheat production (Kadri *et al.*, 2017). In Africa and Asia, sorghum grains of different colors are used for human consumption in the form of bread, fermented or unfermented porridges, couscous, and fried products. In the United States, South

America, and Australia, sorghum is used essentially for animal feed and ethanol production, a smaller quantity of white sorghum is used in the preparation of snacks and gluten-free foods (Taleon *et al.*, 2012).

Sorghum is an increasingly important crop in the developed world, new ways have emerged for its use as human food, it seeks to promote its consumption by considering some technological and nutritional components on foods such as bread, cakes, cookies, tortillas, snacks, and noodles (López-Contreras *et al.*, 2015), or an option of cereals or products for patients with celiac disease who suffer from symptoms related to an immune reaction to gluten proteins which are found in all *Triticum* species and others cereals such as barley and rye (Pontieri *et al.*, 2016). Another alternative includes the production of food for children with germinated sorghum flour, it has been proven that the germination process helps improve the digestibility and nutritional characteristics of the grain by reducing certain anti-nutritional factors such as tannins (López *et al.*, 2011). One more application has been pasta made of a mixture of wheat flour and sorghum. The incorporation of sorghum (5-15%) to bread made with wheat flour increases the nutritional profile of the final product in terms of fiber, fat, carbohydrates, energy value, minerals (ashes) such as Fe, P, Mn, K, Na, and Zn (Surco and Alvarado, 2010). During the last years, the demand for sorghum in the beer production industry has increased (Momanyi, Owino, and Makokha, 2020), it has great importance as a raw material in the production of lager beer and can also be used as raw grains, semolina, or malted material to produce gluten-free beer and other fermented beverages. Lager beer made from sorghum malt can be considered good quality like barley (Singh, Sharma, and Singh, 2017).

3. Chemical composition and nutritional value of sorghum

Sorghum is a product that can easily compete with the most consumed cereals, such as wheat and maize, due to its nutritional and functional characteristics (Salazar-López *et al.*, 2018). Nutritionally, sorghum grain is made up of macronutrients such as carbohydrates, followed by proteins, lipids, crude

fiber, and ashes (Palacios *et al.*, 2020); it also provides micronutrients such as vitamins B complex and minerals such as potassium and phosphorus (Figure 1). Its chemical composition differs between the varieties of the crop. The 100 g content of sorghum provides around 400 calories, like maize and wheat, but with a higher amount of resistant starch (Oseguera-Toledo *et al.*, 2020). Table 1 shows the main nutritional components of the sorghum grain (FAO, 2002a; FAO and WHO, 2017; Palacios *et al.*, 2020; Oseguera-Toledo *et al.*, 2020; Motlhaodi *et al.*, 2018; Althwab *et al.*, 2015).

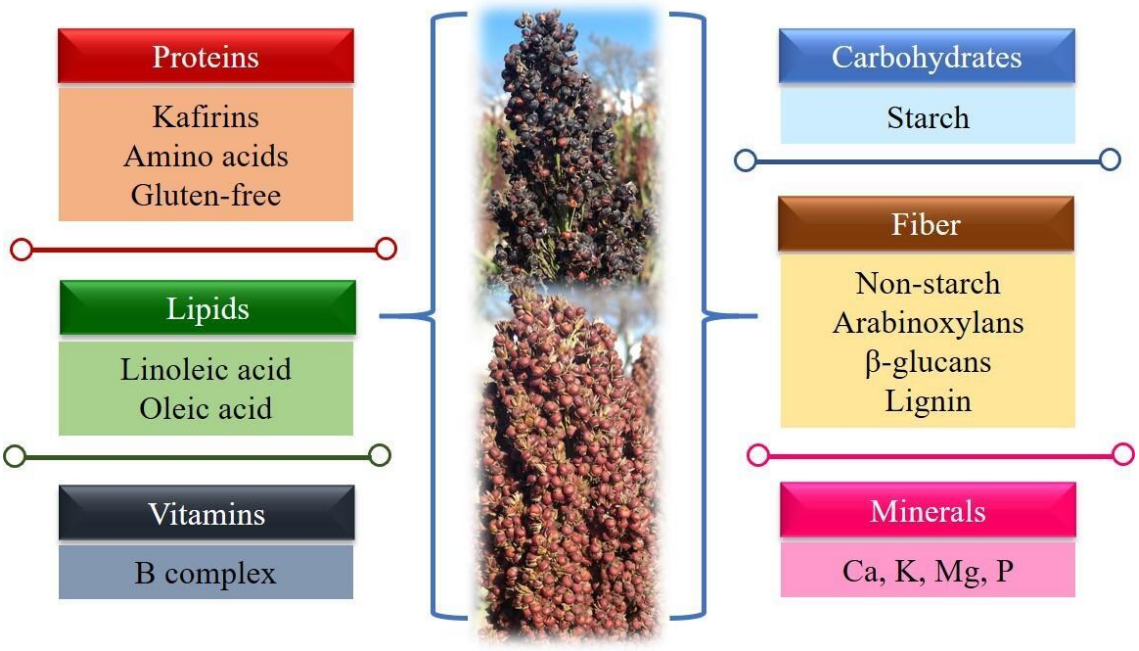

Figure 1. Chemical and nutritional components of sorghum.

Sorghum seed is mainly made up of three basic structures: the outer layer, made up of pericarp and testa, occupying an average of 8% of the total grain; the embryo or germ, which comprises 10% and 80% of the mature seed comprises the endosperm. The germ is where the highest percentage of proteins, lipids, and minerals are concentrated (Carrasco, Zamora, and Melin, 2011).

Table 1. Proximate composition (%), mineral content (mg/100g) and vitamins concentrations (mg/100 g) of sorghum grain.

Macronutrients	Content	Mineral	Content	NRVs-R	Vitamins	Content	NRVs-R
Carbohydrates	50-80.6	Ca	26.00	1000	Niacin	3.30	15.00
Protein	7-21.1	K	287.40	-	Riboflavin	0.15	1.20
Lipids	1.6-7.6	Mg	149.60	310	Thiamin	0.34	1.20
Crude fiber	1-3.4	P	286.50	700	Vitamin B-6	0.59	1.30
Ash	1.3-3.5	Fe	4.50	-	Vitamin E	0.81	9.00

NRVs-R (Nutrient Reference Values-Requirements) mg/100 g.

3.1. Protein

The protein content in sorghum grain differs between 6 to 18% with an average of 11%. Sorghum proteins are classified into prolamins (storage proteins) and non-prolamins (Labuschagne, 2018). Prolamins are the most abundant proteins and are called kafirins, which are found in the protein bodies of the endosperm. Kafirins constitute around 48-70% of the total proteins in whole grain and up to 80% of the proteins in decorticated kernels (Espinosa-Ramírez and Serna-Saldívar, 2016). Sorghum storage proteins are mainly divided into three groups: α-kafirin, β-kafirin, and γ-kafirin, according to their solubility, structure, and molecular weight. In protein bodies, the central nucleus of α-kafirin is enveloped by peripherally located β-kafirin and γ-kafirin components (Selle *et al.*, 2010).

The starch granules in sorghum corneous endosperm are surrounded by a matrix of hydrophobic kafirin protein bodies, which decreases the extent of the swelling of the starch granules during hydrothermal treatment. During cooking, the sorghum β and γ kafirins in the periphery of the protein bodies extensively cross-link, further restricting the starch's ability to swell. Therefore, it can affect the ability of starch to interact with other ingredients during processing to produce foods that are desirable in texture. However, the cross-linking of kafirin in sorghum can also contribute to the reduction of glycemic properties of the endosperm compared to other cereal grains (Girard and Awika, 2018).

A limitation in the consumption of sorghum is the presence of a diversity of antinutritional factors in the grain, such as tannins; these compounds decrease the digestibility of proteins, affecting food efficiency in both animals and humans. In this sense, certain technological processes, such as boiling, cooking, and extrusion, have been tested to decrease antinutritional factors (Luzardo-Ocampo *et al.*, 2019). The application of heat treatment to food grains can modify their texture, nutrient composition, tannin and antinutrient content, antioxidant activity, etc., likewise, these factors determine the digestibility and shelf life of the product once processed (Sharanagat *et al.*, 2019). The nixtamalization process increases the bioaccessibility of sorghum proteins, contributing to the depolymerization of condensed tannins and breaking down the protein-tannin complexes. This process favors its inclusion in the human diet because it improves the nutritional value of sorghum (Cabrera-Ramírez *et al.*, 2020).

It is important to mention that a variety of antifungal proteins have been identified in sorghum grain such as chitinase, glucanase, thionin, defensin, protease inhibitor, and ribosome-inactivating protein. In addition, certain bioactive proteins have been reported, such as amylase inhibitors, protease inhibitors, glycine-rich RNA-binding proteins, putative protein kinases, and glutathione S-transferase isozymes. Other proteins found include lysine 2-oxoglutarate reductase and saccharopine

dehydrogenase involved in lysine catabolism, cationic peroxidase, 2-kDa antiviral peptide, and a homolog of the maize β-glucosidase aggregating factor which is a jacalin-related GalNAc-specific lectin (Lin *et al.*, 2013).

3.1.1. Amino acids

Sorghum prolamins are rich in glutamic acid and nonpolar amino acids such as proline, leucine, and alanine, although they lack essential amino acids such as lysine and tryptophan (Labuschagne, 2018). However, through improvement programs, varieties with a content of 52-115% more lysine than conventional genotypes have been obtained. The result in the increase of this amino acid has been attributed to the decrease in the levels of kafirin proteins and high levels of non-kafirin proteins rich in lysine in the grain endosperm (Morais Cardoso *et al.*, 2017).

Mokrane *et al.* (2010) determined the amino acid content of sorghum grains of seven varieties and the most abundant were alanine, isoleucine, leucine, methionine, phenylalanine, proline, and valine, which are hydrophobic amino acids; the yield in all cultivars was 45% to 50% of the total amino acids. Mohapatra *et al.* (2019) found in sorghum grain amino acids in important quantities, the main ones were glutamic acid (44.38), arginine (28.43), aspartic acid (20.50), glycine (16.22), serine (15.34), and leucine (16.50), all expressed in mg/g of protein.

Branched-chain amino acids such as isoleucine, valine, and leucine have important physiological roles in regulating protein synthesis, metabolism, food intake, and aging (Couteur *et al.*, 2020). Amino acids are essential nutrients for both tumor cells and immune cells. T cells are part of the immune system and help fight cancer cells. T cells are highly dependent on amino acid transport and metabolism for their activation, differentiation, and function; some of these amino acids include alanine, leucine, serine, arginine, methionine, glutamine, cysteine, and cystine (Wang and Zou, 2020).

3.2. Fatty acids

Sorghum grain lipids are present mainly in the germ and it is rich in unsaturated fatty acids (83-88%). Polyunsaturated fatty acids are found in higher amounts in most varieties of sorghum compared to monounsaturated fatty acids. The fatty acids of sorghum are made up of linoleic acid from 45.6 to 51.1%, oleic acid from 32.2 to 42.0% (Morais Cardoso *et al., 2*017), palmitic an average of 15.62%, and 2.94% stearic acid. Linoleic and oleic acids contribute more than 80% of the total fatty acids in sorghum, it has been reported that unsaturated fatty acids have important health benefits and play an important role in the structure and function of biological membranes, in addition to that can reduce the risk of thrombosis and cardiovascular disease (Zhang *et al., 2*019). Sorghum grain could be an alternative source of edible oil due to the presence of all the saturated and unsaturated fatty acids necessary and important for human health (Mehmood *et al., 2*008).

Sorghum lipids have aroused singular interest as bioactive agents for health, especially phytosterols and policosanols, which are present in the oil and wax fraction, respectively. Phytosterols are compounds structurally like cholesterol, while policosanols correspond to a mixture of long-chained aliphatic alcohols. The concentration of phytosterols in sorghum grains is between 46 and 51 mg/100 g (Althwab *et al., 2*015). Phytosterols, in their form of free sterols or esterified fatty acids, can play a very important role in the reduction of plasma cholesterol in humans, mainly in individuals who cannot tolerate cholesterol-lowering drugs (Hoi *et al., 2*009).

Lee *et al.* 2014 made a comparison of two fractionated lipid whole sorghum grains (wax and oil) and showed that oil is the fraction that most reduces cholesterol levels in both plasma and liver, therefore, sorghum has great potential as a functional ingredient in food for heart health.

3.3. Carbohydrates

The main carbohydrate in sorghum grain is starch. The endosperm is made up of starch granules, protein matrix, and protein bodies. Due to its nature and chemical composition, the protein matrix has

an impact on the physical characteristics of the endosperm, essentially in the exposure of the starch granules to enzymatic digestion (Montiel, 2014). The average starch content in sorghum is 69.5%, it consists of two polysaccharides, amylopectin which constitutes 70 to 80% of the starch and the remaining 20-80% is amylose. In this regard, both genetic and environmental factors can influence the amylose content of sorghum (Rao *et al., 2*018). Depending on the type of starch in the endosperm it is classified as waxy and non-waxy. The starch of non-waxy sorghums is composed of amylose (25%) and amylopectin (75%), while in waxy sorghums, amylopectin is close to 100%. The sorghums that show resistance to enzymatic digestion are non-waxy ones, whereas the waxy ones are highly digestible by enzymes and have cooking and gelatinization properties, qualities that are essential in the food industry (Montes García *et al., 2*010). Sorghum is an extraordinary source of energy, comprising about 75% complex carbohydrates that in addition to starches include fibers, which are regularly digested slowly to provide satiety and delay hunger (Rao *et al., 2*018).

3.4. Vitamins and minerals

Sorghum is an excellent source of vitamins, mainly the B complex (thiamin, riboflavin, pyridoxine, etc.), as well as fat-soluble vitamins that include A, D, E, and K (Dicko *et al., 2*006). In addition, the grain contains between 1.30 and 3.30% ash and is rich in minerals such as phosphorus, potassium, and magnesium in various amounts. Likewise, it is an important source of iron and zinc, and it is better than rice and wheat in terms of mineral nutrition (Badigannavar *et al., 2*016). Other outstanding minerals in sorghum are calcium and sodium. Considering its importance as a nutrient-rich staple food, sorghum has also been used for biofortification, which can be further improved to make it more nutrient-dense and benefit populations with micronutrient malnutrition problems (Kotla *et al., 2*019). Calcium and phosphorus in synergy have an essential function as main components of the skeleton. They are also important in metabolic functions, such as muscle function, nerve stimulation, enzymatic and hormonal activities, and oxygen transport (FAO 2002b). Iron and zinc are essential minerals for

life, even though they are required in the body in small quantities (mg), they intervene as part of enzymes that act in various biological processes necessary for the proper functioning of a living organism (Pizarro *et al.*, 2005).

Vitamin A plays an important role in the body's defensive barrier against free radicals, it also prevents blindness and strengthens night vision, maintains skin softness and hydration, and strengthens the immune system. Vitamin D plays an essential role in making the most of the calcium and phosphorus needed to maintain healthy bones and teeth. Vitamin E is a powerful antioxidant, among its main functions is the delay of cellular aging, it has healing action and is one of the least toxic fat-soluble vitamins (Pérez and Ruano, 2004). Vitamin B1, known as thiamin, is a cofactor for several key enzymes that are involved in carbohydrate metabolism, as well as neural nerve function. Vitamin B2 (riboflavin) is a precursor of various nucleotides, mainly flavin mononucleotide and flavin adenine dinucleotide, which act as coenzymes in various metabolic pathways and in the production of energy. Niacin (nicotinic acid or vitamin B3) acts as a functional group of the dinucleotide coenzymes of nicotinamide and adenine, they are essential for oxidative processes (WHO and FAO, 2017).

3.5. Fiber

In sorghum, the bran comprises 10 to 25% of the total whole grain, particularly it is rich in dietary fiber and constitutes an average of 36 to 50% of total dietary fiber and 35 to 48% of insoluble dietary fiber (Miafo *et al., 2*019).

Non-starch polysaccharides are found in sorghum grain in proportions ranging from 2 to 7% depending on the variety, which is found especially in the cell walls of the pericarp and endosperm. These compounds in sorghum are represented mainly by arabinoxylans and β-glucans that constitute 55% and 40% of the total non-starch polysaccharides. Sorghum also contains other non-carbohydrate

polymeric cell wall components such as lignin that comprise 20% of the total cell wall (Dicko *et al.*, *2*006).

Ayala *et al.* (2015) analyzed three types of sorghum bran (white, red, and high in tannins) that showed to be a potential source of glucoarabinoxylans, these sources of soluble dietary fiber with antioxidant capacity could exert an important uptake of radicals in biological systems; therefore, it is possible to extract soluble fibers with antioxidant properties from sorghum and which can be used as food additives with beneficial effects on health.

Arabinoxylan gels help protect certain active ingredients such as lycopene and proteins during passage through the stomach and small intestine so that they are released into the colon during gel breakdown. In this context, sorghum bran arabinoxylan gels could be used to develop innovative functional foods that could provide important physiological benefits or reduce disease risks (Yan *et al., 2*020).

4. Antioxidants compounds

It has been reported that the consumption of sorghum may have important health benefits such as the reduction of oxidative stress and thus the prevention of cancer since it is an abundant source of phenolic compounds (Jiang *et al., 2*020). Grain polyphenols vary in types and levels with different genotypes and mainly comprise phenolic acids, flavonoids, anthocyanins, and condensed tannins, which make the grain ideal for the development of functional foods and nutraceutical (Abdelhalim *et al., 2*020). Several types of anthocyanins have been identified in sorghum. The 3-deoxyanthocyanidins and their derivatives such as apigeninidin and luteolinidin are compounds that are found in large quantities in sorghum, more than in other species (Choi *et al., 2*019). According to the sorghum genotype and the environment where they are grown, they can influence its color, appearance, and nutritional quality, which contributes to the variability in its phenolic compounds. Varieties with pericarp and black testa can have three to four times more 3-deoxyanthocyanidins than

the red and brown genotypes. Flavanones are found in higher concentration in lemon yellow varieties (Shen *et al., 2*018). The pigmented varieties of sorghum with the presence of tannins have high levels of antioxidants that are comparable to those of fruits and vegetables. Tannins are excellent antioxidants that can slow hydrolysis in foods, produce a naturally dark color, and increase dietary fiber levels in food products (Dykes and Rooney, 2006).

Free radicals are an important factor in oxidative stress that induces chronological disorders and diseases such as Alzheimer's, Parkinson's, cancer, cardiovascular diseases, cataracts, diabetes complications, and inflammation. Naturally occurring antioxidants, including polyphenolic compounds, reduce oxidative damage to biomolecules by modulating the effects of reactive oxidants (Kadri *et al., 2*017).

Sorghum is an important agricultural crop considered as a rich source of bioactive compounds (Bradwell *et al., 2*018) which can be used in the formulation of foods with functional improvements or beneficial properties that promote health (Shelembe *et al., 2*014).

5. Conclusions

In relation to the above, it can be concluded that sorghum is a potential source of carbohydrates, proteins, lipids, dietary fiber, and antioxidants; it also provides minerals such as phosphorus, potassium, magnesium, calcium, iron, and zinc; vitamins A, D, E and B complex. Today, with imminent population growth, there is a demand for nutritious foods that not only can improve people's nutritional deficiencies but also influence their health. An alternative to food shortages and malnutrition could be sorghum, as it has significant potential as a nutraceutical ingredient for use in food preparation and fortification, due to its versatility and chemical composition. Finally, it should be added that it is a high-yield, low-cost crop that is highly resistant to drought, which is why it can be used well in regions of the world where poverty reigns.

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7. Author contributions

Espitia writing – original draft manuscript. Ascacio-Valdés, Dávila-Medina, and Teresinha Silva conceptualization. Chávez-González, Flores-Naveda, Ruelas-Chacón, and Sepúlveda writing review and editing manuscript.

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In vitro inhibition of α-glucosidase, α-amylase and pancreatic lipase of quintonil (*Amaranthus hybridus*) processed with household thermal treatments

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Abstract

Quintonil (*Amaranthus hybridus*) belongs to the quelites family, a wild group of plants with comestible foliage. Quintonil is an excellent source of nutrients and bioactive compounds; however, it is considered a neglected, underutilized crop and sparsely collected for human consumption. This research evaluated the effects of household thermal treatments (boiling and steaming) on quintonil to promote its consumption due to its beneficial effects on human health. Aqueous extracts of raw, boiled (water at 98°C for 10 min) and steamed (by the direct incidence of steam for 7 min) of the edible portion of quintonil (leaves) were studied to determine their content of total phenolic content (TPC) and assayed *in vitro* for inhibitory effects on enzymes relevant to carbohydrates (α-amylase and α-glucosidase) and lipids metabolism (pancreatic lipase). Steaming has a significant impact on TPC compared to crude and boiling treatments (45.7 and 27.5 mg GAE/g); then, aqueous extracts from steamed quintonil showed the highest content TPC (92.3 mg GAE/g). All the aqueous extracts could inhibit enzymatic activity with values of 2.44, 10.5 and 20.4% for α-amylase, 17.46, 56.3 and 82.5% for α-glucosidase and 39.7, 42.6 and 58.5%, for lipase in raw, boiled, and steamed process respectively. The results propose encouraging the consumption of quintonil, which have proved to be natural sources of bioactive compounds capable of inhibiting important enzymes in type 2 diabetes and preventing or controlling obesity.

Keywords: *α-amylase, α-glucosidase, Amaranthus hybridus; phenolic compounds; quintonil.*

1. Introduction

Metabolic syndrome is a group of risk factors such as increased fasting plasma glucose, abdominal obesity, and high blood pressure. Additionally, type-2 diabetes mellitus is a chronic condition typified by hyperglycemia (Miglani *et al.*, 2017). The most common medical assessment in managing and controlling metabolic syndrome and diabetes includes acarbose to inhibit α-amylase and α-glucosidase and orlistat for inhibiting pancreatic lipase. However, the continuous ingestion of these medicines may produce adverse effects such as abdominal contractions, flatulence, diarrhea, and oily spotting (Seyedan *et al.*, 2015). Modulation of these enzymes by phenolic based dietary ingredients may help manage the possible syndrome metabolic complications without the undesirable side effects mentioned. Dietary phenolic compounds have been shown to be efficient inhibitors of α-amylase, α-glucosidase, and lipase due to their ability to bind to proteins via hydrogen binding (Ambigaipalan *et al.*, 2016). Vegetables such as quintonil (*Amaranthus hybridus*), a neglected and underutilized crop, offer a natural source of phenolic compounds with different bioactivities (López-García *et al.*, 2017). Quintonil is consumed raw or after household thermal treatments (frying, microwave cooked, boiling, or steaming); these treatments could affect its content of phytochemicals and biological activities (Jiménez-Aguilar & Grusak, 2017; López-García *et al.*, 2017). Therefore, the objective of this study was to evaluate the effects of home cooking treatments (boiling and steaming) on quintonil to focus on its health benefits to promote its consumption.

2. Materials and Methods

2.1. Plant Materials

Quintonil was collected in San Lorenzo Tlacotepec, Estado de México) (19°49´10.84¨N and 99°55´2.19¨W) in 2016. Quintoniles were planted and harvested according to the agronomic practices

of the place. Five weeks after planting, fresh leaves were collected and then identified and authenticated at the National Herbarium of Mexico, with the number of vouchers 1,434,640.

2.2. Cooking treatments

Boiling and steaming of quintonil leaves were conducted according to López-García *et al.* (2017). Boiling was performed with water at 98 °C and atmospheric pressure for 10 min. For steaming, the leaves were cooked by the direct incidence of steam for 7 min. After the cooking, the samples were submerged in cold water at (4 °C) for 30 s to stop cooking. Raw and cooked samples were cut into pieces of 0.5 cm × 0.5 cm.

2.3. Preparation of the extracts

Aqueous extracts for raw and cooked samples were prepared as established by López-García *et al.* (2017). 5 g of raw and cooked quintonil leaves were placed in plastic tubes and 20 mL of distilled water were added, then the tubes were placed in an orbital shaker at 200 rpm for 4 h at 25 ºC in the dark. After this time, the extracts were centrifuged at 11 000 rpm for 15 min. The supernatant was recovered and stored at −20 °C until used.

2.4. Analysis of total phenolic compounds (TPC)

TPC of the aqueous extracts was examined according to Singleton *et al.* (1999) with some modifications. Briefly, 15 µL of the extract were mixed with 240 µL of distilled water and 15 µL of 2 N Folin-Ciocalteu reagent in a 96-well Costar® flat-bottom microplate. After incubation for 3 min, 30 µL of 4 N Na_2CO_3 were added, and the plates were then allowed to stand in the dark for 90 min. The absorbance was determined at 725 nm using a SynergyHT Absorbance Microplate Reader (BioTek Co., USA). A standard calibration curve was prepared using gallic acid and the total phenolic compounds were expressed as mg of Gallic Acid Equivalent per g of sample (mg GAE/g).

2.5. α-glucosidase inhibitory assay

The inhibitory properties of the aqueous extracts against α-glucosidase were evaluated by the method of Matsui *et al.* (2007), with some modifications. 40 µL of extracts and 100 µL of α-glucosidase solution (1.0 U/mL diluted in a 0.1M phosphate buffer, pH 6.9) were incubated at 25ºC for 10 min. After this time, 50 µL of 5mM ρ-nitrophenyl –α-D-glucopyranoside solution (0.1M phosphate buffer pH 6.9) was added mixtures were incubated at 25ºC for 5 min before reading the absorbance at 405 nm. Acarbose (0.44 mg/mL) was used as a reference inhibitor.

2.6. α-amylase inhibition assay

α-amylase inhibitory activity was determined using the method of Nickavar *et al.* (2008). with a slight modification. 40 µL of extracts, 160 µL of distilled water and 400 µL 0.5% starch were mixed in a 2.0 mL centrifuge tube. After adding 200 µL of the enzyme solution (30 U/mL), the tube was incubated at 25 °C for 3 min. Then, 200 µL of the mixture was taken and added into a separate tube, which contained 100 µL DNS solution (96 mM 3,5-dinitrosalicylic acid, 5.3 M sodium potassium tartrate in 2 M NaOH). The tube was incubated at 95 ºC for 10 min. After this time, 900 µL of distilled water were added into the tube and mixed. Then 200 µL of the mixture was taken and added to a 96-well plate. The absorbance of the reaction mixture was measured at 540 nm.

2.7. Lipase inhibition assay

The lipase inhibitory activity was determined using the method of Worsztynowicz *et al.* (2014) with modifications. In the assay, p-nitrophenyl palmitate (pNPP) was used as a substrate hydrolyzed by lipase to generate ρ-nitrophenol (pNP) that can be monitored at 410 nm. Briefly, 20 µL of extract and 20 µL of the lipase enzyme solution (1 mg/mL) in sodium phosphate buffer (0.1 M, pH 6.9) were incubated for 10 min at 37 °C. Then, 1800 µL of 0.1M sodium phosphate buffer containing arabic gum (0.55 mg/mL) and sodium cholate (1.15 mg/mL) and 20 µL of ρNPP (0.01 M in isopropanol)

were added and incubated for 10 min at 37 °C. The released ρNP was monitored at 410 nm using a microplate reader.

The percentage of inhibition of α-glucosidase, α-amylase and lipase was calculated according to the equation:

$$\%\ Inhibition = \left[\left(Abs_{control} - Abs_{sample}\right)/Abs_{sample}\right] x100$$

Abs control =Absorbance of the control (blank, without extract), Abs sample= Absorbance in the presence of the quintonil extracts

2.8. Experimental design

Each experiment was done in triplicate. Data were reported as mean ± standard deviations. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test using the software MINITAB® v. 16. Statistical differences at a level of $p<0.05$ were considered significant.

3. Results and discussion

3.1. Total phenolic content (TPC)

Boiling and steaming showed significant effects on TPC. When quintonil was boiled, samples presented a significant decrease in comparison to raw (60 %) quintonil (45.7 and 27.5 mg GAE/g); conversely, steaming showed an increase over crude quintonil (27.5 and 92.3 mg GAE/g). The loss, retention or increase of phenolic compounds are the results of cooking for different methods; in addition to the reasons mentioned earlier, the chemical structure is a crucial factor. Cooking treatments may release more bound phenolic compounds from the breakdown of cellular components; this disruption also makes available the oxidative enzymes that can destroy the phenolic compounds in vegetables (Chism & Haard, 1996). Steaming seems to deactivate or destroy oxidative enzymes; as a result, the loss of phenolic compounds is less than during boiling.

3.2. Enzymatic inhibitions

Experiments showed that the aqueous extracts of quintonil (raw, boiled and steamed) showed a dose-dependent response in each enzyme inhibition studied. The enzyme α-amylase plays an important role in breaking down complex carbohydrates such as starch, as polysaccharides must break down into monosaccharides to be absorbed (Sultana *et al.*, 2020). The results of the α-amylase inhibition assays were shown in figure 1. Raw extracts of quintonil showed the least inhibitory capacity (2.4 %); in opposition, after cooking, the boiled and steamed extracts showed an increase of (9 and 19 %) respectively. The discrete α-amylase inhibitory activity compared to acarbose (87 %) showed that phenolic present in the aqueous extracts are not very effective inhibitors of α-amylase due to the lower enzyme binding strength by the phenolic compounds.

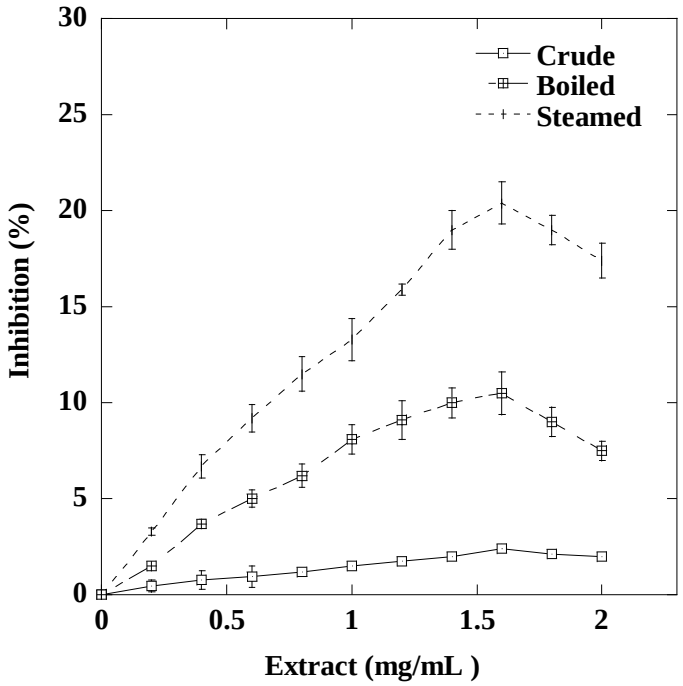

Figure 1. Effect of the quintonil extracts against the inhibition percentage of α amylase. The results represent the mean from three independent replicates.

As shown in Figure 2, all extracts were effective inhibitors of α-glucosidase, which is another enzyme that breaks oligo and disaccharides, making glucose available in the body (Sultana *et al.*, 2020). In

accordance with the α-amylase inhibitory assays, the type of thermal processing had significant effects ($p<0.05$) on the inhibition of α-glucosidase in the order of steamed> boiled>raw. α-glucosidase inhibition by aqueous steamed quintonil extracts (82.6 %) was close to that presented by acarbose (87%). The inhibition of α-amylase and α-glucosidase suggested that quintonil phenolics compounds may have the potential for use in regulating diabetes mainly for the strong glucosidase inhibitory capacity.

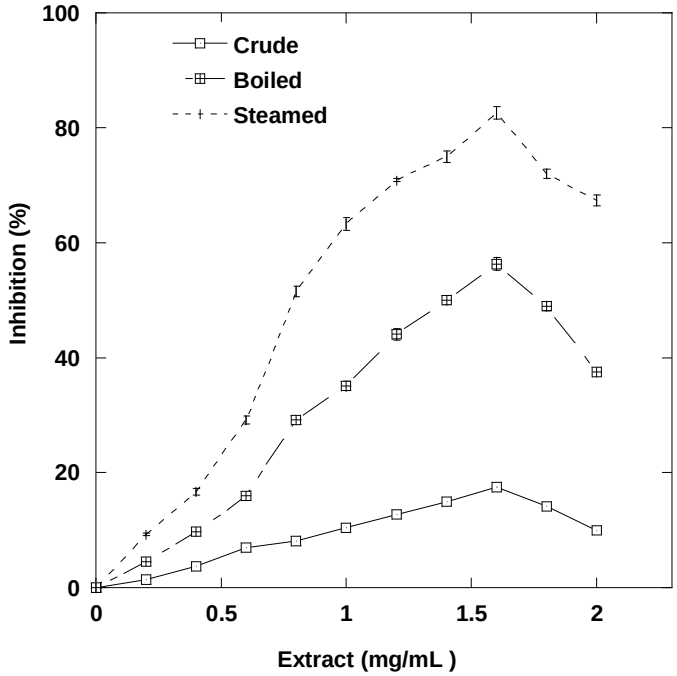

Figure 2. Effect of the quintonil extracts against inhibition percentage of α-glucosidase. The results represent the mean from three independent replicates.

It has been estimated that around 65 % of total dietary fats are hydrolyzed for absorption by the pancreatic lipase (Embleton and Pouton, 1997), highlighting the significance of this enzyme in calorie release from foods. The greater ability to inhibit the activity of α-glucosidase compared to α-amylase exhibited by the aqueous extracts of quintonil, could avoid the side effects of drugs used for the inhibition of this enzymes such as bloating, flatulence , meteorism and diarrhea. These effects can be caused by excessive inhibition of intestinal amylase, leading to abnormal bacterial fermentation of

undigested carbohydrates in the colon (Kwon *et al.*, 2007). Therefore, inhibitors from aqueous extracts of quintonil have low inhibitory activity of α-amylase and strong inhibitory activity α-glucosidase and can be used for the management of postprandial hyperglycemia with side effects minimal.

In the case of pancreatic lipase, the maximum inhibitory concentration was reached at 1.0 mg/mL, which indicates that the aqueous extracts from quintonil are more potent to inhibit this enzyme (Figure 3). The highest inhibitory lipase activity was demonstrated for the steamed (58.8 %) followed by crude (42.6 %) and boiled (39.7 %) extracts. However, no significant difference was shown between the raw and boiled quintonil extracts. The decrease in lipase inhibition at concentrations greater than 1 mg/mL may be due to increased interactions between phenolic molecules, which reduces their interactions with the enzyme protein. Phenolic extracts obtained from natural plants, such as grape seed and oregano, have previously been reported as lipase activity inhibitors (Gutiérrez-Grijalva *et al.*, 2018). The results suggest that the raw, boiled, or steamed quintonil can *in vitro* inhibit enzymes involved in carbohydrate and lipid metabolism. Further studies are needed to examine the potential use of these extracts in the prevention of different pathologies.

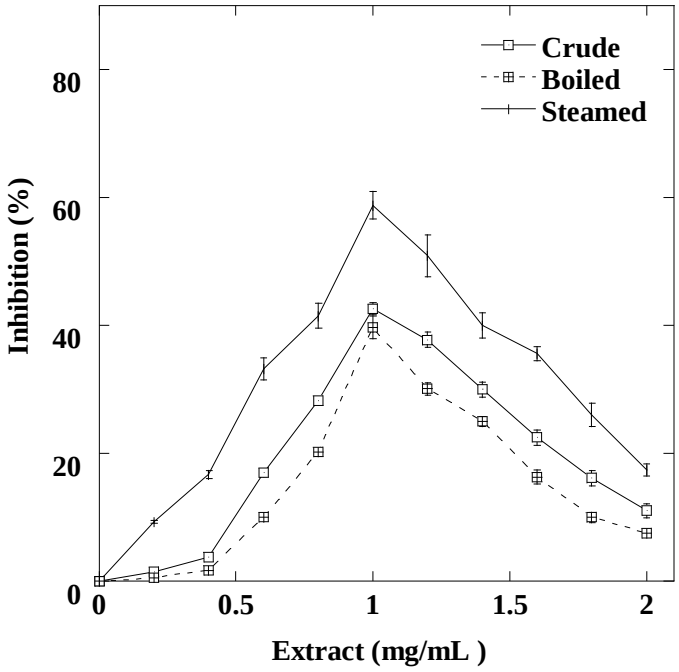

Figure 3. Effect of the quintonil extracts against percentage inhibition of pancreatic lipase. The results represent the mean from three independent replicates.

4. Conclusion

To revalorize an underutilized plant with preventive effects against malnourishment and some chronic diseases such as quintonil, we conducted this research. There are considerable differences in total phenolic content and enzymatic inhibition among raw and cooked quintonil. Steamed quintonil had the highest total phenolic content besides showing high α-glucosidase, lipase pancreatic and moderate α-amylase activities. The content and type of phenolic compounds is strongly related to inhibitory activities; however, non-phenolic compounds may be involved in these inhibitory capacities. The inhibition of pancreatic lipase and anti-diabetic potential (inhibition of α-amylase and α-glucosidase) suggests that quintonil could be used to prevent and manage metabolic syndrome and its complications. Continuing work is essential to identify the compounds involved in the studied activities.

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Physicochemical characterization of vegetable flours for food supplements development

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Abstract

One of the key aspects to develop inexpensive and accessible food supplements for people with malnutrition is knowing the quality and functionality of the raw materials used for their production. Therefore, the objective of this work was to know the composition, physicochemical and functional characteristics of cereal (oats, quinoa, amaranth), legumes (beans, broad beans, chickpeas, and lentils) and dry products (spinach, mushrooms, and grasshoppers) flours used for supplement development. For this, the analysis of density, particle size, solubility, proximal chemical analysis (AQP), water activity (aw), humidity and sorption isotherms and molecular analysis were carried out. It was found that cereals flours were not fluid powders, they presented "cakening" and they were poorly soluble in water. Also, they had low protein quality. Meanwhile, legumes flours showed acceptable flow properties; they were soluble in water and milk, and they had moderate adsorption of water. Particle size established suspensions with homogeneous texture and their sensory attributes were suitable. The AQP added to the biological value provides the nutritional composition of the product with which mixtures of the flours were designed to cover the nutritional needs required in the final products. The a_w, the percentage of humidity and the sorption isotherms were quality parameters to determine the optimal storage conditions. The infrared molecular analysis ATR corroborated that the main functional groups in cereal flours were starch and cellulose while in legume flours were lignin, protein amides, cellulose, and carbohydrates, for mushroom and grasshopper, chitin, and chitosan and for

spinach, cellulose, and chlorophyll. The results obtained allow us to select the raw materials with the biological value and functional characteristics to produce nutritional, stable, and safe supplements.

Keywords: *cereal and legume flours, physicochemical characterization, functionality.*

1. Introduction

Food supplements are products that provide nutrients such as proteins, vitamins and / or minerals. They are used to complement, cover, or increase the intake of these nutrients in the diet, and they can be made with components of plant or animal origin. To know the quality of the supplements that are designed and their functionality, studies are carried out on the composition of each flour used and its physicochemical characteristics. The analysis of a_w and moisture percentage help us to determine the maximum limits allowed in accordance with NOM-247-SSAI-2008, which contributes to the physicochemical and microbiological conservation of the flours as well as the sorption isotherms, that stand quality parameters for optimal storage conditions. The AQP and the amino acid content provide the nutritional composition of the product, with which flour mixtures are designed to fulfil the nutritional needs required in the final products. Infrared ATR analysis confirms that the main functional groups in flours. After all, the particle size of the flours and their solubility in water and milk allow to be aware of the homogeneity of the flour and obtain stable suspensions with agreeable texture. Knowing the composition, physicochemical and functional characteristics of the cereal, legumes and dry products flours used for supplement development allow a better design of high nutritional quality products which could be ready to be used for persons with malnutrition pathologies.

2. Methodology

2.1. Material

Cereals: oats (*Avena sativa*), quinoa, (*Chenopodium quinoa*) and amaranth (*Amaranthus*). Legumes: beans (*Phaseolus vulgaris*), broad bean (*Vicia-faba*), chickpeas (*Cicer-arietinum*) and lentils (*Lens-*

culinaris). All of them were San Lázaro®. Dry products: spinach (*Spinacia oleracea*), mushrooms (*Agaricus bisporus*), and grasshopper (*Sphenarium purpurascens*). Cereals, legumes, and other materials were obtained in bulk from a wholesale supplier, CDMX. All materials were ground in a blender (Vitamix USA Aspire VM0103) and sieved through a # 40 mesh (0.42 mm). The legumes were soaked during 24 h at room temperature. They were subjected to a thermal process for 90 min and dried at 45-56 °C until they reached an a_w of less than 0.4 and a humidity of less than 12%.

2.2. Proximal chemical analysis

Proximal chemical analysis was carried out according to official methods of the AOAC (1990). The determinations were humidity (drying in the oven), ethereal extract (Soxhlet method), ashes (incineration), raw fiber (acid and alkaline digestion) and crude protein (Kjeldahl method). Atomic absorption spectrometry was carried out to determine the minerals content (AOAC 927.02 method).

2.3. Density

Cylinder method with guide. The Hausner relation and the Carr index were calculated.

2.4. Solubility

Solubility in water and skim milk was evaluated following the method proposed by Regiane *et al.* (2014).

2.5. FT-IR: system attenuated total reflectance (ATR)

FT-IR (Fourier-Transform Infrared Spectroscopic Analysis) spectra were collected at 25±2 °C using a Nicolet iS50R spectrometer (Thermo Scientific, USA), with the Smart iS50R ATR sampling accessory. Spectra were collected over the range 4000-525 cm^{-1}. For each sample, 32 scans were averaged with a spectral resolution of 4 cm^{-1}. Baseline corrections and normalization were performed using the OmnicTM Software (Thermo Scientific). A graphical presentation of spectra was performed using the Origin Software (OriginPro8.0).

2.6. Particle size

It was measured by a Malvern Mastersizer 3000 particle size analyzer (Malvern Instruments Ltd., Malvern, UK, using the dry sample dispersion accessory (Aero S, Malvern Instrument Ltd., Malvern, UK).

2.7. Humidity

Moisture content of flours was determined in triplicate using a thermobalance for measuring humidity content at 135 °C (Sartorius MA37-1).

2.8. Water activity

Water activity (a_w) of powders was determined using an AquaLab 4TE apparatus (METER Group; Pullman, WA, USA) at 25 ºC.

2.9. Protein quality aminograms

The determination of the protein quality was carried out with aminograms, which were performed with the ACC-Qtag methods while tryptophan was quantified on a reverse phase HPLC column coupled to a UV spectrophotometer.

2.10. The biological value (PDCAAS)

The PDCAAS for each flour was calculated with WHO recommended intake reference.

2.11. Sorption isotherms

The sorption isotherms were performed at 25 and 45 °C in the VSA (Vapor Sorption Analyzer).

2.12. Statistical analysis

All measurements were performed by triplicate. Statistical analysis was conducted with analysis of variance (ANOVA) using XLSTAT (2014.5.03) to determine whether there were any significant differences between the means of treatments.

3. Results

Table 3.1 shows a comparison between the results obtained with the physicochemical characterization

of the flours (results of ANOVA), where it is shown that there is relationship between all tests proposed to evaluate quality, composition, and functionality of the raw materials for their application in the production of supplements for people with malnutrition. In general, the a_w was below 0.45 and humidity was less than 10 %. The a_w values were between the interval where the monolayer moisture (W_0) represented the content moisture that minimizes biochemical reactions (Ramírez-Miranda *et al.*, 2014) whereas the values of moisture content were below standard required CODEX STAN 171-1989. Thus, all the flours had reduced enzyme reactions and microorganisms lack activity. Even though all the flours had significant differences ($p<0.05$, Table 3.1), there was not much dispersion, in addition that all the parameters were within the norm. Water activity and humidity could be related to the isotherms, which, depending on the adsorption capacity of the particles, the behavior of the raw materials could be predicted. Figure 3.1 illustrates the isotherms of all the flours tested in this work. All the flours showed a significant difference between them ($p<0.05$); at 25 °C and all of them showed hysteresis, as they were describing complex matrices. The material changed its physical characteristics because of the interactions between the food components (Viades, 2013), depending on whether it was absorbing water (adsorption isotherm) or if it was losing water (desorption isotherm) and this change could be reversible, that was, the flours reached conditions like the initial humidity and a_w conditions, which indicated a structural change in the product, mainly for the samples at 25 °C. In the case of the samples at 45 °C, this phenomenon happened in a lower proportion. Eight models, which are widely used in food isotherms, were tested to predict the behavior of isotherms. However, it was observed that all flours fit the Peleg model in common. The flour that had the best water adsorption capacity is mushroom followed by spinach, while the flours with the lowest adsorption were lentil, oatmeal, and chickpea.

Table 3.1. Physicochemical properties of oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom, and grasshopper.

Flour	Density		Particle size			Solubility		a_w	%H	Isotherm	
	Total	*Flux*	*Dv10*	*Dv50*	*Dv90*	*Water*	*Milk*			*25°C*	*45°C*
Oats	F	Poor	E,F	G	B	F	E	A,B	A	C	C,D
Quinoa	G	Poor	D	F	C,D	D,E	E	B	C	B,C	B,C, D
Amaranth	D	Poor	A	A	A	E	E	A	A	B,C	C,D
Bean	D ,E	Acceptable	G	H	B	B	C,D	A,B	D	B,C	B,C, D
Roasted bean	-E	Acceptable	D,E	H	B,C	C	E	B	B,C	B,C	B,C
Broad	B	Acceptable	B	D	B,C	D	B	A,B	C	B,C	C,D
Lentil	A	Excellent	B	C	B,C	E	C	A,B	C,D	C	D
Chickpeas	C	Acceptable	F	F	B,C	D	D	B	B,C	C	C,D
Spinach	F	Acceptable	D,E	E	B,C	A	A	A	A	B	A,B
Mushroom	H	Excellent	C	F	D	A	D	A,B	A	A	A
Grasshopper	C	Acceptable	B	B	B	F	E	A	A,B	B,C	A

Flours with the same letter are not significantly different ($p < 0.05$), test of variance (ANOVA).

On the other hand, at 45 °C a greater dispersion of the data was observed, due to the temperature could promote reactions in some flours. For example, the flour that had the greatest water adsorption capacity was the mushroom followed by grasshopper and spinach while the flour that had less water capacity was lentil.

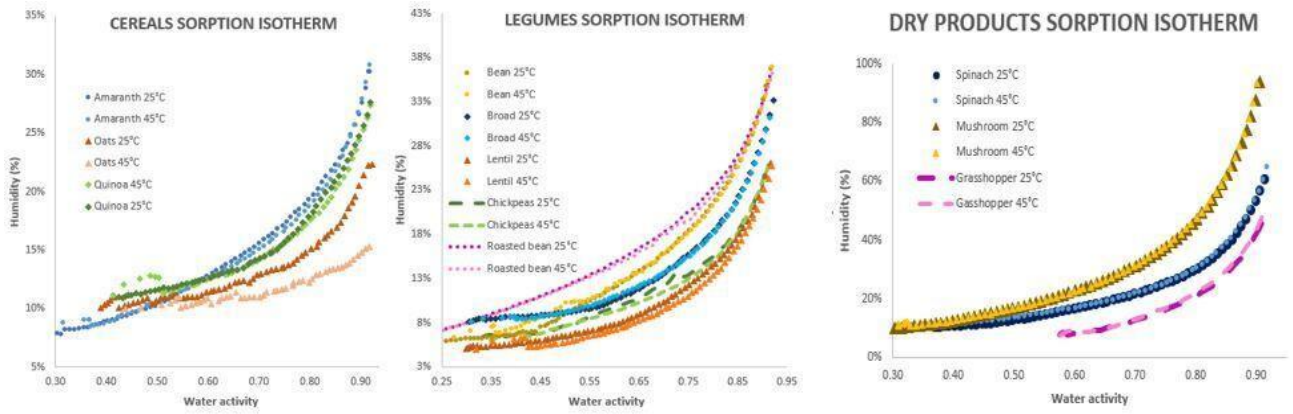

Figure 3.1 Sorption isotherms for flours of oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom, and grasshopper at 25 and 45 °C.

In this work, the nutritional content of the raw materials was determined qualitatively by means of the infrared ATR and quantitatively with the AQP, which revealed the amount of each of the macronutrients contained in each flour and complemented with the biological value. Table 3.2 shows the AQP for all the flours. It was established that the values were agree with that reported in the literature in all cases (Flores *et al.*, 2014; Terán *et al.*, 2015; Zumaran *et al.*, 2017; Asmat, 2016; Machuca *et al.*, 2017; Polo, 2012; Paz, 2014; Ipiales, 2018; Martínez *et al.*, 2018). However, it can see that not all flours have the same amount of protein. Mushrooms (39.49%) and grasshoppers (62.99%) contained more protein, while cereals were low protein (9.8-14.8%). It should be noted that the quality of the protein depends not only on the quantity, also, on the content of essential amino acids and the percentage of digestible protein, which corresponded to the amount of protein absorbed, therefore, metabolized by an organism (Soto, 2019). Table 3.3 shows the results of the amino acid profile, the amino acid score for 100 g of protein and the PDCAAS. It can be observed that cereals contain small amounts of lysine. At that point, the biological value of oats was only 14% while for amaranth, the amino acids that determine the biological value were isoleucine and valine and isoleucine for quinoa, which shows the highest PDCAAS (77.1%). The three flours had high content of tryptophan, methionine and cysteine as described by Carrillo *et al.* (2015). In the case of legumes,

the score was conditioned by the low levels of sulfur amino acids, such as methionine and cysteine. So, the biological value was low (33-44%), as well as isoleucine deficiency. Although, they brought the lysine that cereals did not have (Anchia, 2003). Spinach and mushroom were considered food of high biological value (88.5% and 51.5%) since they contained all the essential amino acids in percentages greater than 80% (Estrada, 2017; Miranda and Sauceda, 2018).

Table 3.2. Proximate chemical analysis of the flours of oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom, and grasshopper.

Flour	Dry matter (%)	Moisture (%)	Protein (%)	Digestible protein (%)	Ether extract (%)	Ash (%)	Fiber (%)	N-free extract (%)	Energy (kcal)
Oats	91.90±0.01	8.30±0.01	9.81±0.02	9.71±0.01	6.08±0.03	1.11±0.02	1.60±0.01	73.30±0.02	387.26±0.02
Quinoa	90.55±0.03	9.45±0.02	11.80±0.03	11.08±0.02	6.13±0.01	2.14±0.02	2.25±0.03	68.22±0.02	375.25±0.03
Amaranth	95.21±0.01	4.79±0.01	14.83±0.02	15.05±0.03	5.75±0.02	2.96±0.03	4.90±0.01	66.77±0.03	378.15±0.02
Bean	97.52±0.02	2.48±0.02	20.65±0.03	20.82±0.01	0.61±0.01	5.13±0.02	5.36±0.03	65.78±0.02	351.21±0.01
Roasted bean	99.03±0.02	0.97±0.02	21.70±0.01	15.44±0.03	0.23±001	5.46±0.03	10.58±0.02	61.1±0.01	333.15±0.03
Broad	99.61±0.03	6.39±0.01	18.53±0.02	27.48±0.02	4.23±0.03	2.68±0.01	3.28±0.02	64.89±0.01	371.75±0.03
Lentil	93.60±0.01	1.98±0.02	23.07±0.03	20.48±0.01	0.29±0.02	2.53±0.01	4.07±0.03	68.06±0.03	367.13±0.02
Chickpeas	98.01±0.02	4.02±0.01	16.85±0.02	13.01±0.02	6.65±0.03	2.73±0.01	0.50±0.01	69.26±0.03	404.29±0.01
Spinach	93.33±0.02	6.70±0.01	20.12±0.02	15.87±0.03	6.40±0.01	17.79±0.01	5.22±0.03	43.81±0.02	313.32±0.02
Mushroom	94.71±0.01	5.29±0.01	39.49±0.02	38.50±0.01	0.38±0.02	11.65±0.03	11.53±0.03	31.66±0.01	288.02±0.03
Grasshopper	97.96±0.03	2.04±0.02	62.99±0.01	21.86±0.02	7.97±0.03	8.70±0.01	9.62±0.02	8.68±0.02	358.41±0.01

Grasshopper flour showed low content of lysine and tryptophan as it was reported in the literature (Ramos *et al.*, 2002). Figure 3.2 illustrates the spectra for all the flours. The spectra presented an overview of the functional groups contained in each of the flours. All flours had similar components such as carbohydrates, proteins, and fiber. However, all contain a large amount of diverse and complex components that prevent having quantitative results directly from the spectra, having band overlap and this could skew the results, that is why a quantitative analysis (in this case the AQP) is needed to corroborate the information. The main functional groups in cereal flours were starch and cellulose even though in legume flours were lignin, protein amides, cellulose; for mushroom and grasshopper were chitin and chitosan, finally, for spinach was cellulose and chlorophyll. In detail, the bands between 1100 and 1000 nm^{-1} are the bands that correspond to cellulose, fiber, saponins and

starch. The presence of these bands is related to 66-73% of the nitrogen-free extract content of the AQP (Table 3.2). Likewise, the amide bands observed close to 1600-1500 nm^{-1} are the bands that correspond to the protein content (9-15%). Whereas in legumes spectra, bands observed in 1600-1500 nm^{-1} correspond to the proteins and lignin and band in 1000 nm^{-1} corresponds to carbohydrates, whose presence was confirmed with data from the AQP analysis (Table 3.2). It is to be observed that in the broad bean and chickpea spectra there are bands with higher absorbance for proteins, lignin and cellulose compared to the spectra of lentil and bean. The spectrum of spinach shows well-defined bands at 1600-1111nm^{-1} (cellulose), 1700-1045nm^{-1} (hemicellulose), 3500-1045nm^{-1} (lignin), some pectin and chlorophyll (2930-2817nm^{-1}, 1626- 1027nm^{-1}). Spinach contains protein, why amide intense bands (1640-1610nm^{-1}) are observed. Regarding the spectrum of the mushroom, the characteristic bands of glucan (940-870nm^{-1}), chitin and chitosan (3260-1018nm^{-1}) as well as the bands of amides and amines (1630-1300nm^{-1}) of the proteins stand out. Finally, the spectrum of grasshopper flour shows defined bands of functional groups of chitin and chitosan (3266-693nm^{-1}), which are the components of the cytoskeleton of insects; On the other hand, the amide and amine bands (3270-1200nm^{-1}) of the proteins are observed, which is corroborated with the highest crude protein content (63%) of all the products.

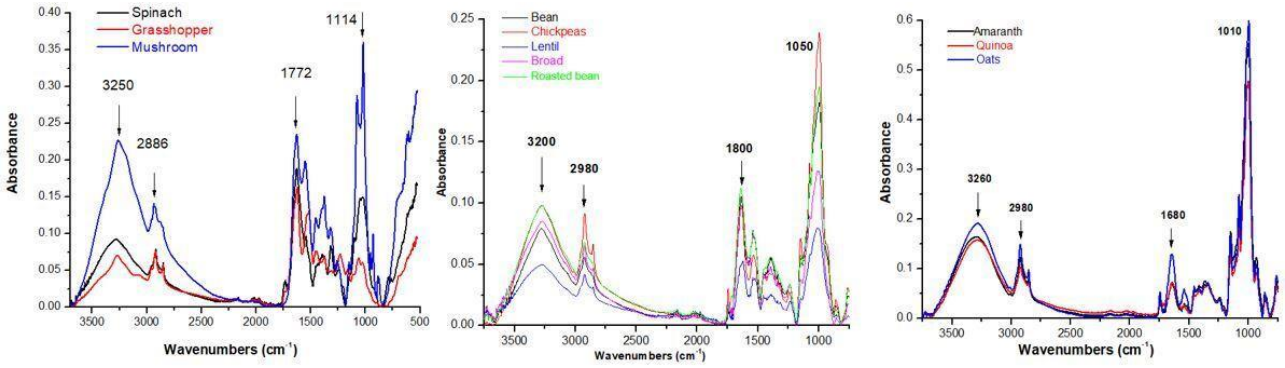

Figure 3.2. FT-IR and UV-Vis spectra obtained for oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom and grasshopper.

Table 3.1 shows the parameters of total, apparent and compacted density, particle size, solubility, moisture, and isotherms. All the parameters presented a significant difference for all the flours. Although, the parameters did not show a significant difference were the compressibility index, Hausner index and a_w. The density displayed the degree of compaction and fluidity of each of the flours; for example, cereal flours had "poor" flow properties (Hausner: 1.4), due to the greater interaction between the particles, therefore, they were more difficult to homogenize and present greater caking. Mushroom and lentil flours had "excellent" properties (Hausner: 1.04 – 1.1), this indicated free fluidity under non external forces application. They had low cohesiveness and intermolecular interactions. The other flours had an acceptable flow behavior since they were considered fluid powders (Hausner ~ 1.2).

Regarding particle size, quinoa, bean, and spinach flours exhibited a normal distribution, thus they were homogeneous samples with little stratification. The other flours had an asymmetric distribution with a positive slant, which indicated that the flours were not homogeneous. Figure 3.3 shows a box plot comparing the particle size distributions of oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom, and grasshopper. All flours presented a lengthening of the distribution curve to the right. Chickpea flour had the largest particle size; therefore, it was the most heterogeneous, being one of the factors that could affect the supplements texture. Also, all flours except quinoa showed that more than 75% of the population is below 420μm, indicating that the sieving process helped to standardize the particle size.

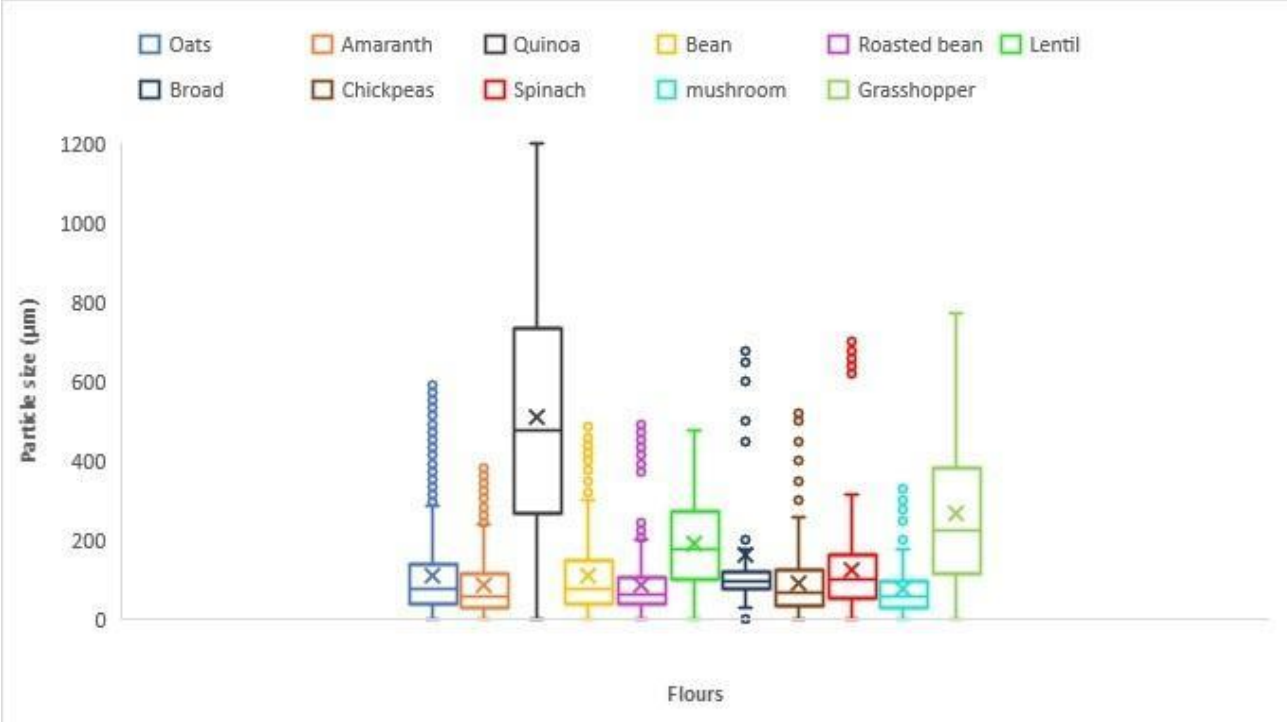

Figure 3.3. Box plot comparing the particle size distributions of oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom, and grasshopper.

Finally, solubility was one of the most important physicochemical properties. Its determination could give information about the behavior of a food during its process; fundamentally in the development of functional properties such as emulsification and gelling. Quinoa, chickpeas, and mushroom flours were more soluble in milk than in water, due to a greater affinity between the micelles of the milk proteins, the flours, and the matrix.

Table 3.3. Amino acid profile, amino acid score for 100g of protein (%) and the PDCAAS of oats, amaranth, quinoa, bean, broad, lentil, chickpea, spinach, mushroom, and grasshopper.

Flour	**Trp** (%)	**Thr** (%)	**Iso** (%)	**Leu** (%)	**Lys** (%)	**M+C** (%)	**F+T** (%)	**Val** (%)	**PDCAAS** (%)
Oats	143±12	219±10	39±5	89±7	14±3	146±9	368±15	82±8	14.3
Quinoa	123±11	197±10	77±6	107±8	150±12	101±9	244±14	100±5	77.1
Amaranth	174±6	30±4	0	45±3	104±12	104±10	109±11	0	0
Bean	90±5	180±13	45±7	70±3	118±8	60±4	200±14	63±2	44.3
Roasted bean	124±11	0	522±15	215±14	110±7	166±8	137±9	68±3	0
Broad	153±12	94±5	74±6	119±9	87±4	18±2	186±7	83±3	17.5
Lentil	81±6	0	0	58±3	117±9	0	52±3	5±3	0
Chickpeas	74±6	129±8	34±4	49±2	62±8	53±3	154±9	42±2	33.5
Spinach	127±12	146±15	100±10	121±9	102±7	89±5	295±16	144±11	88.5
Mushroom	243±12	116±5	67±4	62±3	54±6	52±3	222±14	92±7	51.5
Grasshopper	31±2	83±6	34±5	34±4	4±2	58±3	312±9	49±2	3.5

4. Conclusion

The physicochemical and functional properties of cereals, legumes and dry products determined their behavior in supplements. Almost all the properties of the flours differ significantly at $p < 0.05$, for example: cereals had high caloric content but low in protein, so, they must be mixed with legumes that had a high protein value. Also, cereal flours lacked fluidity, due to the greater interaction between the particles, therefore, they were more difficult to homogenize and present greater «cakening». Quinoa, chickpeas, and mushrooms were soluble in milk. However, the spectra showed similar composition in all of them as carbohydrates, protein, and fiber. All flours except quinoa showed a particle size below 420μm.

All the flours can be used for development of supplements, alone or in combination, due to their intrinsic properties and biological value to create stable suspensions, of nutritional quality, that do not lose their characteristics in extreme environmental conditions.

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Rheological behavior of strawberry jam from the design of simplex mixtures using three citrus pectins

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Abstract

This work presents the rheological behavior of strawberry jam using three citrus pectins, one extracted from persian lime bagasse (*Citrus latifolia* Tanaka), and two commercial ones, one from the Drotasa® brand and the other without a brand name. A simplex mixture design with 10 treatments was used, subsequently its rheological properties (*K* [Pa s] consistency index and n [-] flow behavior index) were measured using a rheometer and adjusting the mathematical model of Power Law. The model obtained $\tau = 228.13\ \gamma^{0.237}$ and an $R^2 = 0.9712$, which represents the mixture nine (0.167 extracted pectin, 0.667 Drotasa® pectin and 0.167 commercial unbranded pectin) with a higher percentage of Drotasa® pectin which presented a pseudoplastic behavior.

Keywords: *strawberry jam, citrus pectins, rheological behavior.*

1. Introduction

Rheology is the science that studies the deformation and flow of materials. In rheological studies, the response of a material to applied stress or deformation is observed, so in food manufacturing, it is essential to understand how a substance moves and behaves to transport and mix it during processing.

Also, these rheological characteristics greatly impact the consumer's sensory experience of a food product, for example, in relation to texture and mouthfeel (Bello-Lara *et al.*, 2014). It is therefore of vital importance to define a set of rheological models that represent the most faithfully possible the flow characteristics of the food that the industry treats; these rheological models are based on the experimental calculation of a series of constants that characterize the flow for each food in the operating variables in which it is found. Rheological parameters have been considered as an analytical tool to provide fundamental information on the structural organization of food (Doğan, 2011). The most widely used mathematical model for rheological characterization is that of the Power Law (Eq. 1) due to its great applicability, where the shear force is related to the variation of the shear gradient (Toralles *et al.*, 2006) to evaluate the profile rheology of polysaccharides, such as starch and pectin (Bello-Lara *et al.*, 2014).

$$\tau = K\dot{\gamma}^{n} \qquad \text{(Eq. 1)}$$

Where: τ [Pa] is the shear stress and γ [s^{-1}] is the shear or shear rate (Toralles *et al.*, 2006). The *K* and n values describe the behavior of the fluid. The consistency *K* is an indicator of the viscous nature of the system, resulting in that the higher *K* the more viscous the material is. The flow index n (dimensionless) is a measure of the degree of non-Newtonian behavior. If n < 1 the fluid is pseudoplastic; if *n* > 1 the fluid is dilatant; and if n = 1 it is a Newtonian fluid (Bello-Lara *et al.*, 2014).

Pectin is one of the main components of the primary and middle cell wall in tissues that has been used as an edible coating because it is a non-toxic, biocompatible, and biodegradable polysaccharide (Bello-Lara *et al.*, 2014). It is a high-value functional ingredient widely used as a stabilizing agent in jam and acidified milk drinks (Muhammad *et al.*, 2014). This multifunctionality of pectin is attributed to the presence of polar and nonpolar regions within a molecule, which allows it to be incorporated into different food systems (Barazarte *et al.*, 2008).

From a technological point of view, food additives play an important role in the development of complex foods (Varela and Fiszman, 2013). The extensive use of hydrocolloids, highly hydrophilic polymers, is due in part to their ability to modify the rheological properties of aqueous feed systems (Soto-Caballero *et al.*, 2016).

A design of experiments with mixtures that have q components or ingredients consists of a set of experiments in which combinations or mixtures of those ingredients are tested. If the proportions in which the components of the mixture participate are denoted by x_1, $x_2 \ldots x_q$, two restrictions must be satisfied: $0 \leq x_i \leq 1$ for each component and $\sum_{i=1}^{q} \quad x_i = 1$ (Ortega-Pérez *et al.*, 2015).

It has been observed that the combination of hydrocolloids can lead to higher viscosity of the solutions. The rheological characterization of different mixtures of hydrocolloids is a fundamental part of the development of new formulations and food products (Soto-Caballero *et al.*, 2016).

Jam is an intermediate moisture food prepared by boiling the pulp of the fruit with sugar (sucrose), pectin, acid, and other ingredients (preservatives, colorings, and degreasing materials) to a reasonably thick consistency, firm enough to keep the tissues of the fruit in position (Younis *et al.*, 2015).

Manayay *et al.* (2013) mention that it is important to highlight that the fruit pulp is fundamentally constituted by a dispersion of solid particles in an aqueous solution of sugars, organic acids, salts and pectins; so it is evident that the rheological behavior is governed by the characteristics of the solid phase (shape, size and concentration of the particles), and by those of the liquid phase (nature, shape, size and concentration of the molecular species that compose it).

As the degree of concentration increases in the fruit pulp, the solid particles, initially individual, will become increasingly close to each other, which facilitates the formation of lumps, which in turn join to form aggregates, these also associate to form a network or mesh that traps the dispersing phase, thus causing a strong increase in the rheological parameters upon reaching a certain critical concentration.

That is why the objective of this work was to evaluate the rheological behavior of strawberry jam using a simplex mixture design with three citrus pectins; the first extracted from the bagasse of persian lime (*Citrus latifolia* Tanaka), and two commercial ones, one from the Drotasa® brand and the other without a brand name.

2. Materials and methods

The strawberry was received in primary packaging, it was selected, washed with running water, and crushed manually. For the characterization of the strawberry pulp, the following physicochemical analysis were carried out: soluble solids (°Brix), measured directly in a refractometer (ATAGO digital pocket PAL-3, China), pH through direct reading in a potentiometer (Conductronic PC 45, México) and total acidity by the NMX-F-102-NORMEX-2010 method.

A formulation for the jam was proposed that consisted of strawberry pulp, sugar, and pectin where the simplex mixture design was used (Gutiérrez and de la Vara, 2012), as shown in Table 1, which consisted of evaluating the three types of citric pectins. The first was pectin extracted at the laboratory scale, obtained through acid hydrolysis under conditions of a temperature of 90 ºC, pH 2 and time of 60 min; the second citrus pectin was a commercial brand named Drotasa® and finally, unbranded commercial pectin (with the legend 'citric pectin') purchased in a store in Córdoba, Veracruz, Mexico.

Table 1. Experimental design proposed to describe the rheological behavior in strawberry jam.

Mixture	Persian lime bagasse pectin (E)	Citrus pectin Drotasa ® (D)	Commercial pectin without brand (C)
1	1	0	0
2	0	1	0
3	0	0	1
4	0.5	0.5	0
5	0.5	0	0.5
6	0	0.5	0.5
7	0.333	0.333	0.333
8	0.667	0.167	0.167
9	0.167	0.667	0.167
10	0.167	0.167	0.667

The rheological properties *K* and n were measured with a rheometer (Anton Paar, model MCR301, Austria). A cutting rate range from 2 s^{-1} to 1000 s^{-1} was used, using a straight vane geometry that is suitable for characterizing fluids with suspended particles. All these elements were controlled with the Reoplus/32 software version 2.81 and the results were obtained directly in computer format.

3. Results and discussion

The strawberry pulp presented a pH = 3.35, ºBrix = 4.76 and titratable acidity = 0.0087%; these values are low compared to that reported by Siche *et al.* (2012) in clarified and depectinized orange juice ready to drink, which presented 12 ºBrix and showed Newtonian behavior. Andrade *et al.* (2010) in

zapote pulp showed higher values than that shown in this study, which represents an advantage for the use of this fruit at an agro-industrial level.

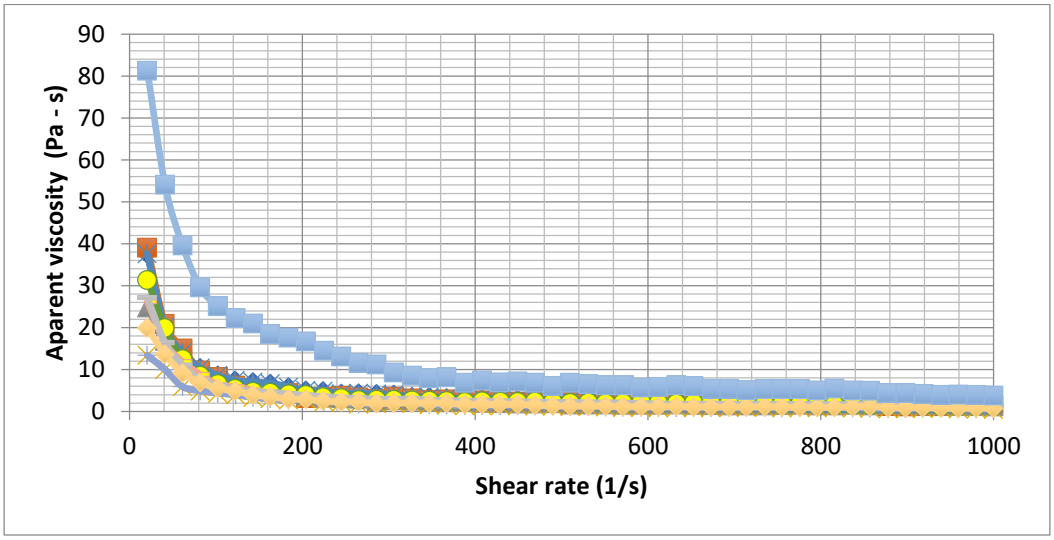

Figure 1. Phenomenological profile of jam viscosity

In Figure 1, a phenomenological profile shows that mixture 10 (0.167 E, 0.167 D y 0.667 C) has a higher viscosity than the rest of the mixtures. All mixtures showed non-Newtonian behavior, in which the relationship between shear stress and shear speed did not remain constant as shear speed increased. The viscosity of all jam mixtures decreases with increasing shear speed (s^{-1}); this behavior is defined as "thinning shear" or pseudoplastic, which occurs when stress disrupts the arrangement of macromolecules within a structural matrix.

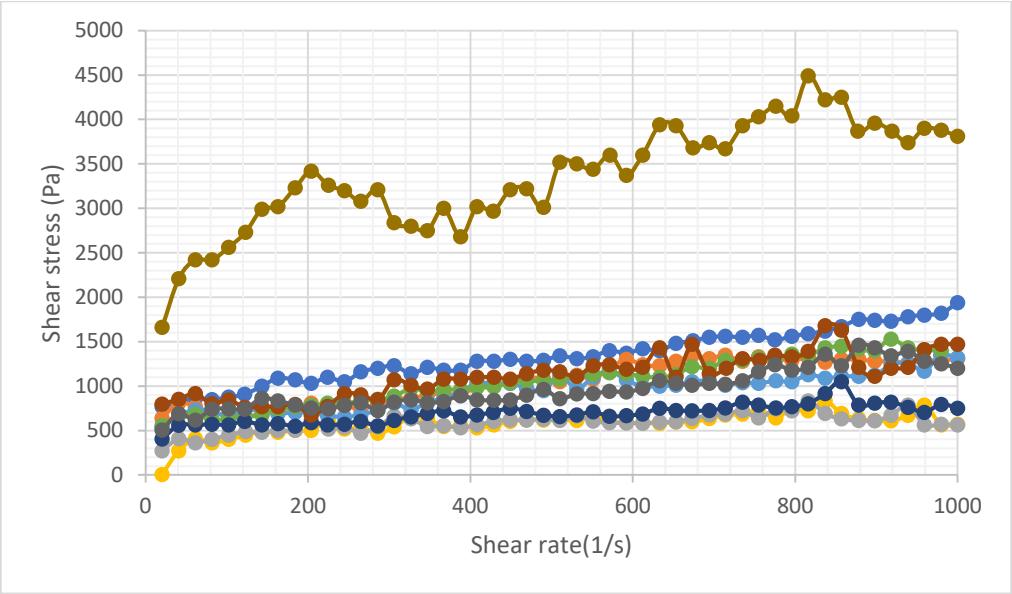

Figure 2. Rheogram of the best strawberry jam settings

All reproductions show non-Newtonian behavior, in which the relationship between shear stress and shear speed does not remain constant as shear speed increases as shown in Figure 2. This indicated that the viscosity decreased when increased cutting speed; this behavior is defined as "thinning shear" or pseudoplastic, which occurs when stress disrupts the arrangement of macromolecules within a structural matrix. The power law model was adequate to fit the experimental data ($R > 0.9573$) and mix nine with a ratio of (0.167 E, 0.667 D y 0.167 C) showing a higher percentage of Drotasa® pectin and showing pseudoplastic behavior.

Bello-Lara *et al.* (2014) in starch and pectin at 1% showed data between 0.7251 and 0.78 in the flow index; these values are higher than those shown in this study. On the other hand, in the consistency index the authors obtained values between 0.0325 and 0.014, which are low compared to the consistency index of the jam mixtures in this study (93.394 to 1022), in the case of the mixture eight with 298.85 and the nine with 228.13.

Strawberry jam presented a strong pseudoplastic behavior since in all treatments the value of the flow behavior index (n) was less than 1, as shown in Table 2. Andrade *et al.* (2010) reported a very small flow behavior index for zapote pulp, which presented a gummy appearance very similar to strawberry

jam. From an industrial point of view, this pseudoplastic behavior facilitates pulp flow and heat exchange during processing, because a decrease in apparent viscosity causes less pressure drop during flow, resulting in less energy demand for processing.

Table 2. Power Law constants for strawberry jam.

Mixture	*K* (Consistency index) [Pa s]	*n* (Behavior index) [-]	R^2
1	93.394	0.3564	0.8443
2	292.9	0.2501	0.9138
3	233.98	0.2474	0.7912
4	179.16	0.1955	0.7621
5	240.31	0.2257	0.7849
6	180.17	0.2918	0.9014
7	265.42	0.1577	0.745
8	298.85	0.217	0.9573
9	228.13	0.2376	0.9712
10	1022	0.197	0.8162

The Power Law model was adequate to fit the experimental data. An $R^2 > 0.9712$ was obtained for mixture 9 (0.167 E, 0.667 D y 0.167 C) which contained a higher proportion of Drotasa® pectin. Furthermore, mix 8 with proportion values of 0.667 E, 0.167 D and 0.167 C, presented R^2 values above 0.9573 Andrade *et al.* (2010) applied the Power Law to zapote pulp ($R^2 \geq 0.9958$), presenting a strong pseudoplastic behavior, since it all treatments the value of the flow behavior index (*n*) was less than 1. Usually, fruit pulp homogenates exhibit this behavior, and in many cases, it can be

attributed to the presence of high molecular weight substances in solution and/or too dispersed solids in the liquid phase.

Doğan (2011) reported values from 0.3711 to 0.4691 in the behavior index (*n*) in kefir samples with honey or pine honey, which are slightly higher than the indices of strawberry jam; and in the consistency index (*K*) presented values of 130 to 204 mPa s in kefir, values much lower than what was obtained in the present study.

Manayay *et al.* (2013) reported values in simple apple pulp of 11 ºBrix and concentrated pulp of 17 ºBrix, with consistency indexes (*K*) (Pa s) from 45.0229 to 112.2320 for simple pulp and 75.0188 to 171.2071 for concentrated pulp, while for the flow behavior index (*n*) it was 0.1685 to 0.2840 for simple pulp and 0.1110 to 0.2304 for concentrated. According to the previous data, it can be seen that mixture 1 of strawberry jam, to which only pectin extracted from Persian lime bagasse was added, showed a similar viscosity to simple apple pulp, and mixtures 4 (0.5 E, 0.5 D, 0 C) and 6 (0 E, 0.5 D, 0.5 C) are more similar to the viscosity of concentrated apple pulp; a flow behavior index was also presented below 1 in both, apple pulp and strawberry jam.

Drotasa® pectin showed better properties in the strawberry jam and later the pectin obtained during the extraction from Persian lime bagasse. It is observed that jam 9 has the best properties and that the adjustment shows a value of $R^2 = 0.9712$, which can be proposed as a good option for the elaboration of jams.

7. Conclusions

All the strawberry jam mixtures presented pseudoplastic behavior; the best fit of the mathematical model was presented in mixtures 8 (with a higher proportion of pectin based on persian lime bagasse) and 9 (with a higher proportion in the Drotasa® pectin), therefore both pectins are viable to be used as an optimal mix and thereby improve the viscosity in confectionery.

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Extraction and physicochemical characterization of pectin from persian lime bagasse (*Citrus latifolia* Tanaka)

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Abstract

Large-scale consumption of citrus produces large amounts of peel as waste that pollutes the environment; these residues are rich in pectin. Pectins are used in the food industry as gelling agents, thickeners, texturizers, emulsifiers and stabilizers, as fat substitutes in low-calorie foods. The objective of this work is to present the pectin extraction process from persian lime bagasse (*Citrus Latifolia* Tanaka) using an acid hydrolysis method, as well as the physicochemical characterization. The methodology was the following: selection of raw material, washing, weighing, enzymatic inactivation, acid hydrolysis; For the analysis of results, a Box-Behnken experimental design with three factors (temperature, time, and pH) was used, followed by filtration, precipitation, drying, grinding, and packaging. Likewise, the quality of the extracted pectin was determined through the equivalent weight, anhydro-uronic acid (AGA) and methoxyls. The yield of the process was 16.19% on a dry basis and the product presented a green coloration. The pectin obtained according to the results presented for AGA and methoxyls, is a low methoxy pectin. With this, it is concluded that pectin extraction is a viable option for the use of Persian lime bagasse, whose conditions that

maximize its performance are: 90 °C hydrolysis with a pH adjusted to 2 and a time of 60 min. The pectin obtained is viable in combination with other citrus pectins.

Keywords: *acid hydrolysis method, anhydro-uronic acid, Box-Behnken experimental design*

1. Introduction

The persian lime (*Citrus latifolia* Tanaka), better known as the seedless lemon, is an oblong to ovoid citrus, with a not very pronounced wide terminal paila 3.8 to 6.6 cm long and even larger, bright yellow when ripe, with slight roughness with 8 to 10 segments (Carrillo *et al.*, 2011). Citrus fruits are cultivated on the five continents; In many producing countries, it is considered the most important fruit, both for the cultivated area, as well as for production and per-capita consumption (Acosta *et al.*, 2012). In 2020, approximately 1.5 million tons of persian lime were produced in Mexico (SIAP, 2021). The state of Veracruz occupies the first national place in terms of production volume of this crop with 787 thousand tons (Contreras *et al.*, 2008; SIAP, 2021). The large-scale use of this fruit inevitably leads to the generation of large quantities of fruit peels, which constitute about half of the fruit's mass (Seixas *et al.*, 2014). These fruit peels are discarded as major waste, causing a substantial burden on the environment. Therefore, it is necessary to find the proper arrangement or means to convert the shells into useful products. Recent studies show that the pericarp, although processed and stored, could be used as raw material for obtaining by-products in the food industry such as dietary fiber and other bioactive components (Seixas *et al.*, 2014). In Mexico, the persian lime by-product industry is especially dedicated to the production of essential oils, while the dehydrated peel is sold abroad where the pectin is extracted. For other citrus fruits, pectin shows an increase of more than 200% in the value of the dry peel, therefore it represents a great profit potential, even higher than clarified concentrated juice and simple juice (Secretaría de Economía, 2002). Additionally, the

process of obtaining pectins is simple so that persian lime juice producers and essential oil extractors could easily implement it and increase their profits by diversifying their products.

The interesting thing about citrus fruits is that they are rich in high methoxyl pectins, which are widely used in industry, not only in food, but also in other areas such as pharmaceuticals and cosmetics (Barazarte *et al.*, 2008).

There are different techniques for the extraction of pectin from plant tissues, which use physical-chemical, microbiological, or enzymatic procedures (Zapata *et al.*, 2009).

Pectins are a group of plant polysaccharides basically structured by D-galacturonic acid molecules linked by glucosidic bonds, where some of the carboxyls can be esterified with methyl or in the form of salt (Barazarte *et al.*, 2008). Pectin is a high-value functional ingredient widely used as a stabilizing agent in jam and acidified milk drinks, for example (Muhammad *et al.*, 2014). This multifunctionality of pectin is attributed to the presence of polar and apolar regions within a molecule, which allows it to be incorporated into different food systems (Barazarte *et al.*, 2008).

Today there is a wide range of microscopy techniques to investigate the structure of biomaterials and thus have a process control to improve the quality of the final products. The use of image processing techniques can provide quantitative information about the microstructure and morphology of biological materials from the obtained micrographs (Domínguez–Fernández *et al.*, 2012).

In this sense, the present work aims at the extraction and physicochemical characterization of the pectin extracted from the bagasse of persian lime (*Citrus Latifolia* Tanaka), not reported until now for our knowledge.

2. Materials and methods

The persian lime (*Citrus Latifolia* Tanaka) was acquired in the municipal market of Cuitláhuac, Veracruz. The characteristics were size 5-7 cm long and 4-6 cm in diameter, weight about 50 g, thin

peel with a slightly yellow-green color free from bruising and mechanical damage and with an optimal degree of maturity. The selected persian lime was washed, cut and the juice was extracted, the bagasse obtained was cut into 1 cm squares.

The bagasse was placed on 30x30 cm stainless steel meshes inside a pilot plant type tray dryer at a temperature of 60 °C for a time of 210 min. The raw material was placed in distilled water in a ratio of 1:6 at a temperature of 98 °C for 15 min. Subsequently, it was filtered and washed with distilled water at a temperature of 40-60 °C. The mixture of the shell was then heated with acidified water in a 1:4 ratio for 40 min, using HCl (Baker) to adjust the pH of the solution. The mixture was then filtered through a 100 mesh (0.149 mm) screen. Following this, isopropyl alcohol (Drotassa®) was added in a 1:2 ratio to precipitate the pectin and allowed to stand for 2 h at a temperature of 4.5 °C. Once the precipitation time had elapsed, the liquid pectin and the supernatant were separated by filtration with a 100-mesh sieve (0.149 mm). This procedure was carried out several times to avoid high concentrations of water and alcohol and consequently decrease the drying time. Finally, for drying, a Felisa® digital oven was used at a temperature of 75 °C for a time of 3 h. To homogenize the size of the particle, it was crushed with the help of a mortar to later sift with a 100-mesh sieve (0.149 mm) to later pack in hermetic bags and finally store them in a dry place, thus avoiding a change in appearance and quality of pectin due to moisture absorption.

To improve the performance of pectin in the acid hydrolysis stage, a Box-Behnken design (Montgomery, 2013) with three factors was used; the first factor was temperature, with levels of 70, 80 and 90 °C. The second factor was pH, with levels of 1.5, 2 and 3. The third factor was time with levels of 40, 50 and 60 min, As shown in Table 1.

Table 1. Factors studied and levels for extraction using Box Henken.

Factors / Levels		-1	0	1
Temperature	X_1	70	80	90
pH	X_2	1.5	2	3
Time	X_3	40	50	60

The response variables were performance, AGA, methoxyls, equivalent weight.

The yields of each treatment were determined, and the pectin samples obtained were characterized according to their physicochemical properties as; equivalent weight% methoxy and% AGA, as indicated by Ismail *et al.* (2012). It was calculated based on the following formula shown below (Eq. 1).

$$Yield\ (\%) = 100 * [\frac{Dry\ pectin\ weight}{Weight\ of\ Persian\ lime\ bagasse}] \quad \text{(Eq 1)}$$

It was determined using the volume of NaOH (Fermont) spent to assess the total titratable acidity, considering that when the indicator (phenolphthalein) turns from pale pink to blue, neutralization has been achieved, and the numbers of equivalent weights are equalized. of the acid component (pectin) and the base (NaOH). Subsequently, the weight of the sample used (0.5 g) and the NaOH equivalents spent in the titration were related.

It was calculated based on the evaluation method of Schultz (1965). For this, the volume of 0.1 N NaOH spent during titratable acidity in the tested sample was considered as titration A. Subsequently, 20 mL of 0.5 N NaOH were added, and they were left to stand for 30 min until the deterification of the pectin occurred. Then 20 mL of 0.5 N HCl were added to neutralize the NaOH. Finally, the dilution was titrated with 0.1 N NaOH (titration B), (Eq. 2).

$$Degree\ of\ esterification\ (DE) = \left[\frac{B}{(A+B)}\right] * 100 \qquad \text{(Eq 2)}$$

It was based on the determinations of the assessment B of the degree of esterification (Ferreira, 1976), and it was calculated by means of the following formula, (Eq. 3):

$$\%\ Me = \left[\frac{meq\ of\ sodium\ hydroxide * PM\ CH_3O^- * 100}{Sample\ weight\ (mg)}\right] \qquad \text{(Eq 3)}$$

Where 31 is the molecular weight (MW) of methoxy (CH_3O^-)

It is based on the valuation method of Schultz (1965); described in section 2.6.3, in this way the esterified uronic carboxyls and total uronic carboxyls were established; anhydro-uronic acid was calculated by the formula described by McComb and McCready (1957), (Eq. 4).

$$(\%)AUA = [\frac{176 * 100 * total\ uronic\ carboxyls\ (meq-g)}{1000}] \qquad \text{(Eq 4)}$$

where 176 = equivalent weight of anhydro-uronic acid (AUA)

3. Results and Discussion

Of the two kilograms of bagasse that were placed on the trays, 200 g of the same were obtained after being exposed to drying. The results of the pectin extraction through the different treatments and the characterization of each product are presented in Table 2.

Table 2. Results obtained in pectin characterization.

E	T (ºC)	pH	t (min)	Rend (%)	Metx (%)	AGA (%)	PE	GE (%)
1	70	1.5	50	0	0	0	0	0
2	70	3.0	50	0	0	0	0	0
3	90	1.5	50	13.02	6.3	68.3	561.3	52.36
4	90	3.0	50	7.58	7.2	66.4	502.6	61.56
5	70	2.0	40	0	0	0	0	0
6	70	2.0	60	0	0	0	0	0
7	90	2.0	40	9.38	4.8	66.2	542.6	41.16
8	90	2.0	60	16.19	5.6	72.1	537.3	44.09
9	80	1.5	40	8.13	3.6	73.6	495.8	27.76
10	80	1.5	60	9.30	4.3	69.8	498.4	34.97
11	80	3.0	40	4.59	7.9	81.3	523.8	55.16
12	80	3.0	60	5.82	8.2	70.2	517.6	66.31
13	80	2.0	50	10.26	13.02	91.3	2500	80.96
14	80	2.0	50	13.48	13.64	95.65	5000	80.96
15	80	2.0	50	9.82	31.62	96.22	2500	186.57

Experiment (E), Temperature (T), Time (t), Yield (Rend), Methoxy (Metx), Galacturonic acid (AGA), Equivalent weight (PE) and Degree of esterification (GE)

The pectin extraction with the best yield from the Persian lime bagasse was 8, carried out at 90 °C for a time of 60 min and pH of 2 with 16.19% on a dry basis. Compared with what Muhammad *et al*.

(2014), in the extraction of pectin from the inner layer of the fresh shell, under conditions of temperature of 73 ºC, time of 67 min, pH 2 and using citric acid as extractant, the yield they obtained was 26.38% but with high humidity value (91.68%). Seixas *et al.* (2014) found that tartaric acid was the best extracting agent with yields between 15.32 and 30.29%. For acetic acid, the yield remained between 9.43 and 12.91% and nitric acid between 9.5 and 13.1%. These results show that the pectin extraction performance not only influences the concentration but also the type of acid. Specifically for this extracted pectin, the methoxyl content is 5.6% which indicates that it is a low methoxyl content pectin, the equivalent weight was 537.3, the AGA value of 72.1% and the color of the pectin obtained was green. On the other hand, in treatment 14, higher values of methoxyl, AGA and equivalent weight were observed, but with a yield of 13.48% on a dry basis. These results indicate that the pectin obtained at 80 °C, time of 50 min and a pH of 2 (experiment 13, 14 and 15) has the property of forming a firmer gel, since according to Chacín *et al.* (2010), the greater the equivalent weight, the greater the gel strength. For methoxy values, a value above 7 indicates that it is high methoxyl pectin.

4. Conclusions

The conditions that maximize the pectin extraction yield from Persian lime bagasse are temperature of 90 °C, pH of 2 and time of 60 min, during the acid hydrolysis step. This treatment showed to have a yield with a recovery percentage of 16.19% on a dry basis but obtaining a low methoxyl pectin. Likewise, it was observed that in the conditions of temperature of 80 °C, pH of 2 and time of 50 min, the equivalent weight, the percentage of methoxyls and the percentage of anhydro-uronic acid (AGA) are like other pectins extracted from various fruits and used commercially but with a high content of methoxyls. Therefore, it is concluded that the extraction of pectin from persian lime bagasse is a viable option for its application in the food industry.

5.Acknowledgments

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Stability, fatty acid profile, and antioxidant capacity of a macadamia nut beverage

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Abstract

This study aimed to develop vegetable beverages based on a macadamia nut and evaluate the beverage's stability and sensory acceptance. Methodology. Macadamia nut beverages were prepared by using an experimental design with a response surface methodology. The independent variables were % macadamia nut, % soy lecithin, % xanthan gum; the dependent variables were particle size distribution [D(3,2), D(4,3), span], and zeta potential. The antioxidant capacity (ABTS and DPPH) and fatty acid (FA) content of the most stable beverage were obtained. Results. Beverages showed a polydisperse distribution, with the presence of a narrower peak and a secondary group of bigger-sized particles. The D(3,2), D(4,3), and span values ranged from 4.5 to 6.1 µm, 14.1 to 41.2 µm, and 3.2 to 17.7, respectively. The zeta potential ranged from -37.3 mV to -41.3 mV. The optimized formulations showed good stability and sensory acceptance in flavor and aroma. The content of saturated FA, monounsaturated FA, and polyunsaturated FA were 16.62, 78.38, and 3.52%, respectively. The % inhibition with ABTS was 16.85% and with DPPH was 4.77%. Conclusion. The macadamia beverages showed acceptable stability and nutritional properties.

***Keywords**: Macadamia nut, vegetable beverage, zeta potential, particle size, antioxidant capacity*

1. Introduction

Even though cow's milk is one of the staples in the global and national diet, the number of people who avoid its consumption increases, either because they follow a diet free of animal proteins or because the milk intake causes consumers certain pathologies (AINIA, 2018).

Nowadays, vegetable beverages consumption has been increasing worldwide (Alcántar, 2017), as they are an alternative to milk for a population sector that desires a diet without animal products or more sustainable, or products with functional properties. That is why new products of vegetable beverages are a business opportunity for the food industry.

In the market, there are some vegetable beverages based on legumes, cereals, pseudo-cereals, seeds, dried, and fresh fruits (Trejo, 2015). The amount of nutrients in vegetable beverages varies considerably between the different types. Their nutritional properties (lactose-free, low in saturated fat, among others) depend on the raw material used, processing, and fortification with vitamins and minerals, and the addition of other ingredients such as sugar and oil (Thorning *et al.*, 2016).

The macadamia nut is an option to produce vegetable beverages. It is a food with high monounsaturated fatty acid content and antioxidant compounds (tocopherols and polyphenols). Due to this, it is a food of excellent quality and nutritional value (Wall, 2010).

Therefore, the present work's objective is to formulate a vegetable beverage made with macadamia nuts, besides evaluating its stability and sensory characteristics, and quantifying its antioxidant capacity and total fatty acid content.

2. Materials and methods

2.1 Raw materials

Macadamia nut (*Macadamia integrifolia*) acquired in bulk in Mexico City; soy lecithin powder brand G-EX®; and xanthan gum brand Maprysa®.

2.2 Formulation and experimental design

In the first stage, a Taguchi experimental design L9 3^3 was used to obtain the optimum operating conditions. The independent variables were macadamia nut (4.0, 6.0 y 8.0%), soy lecithin (0.1, 0.2 y 0.3%), and the grinding temperature (50, 60, 70 °C). In a second stage, a new mix design was considered with macadamia nuts (4-6%), soy lecithin (0.3-2%) and xanthan gum (0.08-0.50%). The experimental design and optimization procedure was carried out through the Minitab® software version 17. The mix processing was done in a commercial blender. Nine formulations were prepared in duplicate.

2.3 Stability parameters

The particle size distribution was studied through the parameters Sauter diameter [D(3,2)], Brouckere diameter [D(4,3)], and polydispersity index (span) measured with a particle size analyzer (Malvern IM 026). Moreover, the zeta potential (ζ) was measured with a zeta potential analyzer (ZetaPlus 21471), according to Bernat *et al.* (2015).

2.4 Sensory analysis

Affective testing was carried out with 11 untrained judges (7 females, 4 males) to evaluate the higher preference (3) and lower preference (1) for consistency (like cow's milk), aroma, and flavor of macadamia nut beverages. The analysis was made for the three most stable formulations according to stability parameters. The results were analyzed using the Kramer Table of total categories necessary for a significance of 5% ($p < 0.05$).

2.5 Total fatty acid content

The methyl esters of fatty acids from the lyophilized sample were analyzed by gas chromatograph (PerkinElmer Clarus 500), according to Hernández-Martínez *et al.* (2013). Only the macadamia nut optimized beverage was analyzed.

2.6 Antioxidant capacity

The antioxidant capacity of the lyophilized sample was analyzed by using DPPH radical (1,1-diphenyl-2-picrylhydrazyl) (Brand-Williams *et al.*, 1995) and by ABTS radical cation (2,2'azinobis-(3-ethylbenzothiazoline -6-sulfonate) (Re *et al.*, 1999). Trolox's standard curve was used to calculate the antioxidant activity, and results were reported as the percent of radical inhibition.

3. Results and discussion

3.1 Particle size distribution and zeta potential

The particle size distribution of vegetable beverages shows two populations (bimodal distribution) with a heterogeneous size particle population (polydisperse distribution). One population with a particle size between 2.19 µm and 2.55 µm, and another between 11.10 µm and 15.00 µm. In formulations, the Sauter diameter [D(3,2)] values ranged from 4.39 µm to 16.01 µm, and the Brouckere diameter [D(4,3)] values ranged from 7.24 µm to 31.16 µm. The lowest D(3,2) and D(4,3) values were presented in the formulations with a lower macadamia amount. The polydispersity index ranged from 1.58 to 8.08.

Considering the particle size, the beverage's stability is mainly influenced by the percentage of macadamia nut and soy lecithin and not so much by the temperature of mixing. Figure 1 shows the main effects of independent variables over the dependent variables.

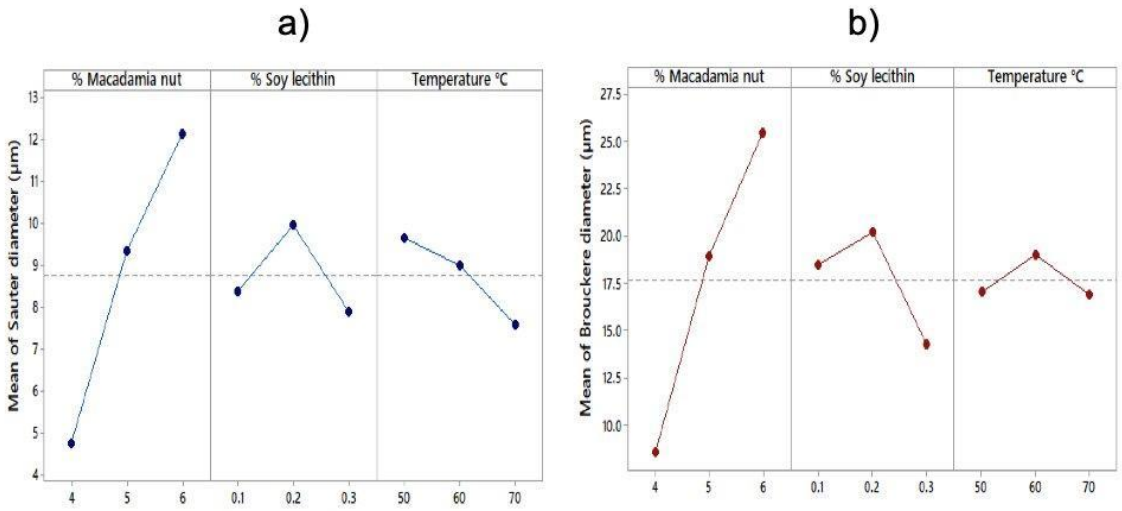

Figure 1. Main effects plot for a) Sauter diameter D(3,2) b) Brouckere diameter D(4,3) of macadamia nut beverages.

However, after a week of refrigerated storage, the beverages were not stable. Thus, a new mix design was proposed (see methodology) that included xanthan gum, and the grinding temperature was set at 70 ºC. Figure 2 shows the results of analyzing the mixture design.

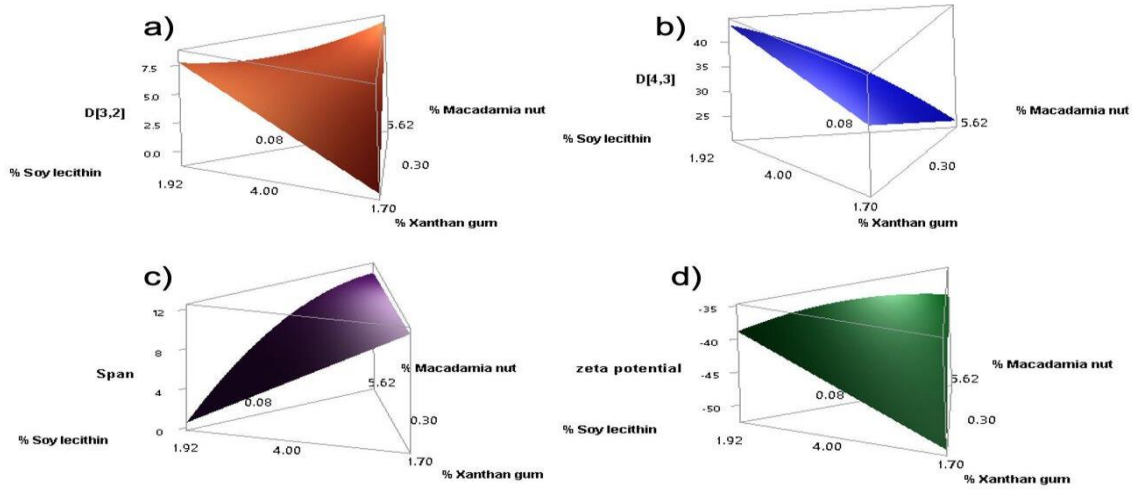

Figure 2. Effect of macadamia beverage ingredients on a) Sauter diameter [D(3,2)], b) Brouckere diameter [D(4,3)], c) polydispersity index (Span), and d) zeta potential.

The D(3,2), D(4,3), and Span values ranged from 4.5 to 6.1 µm, 14.1 to 41.2 µm, and 3.2 to 17.7, respectively. In general, the stability increases with low macadamia nut and lecithin content and a high amount of xanthan gum. According to Stoke's law, a decrease in the particle's average diameter can decrease the coalescence rate (Bergenstahl and Claesson, 1990).

Regarding the zeta potential (ζ), all the formulations presented values lower than -30 mV; it ranged from -37.3 mV to -41.3 mV. According to Roland *et al.* (2003), these values guarantee the stability of the emulsions.

Analyzing the particle size in Minitab®, the optimized mixture design indicated that the formulation containing 4.39% macadamia nut, 1.13% soy lecithin, and 0.48% xanthan gum provides the lowest values for D(3,2), D(4,3), and span. This optimization proposal is a successful approach to have a product that can be stable for several months in storage.

3.2 Sensory analysis

Sensory analysis was carried out for the three most stable formulations. Table 1 shows the three formulations tested; the code in the table corresponds to the code presented to judges.

Table 1. The three most stable macadamia nut beverage formulations.

Code	Macadamia nut (%)	Soy lecithin (%)	Xanthan gum (%)
HI2	4.39	1.13	0.48
KL3	4.95	0.65	0.40
ML1	4.71	1.01	0.29

Figure 3 shows the sensory analysis results. The attributes of consistency, flavor, and aroma did not show a significant difference. However, the degree of acceptance of the beverages was HI2> KL3> ML1 for consistency; KL3> ML1> HI2 for flavor; and KL3> ML1> HI2 for aroma. The judges mentioned that the beverages had a pleasant flavor, but the consistency was viscose, especially in the

sample ML1 (optimized formulation). Thus, the emulsifiers could be modified to improve consistency.

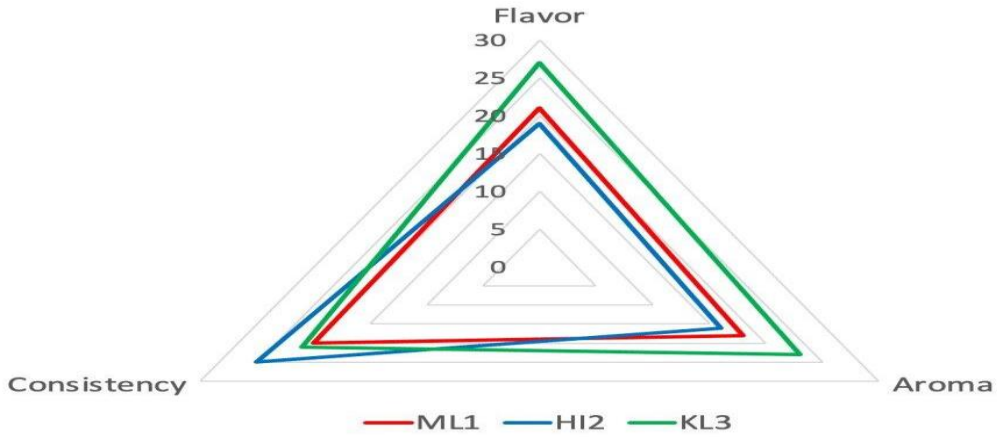

Figure 3. Sensory evaluation of macadamia nut beverages.

3.3 Total fatty acid content

The total fat of macadamia nut optimized beverage was 2.9 g/100 g. Regarding fatty acid content reported as % of total fatty acids, the percentage of saturated fatty acids (SFA) was 16.62%, and the main fatty acid was the palmitic fatty acid (C16:0); the percentage of monounsaturated fatty acids (MUFA) was 78.38%, and the main was oleic fatty acid (C18:0); the percentage of polyunsaturated fatty acids (PUFA) was 3.52%, and the main was linoleic fatty acid (C18:2). Compared with an almond beverage (Gallier *et al.*, 2012), the macadamia nut beverage has a higher MUFA content but a lower PUFA content.

3.4 Antioxidant capacity

Regarding the antioxidant capacity, for ABTS, the macadamia nut beverage showed 16.85 ± 1.44% of radical inhibition; and for DPPH, values of 4.77 ± 0.17% of radical inhibition. Other vegetable beverages showed a lower antioxidant capacity by DPPH. Wansutha *et al.* (2018) in almond beverages reported 3.16% inhibition of DPPH radical and 44.6% inhibition of ABTS radical.

4. Conclusion

The macadamia nut beverage is a good choice of vegetable beverage due to its nutritional properties, such as the high content of oleic fatty acid and its antioxidant capacity. This beverage is an unstable diluted emulsion; thus, it must maintain its stability as processed food. The present work provides information on formulation possibilities. Based on particle size analysis, the most stable formulation for the beverage was 4.39% macadamia nut, 1.13% soy lecithin, and 0.48% xanthan gum. However, the beverage's adequate stability does not guarantee sensory acceptance, so this last attribute must be considered a response variable in the optimization process. The beverage's sensory acceptance is adequate, although other formulations could be tried to decrease viscosity and increase sensory acceptance. Formulation with less xanthan gum could be considered; this reduces stability but gains sensory acceptance. The use of other hydrocolloids such as arabic gum, guar gum, or carrageenan could be tested in future research.

5.Acknowledgements

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Physicochemical composition and nutrient value of brewers spent grain (BSG) for products formulation

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Abstract

One of the main byproducts from the craft brewing industry are the brewers' spent grain (BSG) obtained after the craft brewing processes. Its potential use to formulate added value animal nutrition products, represent an attractive opportunity for the agro-industry; however, the main usage in Costa Rica is for cattle feeding in its raw presentation, due to the material's high moisture and its rapid degradation. The present investigation focuses on the nutritional characterization and drying study of BSG from a commercial microbrewery process, and a pet snack formulation. The drying conditions were determined by a factorial design based on the operating characteristics of an automated air circulation dryer. The nutritional composition of the BSG and the formulation designed was determined by means of an NIR chemical analysis of the dry products. The best drying conditions, to obtain a product with a moisture percentage less than 10 %, were determined as a mechanical pressing at 800 lb (55.3 psi, 3 min), followed by a drying operation at 60°C, a speed of 1.4 m/s and a layer thickness for the sample of 0.030 m. NIR chemical analysis of the dry grains resulted in 14.2% proteins, 2.44% fats y 9.51% fiber (% m/m of dry mass). NIR chemical analysis of the final product design resulted in 12.36% crude protein, 7.12% ethereal extract and 5.6% crude fiber (% m/m of dry

mass). Results obtained demonstrate that the proposed drying conditions allow to obtain a dry barley byproduct malted that constitutes a valuable material for the animal feed product formulation.

Keywords: *Composition and Nutrient Value, Brewers spent grain (BSG), agro-industrial byproduct.*

1.1 Introduction

Brewers` spent grain (BSG) is one of the main byproducts from the craft brewing industry, these are obtained from the process of extracting fermentable sugars from grains, mainly barley, at a stage known as mashing. This process is complex, and it is one of the main steps in defining the body and alcohol content of beer (Palmer, 2006).

BSG have a high content of protein (19-30% w/w), lipids (10% w/w) and fiber (30-50% w/w), and they are also made up of other molecules of nutritional interest such as amino acids, vitamins, and minerals (Lynch *et al.*, 2016). Studies have even identified the presence of other bioactive compounds including alkaloids, antibiotics, plant growth factors, food-grade pigments, and phenolic compounds, which have been related to the prevention of chronic diseases (Ikram *et al.*, 2017).

Estimations show that from 100 kg of malt, there is 100 to 130 kg of wet BSG (Bianco *et al.*, 2020), which makes it a highly valued by-product, especially if you consider its nutritional contribution and low cost. However, since it has a high moisture that varies between 70% and 80% (Farcas *et al.*, 2014), it is susceptible to deterioration in short periods of time, which is why it is mainly used in cattle feeding and composting (Farcas *et al.*, 2014), or simply discarded when small quantities come from small-scale breweries.

One of the strategies to improve storage time is to dry grains up to moisture percentages below 10%; several drying mechanisms have been currently studied, including direct rotary-drum dryers, steam drying, freeze drying, oven drying, superheated steam and freezing methods (Aliyu and Bala, 2011; Ikram *et al.*, 2017; Tang and Cenkowski, 2001), most of these techniques apply as a first step the

pressing of the material to reduce the moisture percentage to values close to 65% and it is estimated that dry BSG is stable for up to 6 months after the final drying process (Ikram *et al.*, 2017).

Despite advances in the characterization of the drying process, small breweries cannot cover costs and waste continues to be undervalued. Costa Rica is not an exception, with more than 80 craft breweries, BSG production is mainly used for livestock feed. To propose an option for the BSG reuse produced by a craft brewery, this project was carried out, with two main objectives; one objective was to identify the nutritional and drying characteristics of BSG, the other objective was the design of a snack for dogs based on it.

2.1 Materials and Methods

2.1.1 Materials

The BSG used in this study was collected from a craft brewery located in Heredia (Costa Rica).

2.1.2 Drying Analysis

2.1.2.1 Moisture

The method established by AOAC number 981.05 was followed.

2.1.2.2 Press Tests

A Carver brand hydraulic press was used, the tests were done, in triplicate, applying loads of 1800 lb, 1600 lb, 1400 lb, 1200 lb, 1000 lb, 800lb, 600 lb, 400 lb, 200 lb and a manual force for a time of 3 min, 600 g of fresh BSG was used for each test.

2.1.2.3 Experimental design to determine drying conditions

An automated air circulation tray dryer was used through the solid. Measurements were made to reach 7% of residual wet. A factorial design 2^3 was applied considering as experimental factors the inlet air temperature (T_0, levels 50 °C and 60 °C), the speed of the inlet air (v_0, levels 1.0 m/s and 1.4 m/s) and the thickness of the BSG layer (δ, levels 0.015 m and 0.030 m). The temperature was selected as described by various authors such as Ikram *et al.* (2017), which determined that temperatures above

60 °C affect the taste of the grain and an undesired brown coloration is generated in the dry product. As a response variable, the drying time(s) was selected, considering as independent variables the ambient temperature, the moisture of the inlet air and the moisture of the outlet air. Based on the results of the press tests, it was decided to use two loads, manual and 800 lb. The data were statistically analyzed with the Minitab software and normality, variance and independence were verified, as well as the effects of the variables were analyzed.

2.1.3 Nutritional characterization

2.1.3.1 Proximal analysis of the dry sample

Proximal analysis, including the determination of protein, fiber, and ethereal extract, was performed using the NIR technique (Foss Model 5000 equipment).

2.1.3.2 Wet chemistry testing

The wet chemistry analysis was performed at the Research Center on Animal Nutrition (CINA) of the University of Costa Rica, to verify the results obtained by the NIR method. The laboratory performed the following determinations: crude protein (AOAC 2001.11), ether extract (Misir *et al.*, 1985), ash (AOAC 942.05), crude fiber (AOAC 962.09).

2.1.4 Design of a snack for adult dogs

The ALLIX formulation program, developed by A-Systems (2018), was used, whereas it was intended to develop a snack for adult dogs, certain nutritional indicators required by the species and accepted for that type of product were defined as formulation criteria: fatty acids, minerals, amino acids, ashes, dry matter content, crude fiber, ethereal extract, starch content, and others (Murillo 2018).

3.1 Results and discussion

3.1.1 Influence of the use of a hydraulic press

According to Geankoplis (2006) in some types of materials, a treatment prior to drying can be carried out through the extraction of water present in the material by means of mechanical methods, such as the use of presses and centrifuges, among others. Therefore, reducing the amount of water to be extracted by thermal methods is reflected in greater energy savings and, consequently, in an economic and environmental benefit.

The initial mechanical dewatering stage of spent grains offers a cost-effective method to remove free moisture to optimize the drying conditions as described in previous research (Orsat *et al.*, 1996). Many breweries have plants for BSG processing using a two-step drying technique, where the water content is first reduced to less than 60% by pressing, followed by drying to ensure the moisture content is below 10% (Santos *et al.*, 2003).

The initial moisture of the BSG samples in this study was estimated at 67.5±0.8%, coinciding with what was reported in the literature (Farcas *et al.*, 2014).

With the application of hydraulic pressing no statistical difference was found with the use of forces greater than 800 lb and a holding time of 3 minutes, since between 800 lb and 1800 lb the variation in moisture was 45% to 43.6% respectively.

These results exceed those obtained by Crowe (2019) who found the optimal condition to reduce the maximum amount of water through lab-scale mechanical pressing. As a maximum, water content was reduced by 35%, which resulted from a pressure of 1.19 MPa and holding time of 2 min and 44 s. The variations may be due to the type of mechanical press used and brew variety.

A press with vacuum drying may be considered since water loss efficiency has been shown to increase to 59% water removal using a filter press and membrane planes with vacuum drying (Machado *et al.*,

2016) however, due to the availability of laboratory equipment, a hydraulic press was used in this study.

3.1.2 Drying conditions

The results obtained showed the best working conditions, with a mechanical pressing at 800 lb (55.3 psi) for 3 min, followed by a drying operation with an air temperature at 60 °C, a speed of 1.4 m/s and a layer thickness for the sample of 0.030 m. Response times were measured when moisture percentages reached 7%, the above in order to give a safety margin and ensure that a moisture percentage less than 10%. Response times varied between 19.00±2.83 s and 41.50 ± 4.95 s.

The study showed that significant variables are the air temperature and the thickness of the sample. The main effects analysis allows us to appreciate that at high values of temperature and air velocity, using low sample thickness, the best conditions are obtained in response to the BSG drying process. As expected, optimal conditions are high air velocity (1.4 m/s), high air temperature (60 °C), and low BSG layer thickness (0.015 m). Analyzing the behavior of the response time considering the thickness, it was identified that by doubling the thickness from 0.015 m to 0.030 m, the response increases in 1 min, which represents an acceptable working condition.

This behavior in the data coincides with the studies carried out by Arranz (2018) who carried out a drying analysis for BSG for various temperatures and inlet air flow by means of a convective dryer. The thickness of the sample on each tray was less than 1 cm. Several tests were carried out, with different drying air velocities (v = 0.8, 1.2 and 1.6 m/s) and temperatures (T = 30, 40 and 50 °C). As expected, drying of BSG is produced in a shorter period the higher the temperature of the experiment is. For the same temperature, the drying process is slower the lower the air flow is. In this way the highest speed in the decrease in moisture ratio is achieved in 1.6 m/s and 50 °C.

Another study conducted by Dominguez (2019) compared the drying kinetics of BSG in a tray dryer and in a pulsed fluidized bed (PFB) dryer with different combinations of temperature and air velocity

and found that a moisture reduction of 90% was four times faster at 90°C and 1.7 m/s than a 70 °C and 1.0 m/s in the tray dryer.

3.1.3 Nutritional characterization of the BSG

Table 3.1 shows the results of the characterization of the five types of beer produced in the brewery. The statistical analysis of the results showed that there are significant differences in two of the four variables studied, specifically between the protein and fiber content of two beers. For the comparison of the mean values of the samples, a single factor ANOVA was applied, by means of the Tukey test at 95%. It is established that there is a significant difference between the protein value in beer style 1 residue and the remaining ones. In addition, there is a difference in the fiber value of the beer style 3 residue compared to the other samples.

Table 3.1 Proximal NIR analysis of dry residue samples by beer type

Beer style	Crude Protein (% w/w)	Fat / Ether extract (% w/w)	Crude Fiber (% w/w)	Moisture (% w/w)
1	13.42 ± 0.10	2.43 ± 0.10	9.10 ± 0.07	10.18 ± 0.02
2	14.27 ± 0.10	2.52 ± 0.06	10.01 ± 0.14	9.98 ± 0.19
3	14.18 ± 0.11	2.02 ± 0.27	9.52 ± 0.25	9.73 ± 0.32
4	14.76 ± 0.16	2.53 ± 0.21	9.74 ± 0.14	9.63 ± 0.15
5	14.35 ± 0.24	2.68 ± 0.11	9.17 ± 0.09	9.94 ± 0.19

Considering the observed variability, it was decided to characterize a sample composed of grain residues in equal proportions, to carry out the development of the snack from equal mixtures of waste. Table 3.2 shows the results obtained by the NIR method and the characterization by wet chemistry, proving that there is a similar trend. Additionally, and considering the importance of fatty acids for animal nutrition, a characterization of these in the composite sample was performed. As shown in Table 3.2, linoleic acid content was found that has been related to functions such as strengthening the

immune system, synthesis of hormones, reduction of blood cholesterol level, antioxidant capacity and increase of muscle mass (Hand *et al.*, 2000). Glycine also plays an important role in acting as a neurotransmitter and helping to improve physical performance and slow muscle degeneration (Case *et al.*, 2013).

Table 3.2 Results of the nutritional characterization of the BSG composite sample

Proximate Analysis			Fatty acid profile	
Assay	Wet chemistry %w/w	NIR %w/w	Assay	%w/w
Crude Protein	15.34 ± 0.25	15.08 ± 0.16	Linoleic acid	13.10 ± 0.08
Crude Fiber	9.23 ± 0.12	9.27 ± 0.09	Benzoic acid	15.37 ± 0.08
Ash	2.20 ± 0.10	-	Saturated	39.74 ± 0.50
Ether extract	-	2.9 ± 0.12	Monounsaturated	35.29 ± 0.29
Moisture	8.71± 0.39	8.56 ± 0.17	Polyunsaturated	24.98 ± 0.18
-	-	-	Glycine	5.73 ± 0.10

3.1.4 Design and nutritional composition of a snack

To formulate the final snack, the barley residue was chosen as the highest proportion in the diet (56% w/w), considering the nutritional contribution determined. In addition, the formulation included other raw materials such as rice flour, wheat flour, chicken fat, hydrolyzed chicken liver, source of calcium, salt, eggs and alquermold. The design formulation was analyzed to determine its composition, obtaining the values shown in Figure 3.1.

Figure 3.1. Design and nutritional composition of the formulated snack.

In this study, the BSG mainly replaced 100% of the yellow corn, soybean hull, soybean meal and a portion of the wheat flour of the standard pet snack formulation designed with ALLIX formulation software, maintaining a minimum of 12% of crude protein and going from 17 to 9 ingredients.

4.1 Conclusion

The best drying conditions determined (manual hydraulic pressing at 800 lb per 3 min and drying operation at a flow rate of 1 m/s, thickness of 0.03 m for grain bed and an air temperature of 60 °C), allow to have a dry by-product with humidity percentages less than 10% w/w that facilitates its handling and subsequent use. The results are comparable with those obtained by other authors.

The nutritional characterization of the product demonstrated that it is a valuable complement in the design of snack for dogs, adding in amounts greater than 50% w/w of dry product to the formulation, allows to replace other grains, such as rice and corn, normally used in snack formulations as a source of fiber and protein, which simplifies the production process and eliminates costs associated with the purchase of other ingredients.

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Algmatine reduces the toxicity of the β-amyloid peptide (25-35) in human neuroblastoma cells: A preliminary report

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Abstract

Alzheimer's disease is associated with a pattern of pathological changes such as the presence of senile plaques and neurofibrillary tangles that occur in the brain, usually in older adults which results in neurodegeneration and progressive development of senile dementia. The β-amyloid peptide (Aβ) is the main component of senile plaques, this peptide from 39 to 42 amino acids has a folding that allows it to start an aggregation process until it forms intra or extracellular molecular aggregates that lead to the deterioration of cellular functions and eventually to cellular death probably through NF-κB pathway. Furthermore, agmatine is an endogenous ligand of imidazoline receptors. It has been shown that stimulation of the imidazoline receptors protects against cell death induced by stress stimuli. In this work, we analyzed the effect of AB on SH-SY5Y cells treated with agmatine and the expression of NF-κB by western blot. This work demonstrates that agmatine protects SH-SY5Y of the cell death induced by AB probably via inhibition of NF-κB.

Keywords: *Alzheimer, Agmatine, β-amyloid peptide*

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is the leading cause of dementia in older adults. Symptoms of this disease include progressive memory loss, difficulties in planning and solving problems, confusion in time and place, alterations in oral and written expression, difficulties in making a judgment, and alterations in mood and personality (Schachter and Davis, 2000). In 1906, when the German neuropsychiatrist Alois Alzheimer analyzed the brain of a patient, found two main histopathological features: the formation of neurofibrillary tangles at the intraneuronal level and the appearance of senile plaques in the extra neuronal space (Hippius and Neundorfer, 2003). Later, neurofibrillary tangles were found to consist primarily of a microtubule-associated protein, tau protein, in an abnormally phosphorylated state. In contrast, senile plaques were primarily composed of the Aβ peptide, which is why they are also called amyloid plaques. In 1992, Hardy and Higgins suggested that the key step for the development of this disease is the production and aggregation of the Aβ peptide, a hypothesis known as the amyloid cascade. Currently, these histopathological changes are the clinical features of AD

The β-amyloid peptide (Aβ) is the main component of senile plaques, this is a peptide of 39 to 42 amino acids that, once formed, acquires a β-folded formation. The folding that this peptide possesses allows it to begin an aggregation process that is stabilized by hydrogen bonds to form intra- or extracellular molecular aggregates that lead to the deterioration of cellular functions and eventually to cell death (Gómez, 2002).

The production of Aβ peptide is a normal process, but in a small number of individuals, overproduction of all Aβ peptides, or a greater proportion of the 42 amino acid form, appears enough to cause the early onset of Alzheimer's disease (Dominic *et al.*, 2007).

The nuclear factor kappa B (NF-κB) is a dimeric protein, considered as a regulator of a wide range of genes involved in cellular responses to inflammatory and stress signals, and it also plays a key role

in physiological processes, such as development or immune function. It is produced in numerous cells and is activated by a series of stimuli. (Cristina *et al.*, 2016), curiously NF-κB increases the expression of the APP protein, which is necessary for the formation of senile plaques (Tilstra *et al.*, 2011), and the stimulation of NF-κB via the Aβ peptide increases cytokines levels in AD. Contrastingly, the suppression of NF-κB in microglial cells decreased neurotoxicity. The evidence described above indicates that NF-κB plays an important role in the formation of senile plaques in AD.

Agmatine is an endogenous polyamine synthesized by decarboxylation of L-arginine by arginine decarboxylase (Aricioglu-Kartal *et al.*, 2002; Battaglia *et al.*, 2010), binds to high-affinity receptors located on the cell surface such as α_2 adrenergic receptors, imidazoline receptors, N-methyl-D-aspartate (NMDA) receptors, among others (Molderings *et al.*, 1999). Currently, it is known that this amine induces neuroprotective effects at 50 or 100 mg/kg in experiments carried out in vivo or experiments carried out in cultured neurons. Also, this amine can inhibit the formation of some end products of glycation, which is a process that is involved in damage to extracellular matrix proteins, and this damage is involved in the pathology of neurodegenerative diseases (Vlassara *et al.*, 1994). On the other hand, it has been shown that exogenous agmatine showed a beneficial effect, for example by inhibiting neuronal death, in models in which animals with neurodegenerative disorders such as Parkinson's and Alzheimer's were used (Condello *et al.*, 2011).

In 1984, Bousquet *et al.* (1984) described the existence of cerebral sites whose stimulation with imidazoline drugs induced hypotensive effects (Bousquet *et al.*, 1984). Due to this peculiarity, these sites were called imidazoline receptors. Pharmacological studies have allowed the characterization of three main classes of imidazoline receptors: I) I_1 receptors, located in the RVLM, involved in the inhibition of cardiovascular sympathetic tone to lower blood pressure, II) I_2 receptors are widely distributed in the Central Nervous System, and III) I_3 receptors located in pancreatic cells. I_1 receptors bind moxonidine with high affinity, I_2 receptors have a high affinity to idazoxan, and I_3 receptors bind

efaroxan with high affinity (Head *et al.*, 2006). Specific antagonists such as AGN192403 (I_1), BU224 (I_2), KU14R (I_3) are known (Cobos-Puc *et al.*, 2009; Mayer *et al.*, 2002).

2. Objective

The aim of this work was to analyze the effect of agmatine administration on the neuronal death induced by the β-amyloid peptide$_{(25-35)}$ in human neuroblastoma cells (SH-SY5Y).

3. Methodology

The growth medium DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% Streptomycin/Penicillin was used. SH-SY5Y cells were seeded in a complete medium (DMEM/F12-10% FBS-1% Streptomycin/Penicillin) under optimal conditions for culture. After a previous analysis of cell density, SH-SY5Y cells were seeded in 96-well plates at a rate of 10,000 cells per well. After 24 h, the culture medium was removed from each of the wells of the plate by absorption, and then 100 µL of the following treatments were added: agmatine (500, 1000, and 2000 nM), Aβ peptide (0.1, 1, and 10 µM) and/or their combination, were incubated for 24 h at 37 °C with 5% CO_2. Cell viability was determined by the neutral red recapture assay.

A Western Blot analysis was performed, for this, $2.5x10^5$ cells were seeded in 6-well plates, and they were pre-treated with agmatine (500, 1000, and 2000 nM) and Aβ peptide (0.1, 1, and 10 µM). Human NF-κB p65 (rabbit: 1: 2000), the human anti-β-actin loading control (rabbit: 1: 10000), and the mouse anti-rabbit IgG secondary antibody (1: 20000). Bands were visualized by chemiluminescence. The bands obtained were subjected to densitometric analysis using ImageQuant v8.1 software. All treatments were corrected using load control.

The data were analyzed using an analysis of variance (ANOVA) and a Tukey post-hoc test was applied using GraphPad Prism v7.0 software. When the probability value (p) was ≤ 0.05, it was considered statistically significant.

4. Results and discussion

We observed that the deprivation of the culture medium (1% FBS) significantly reduced cell viability compared to the culture medium with 10% FBS (Figure 1). On the other hand, cell viability was not modified in cells treated with agmatine and culture medium supplemented with 10% FBS. However, when agmatine was administered in a culture medium deprived of FBS (1%), the cell viability increased in a concentration-dependent manner (Figure 1).

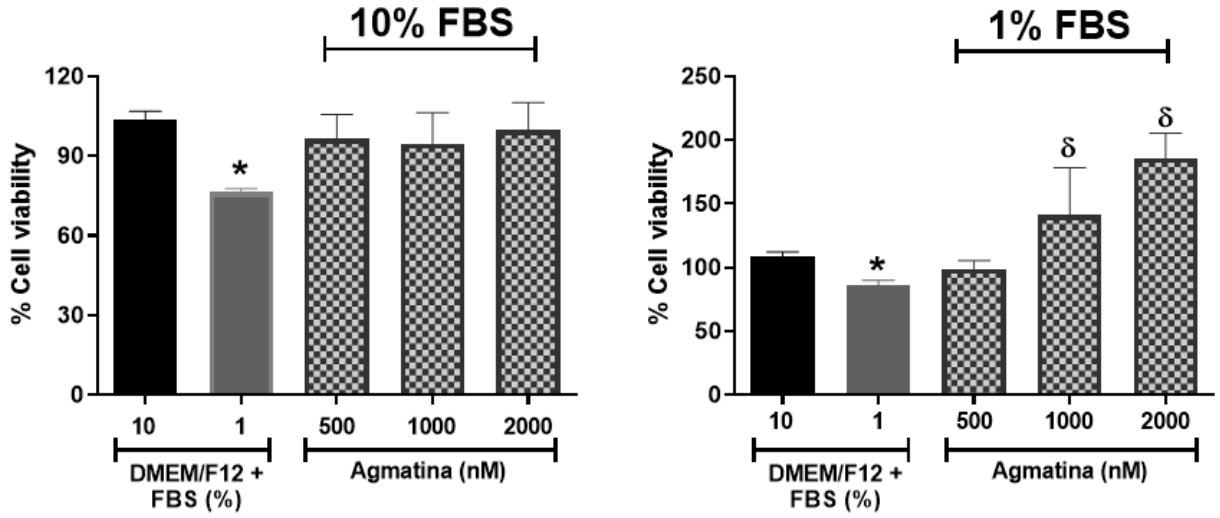

Figure 1. Effect of agmatine on cell viability in the presence of control conditions (10% FBS) and FBS-deprived SH-SY5Y cells.

Under conditions that promote the toxicity of SH-SY5Y cells (culture medium supplemented with 1% FBS), the effect of the β-amyloid peptide$_{(25-35)}$ (0.1-10 µM) on cell viability was evaluated. Here, we observed that the administration of the β-amyloid peptide$_{(25-35)}$ (1 and 10 µM) further reduced cell viability. The neurotoxic effect observed with the β-amyloid peptide $_{(25-35)}$ was dependent on the concentration (Figure 2).

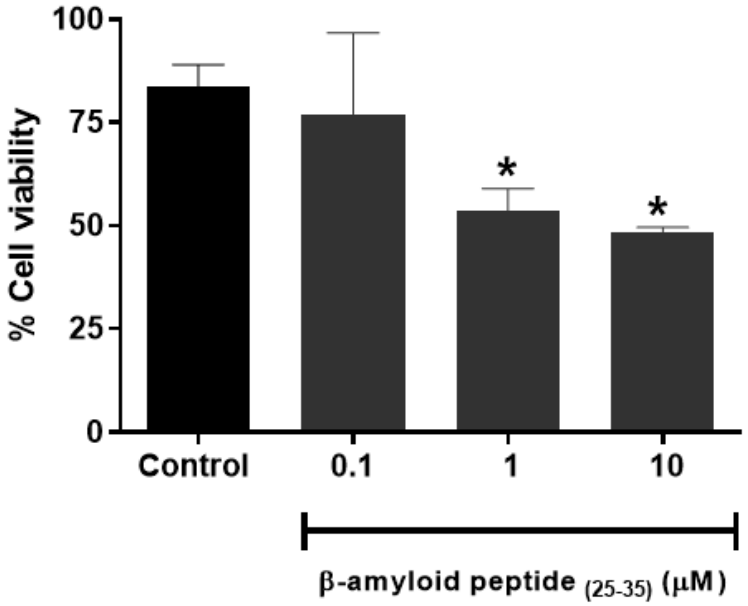

Figure 2. Effect of the β-amyloid peptide on the viability of SH-SY5Y cells deprived of FBS

Figure 3 shows the effects of agmatine (500-2000 nM) on the toxic effect of the 10 μM β-amyloid peptide$_{(25-35)}$. Agmatine reverses the neurotoxic effect of the β-amyloid peptide $_{(25-35)}$ from 1000 nM in a concentration-dependent manner.

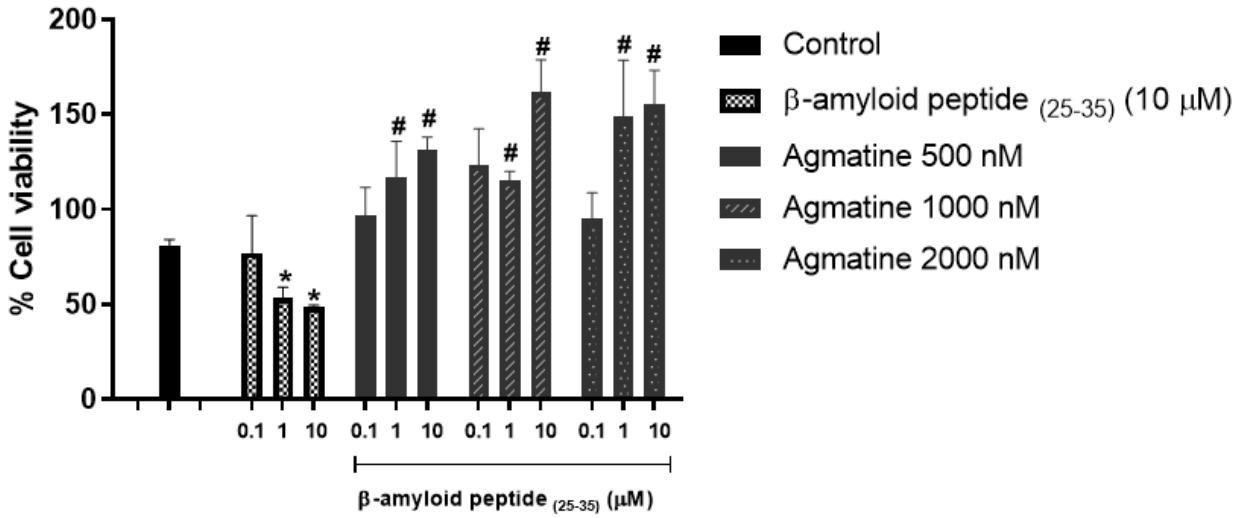

Figure 3. Effect of agmatine on the neurotoxicity of the β-amyloid peptide in SH-SY5Y cells.

Expression of NF-κB was evaluated to identify the possible intracellular signaling mechanisms that are involved in the effect of agmatine (2000 nM) on the viability of SH-SY5Y cells treated with β-amyloid peptide $_{(25-35)}$ (10 µM). The results obtained show that agmatine reduced the expression of NF-κB. As expected, the β-amyloid peptide$_{(25-35)}$ (Figure 4) individually increased the expression of NF-κB. Interestingly, this effect was reversed by the treatment with agmatine.

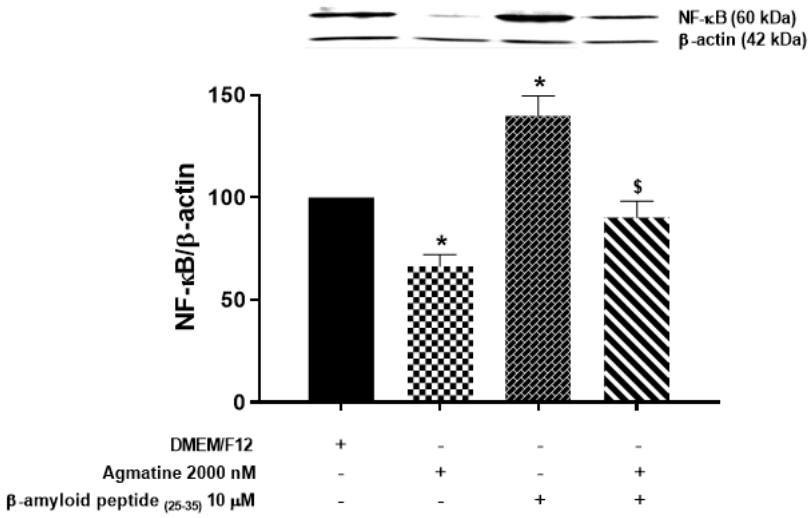

Figure 4. Effect of agmatine, the b-amyloid peptide, and its combination on the expression of NF-kB in SH-SY5Y cells

The results of this work demonstrate that agmatine increased the viability of SH-SY5Y cells particularly when the cells were deprived of FBS (1%). This result is likely related to the fact that the basal expression of imidazoline receptors under control conditions (10% FBS) is low and that under conditions such as FBS deprivation, the expression of imidazoline receptors is increased.

The fact that the β-amyloid peptide$_{(25-35)}$ reduced the viability of SH-SY5Y cells to a greater degree than the reduction induced by FBS deprivation indicates that this compound potentiates the deleterious effects of the reduction of nutrients required by this cell type. Although cell death was not determined in the present work, cell viability is an indirect measurement of this phenomenon, since

those stimuli that reduce cell viability are toxic and those that induce the opposite effect are beneficial affecting in both cases the degree of cell survival.

Some experimental evidence indicates that different deleterious systems are increased in SH-SY5Y cells. What is interesting is that most of these systems are activated by NF-κB, which is regulated by mitogen-activated kinases (MAP kinases), particularly ERK and p38 (Jang and Surh, 2005) and caspases (Allen *et al*., 2001). On the other hand, it has been shown that, at the systemic level, the β-amyloid peptide induces the activation of microglial cells, which induces the synthesis and release of pro-inflammatory and pro-apoptotic factors (Martinez and Pascual, 2007). In agreement with these, the β-amyloid peptide produced neurotoxic responses probably mediated by the stimulation of NF-κB, as shown by the western blot studies carried out in the present work.

The neurotoxic effects of the β-amyloid peptide were reversed and prevented by agmatine. This evidence suggests that agmatine has neuroprotective properties against toxic stimuli capable of causing neuronal death.

5. Conclusions

The neuroprotective effect that agmatine exerts against the neurotoxicity induced by the β-amyloid peptide involves the inhibition of the NF-κB pathway.

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Effects on the production of short chain fatty acids (SCFA) and inflammation and oxidative stress biomarkers of polysaccharides associated to *Opuntia ficus-indica* (L.)

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Abstract

Hypercholesterolemia is a metabolic disorder that affects a large part of the world's population, which is characterized by an increase in the levels of inflammatory markers accompanied by the production of oxidized species. *Opuntia ficus-indica* (OFI) is a food rich in antioxidant compounds and both insoluble and soluble dietary fibers that is widely used for its nutritional and favorable effects on human health. This study was designed to evaluate the effects of a polysaccharide-rich fraction isolated from *O. ficus-indica* on the production of short chain fatty acids (SCFA) and on important inflammatory biomarkers and oxidative stress. The OFI polysaccharide-rich fraction was characterized by FTIR and SEM. The OFI polysaccharide-rich fraction and flour were subjected to a simulated gastrointestinal digestion process and colonic fermentation during 72 h. Inulin was used as positive control for the SCFA production. The OFI polysaccharide-rich fraction and flour exerted the highest concentration of acetic, propionic, and butyric acids after 72 h of colonic fermentation. In addition, a significant reduction of 90% of nitric oxide compared to the control was observed in samples obtained after colonic fermentation. In terms of antioxidant capacity, the polysaccharide-rich fraction diminished reactive oxygen species between 36% to 40%. Results herein showed that the

OFI polysaccharides-rich fraction is a healthy alternative to control hypercholesterolemia by modulating inflammatory biomarkers, oxidative stress, and production of colonic SCFA.

Keywords: *Short chain fatty acids, hypercholesterolemia, Opuntia ficus-indica, inflammation, oxidative stress.*

1. Introduction

Hypercholesterolemia is a metabolic disorder characterized by high serum levels of cholesterol. It is estimated that around 2.6 million deaths in the world are due to high concentrations of cholesterol in the blood (WHO, 2020). The development of this chronic disease has been associated with a poor diet, lack of exercise, tobacco use, obesity, family history and prevalence of other chronic diseases (Csonka *et al.*, 2016). High levels of cholesterol are closely related to a high risk of developing coronary heart disease, heart attacks, atherosclerosis, Alzheimer's, and Parkinson's (Shepardson 2011). The first-line therapeutic strategy to lower cholesterol levels focuses on treating the main pathology through changes in lifestyle and dietary patterns. The markers of this disease improve with the higher consumption of fruits and vegetables and reduction of the consumption of total fats, saturated fats, and dietary cholesterol. In terms of lifestyle, patients that are sedentary, smoke and consume large amounts of alcohol are more prone to hypercholesterolemia. From the pharmacological viewpoint, the levels of total and LDL-cholesterol are significantly lowered when the patient consumes drugs such as statins. These compounds significantly inhibit the hepatic enzyme HMG CoA reductase responsible to produce endogenous cholesterol (Pascual-Fuster, 2016).

Opuntia ficus-indica (OFI) is an indigenous Mexican food, which has been used in traditional medicine since prehispanic times for the treatment of burns, wounds, edema, hyperlipidemia, obesity, and gastritis. Its beneficial health properties are attributed to the presence of phytochemicals such as polyphenols, flavonoids, vitamins, polyunsaturated fatty acids, and amino acids (Aragona *et al.*, 2018). *Opuntia ficus-indica* diminishes serum cholesterol levels through a reduction in the expression

of cholesterol transporting proteins (Ressaissi *et al*., 2020; Zhao *et al*., 2012). In addition, *Opuntia* cladodes have a high content of pectin, mucilage, cellulose, and hemicellulose, which exert benefits in the metabolism of both lipids and glucose (Padilla-Camberos *et al.*, 2015). It has been reported that these polysaccharides reduce cholesterol levels through an increase in the sensitivity of LDL receptors and a reduction in oxidative damage, as well as a higher expression of genes and proteins of the enzyme superoxide dismutase (Huang *et al.*, 2010). The aim of the study was to evaluate the effect of an isolated fraction of polysaccharides from *O. ficus-indica* on the production of short chain fatty acids and its effects on the biomarkers of inflammation and oxidative stress after a process of gastrointestinal digestion and colonic fermentation.

2. Material and methods

2.1 Biological material

Opuntia ficus-indica L. (Mill) (OFI) cladodes were harvested in Nuevo León, Mexico and then dehydrated and ground into an OFI flour as previously reported (Antunes-Ricardo, Gutiérrez-Uribe, López-Pacheco, et al. 2015). The OFI flour used in this study was kindly donated by Alimentos Funcionales S. de R.L.M.I. The flour was packed in aluminized bags to protect from light and taken to the laboratory for analysis.

2.2 Extraction of *O. ficus-indica* polysaccharides-rich fraction

The polysaccharides of the OFI were extracted following the method described by Bayar, Kriaa, and Kammoun (2016) with some modifications. One gram of OFI flour was weighed and dissolved in 10 mL of Milli-Q water (1:10 w/v). The flour was dissolved and then mixed using a magnetic stirring plate (Super-Nuova ™ Multi-place, Thermo Fisher Scientific, MA, USA) at 150 rpm and 80 °C for one hour. Thereafter, contents were centrifuged at 1,000 xg for 20 min at 25 °C (SL16R model, Thermo Scientific, Osterode, Germany) to separate the supernatant from the pellet. The supernatant was further treated with absolute ethanol for 48 hours at 4 °C and centrifuged again to recover the

pellet or precipitate which was first lyophilized and then the resulting dry material stored at -20 °C until analysis.

2.3 Characterization of the polysaccharide-rich fraction obtained from Opuntia ficus-indica

2.3.1 Analysis of functional groups by Fourier transform infrared transmission spectroscopy (FTIR)

The functional groups present in the polysaccharide-rich fraction extracted from OFI flour were analyzed using an infrared spectrum (FTIR, Perkin Elmer Spectrum ONE, USA) equipped with an attenuated total reflectance accessory (ATR). The dried and powdered polysaccharide-rich fraction was analyzed at a resolution of 4.0 cm^{-1} in a range of 4000 to 500 cm^{-1} at 25 °C (10 scans).

2.3.2 Morphological analysis by scanning electron microscopy (SEM)

The dried polysaccharide-rich fraction was mounted on a copper sample-holder, using a double-sided carbon tape, and coated with gold (5–10 nm thicknesses) to allow conductivity through the polysaccharide granules. The sample was viewed and analyzed using a scanning electron microscopy (ZEISS EVO®MA 25, Germany) at a voltage of 15Kv.

2.4 *In vitro* simulated gastrointestinal digestion and colonic fermentation

For this stage, a static model reported by Flores et al. (2014) was adapted to simulate digestion in the mouth, stomach, and intestine. Different solutions were prepared that simulate the composition of biological fluids of each digestion stage as shown in **Table 1.** About 0.5 g of dry polysaccharide-rich fraction, OFI flour, or inulin (positive control) were placed in 250 mL flasks and incubated in an orbital shaking chamber (VWR, Incubating Orbital Shaker, Radnor, PA, USA) at 37 ºC and 200 rpm.

2.5 *In vitro* simulated colonic fermentation

After *in vitro* gastrointestinal digestion, the resulting intestinal fraction was incubated in the presence of human microbiota obtained from the feces of five healthy adults (27-35 years), who reported not having any gastrointestinal diseases nor the consumption of antibiotics in a minimum period of 6

months. In addition, the subjects should have not consumed any kind of probiotics and prebiotics before the study. The fermentation medium was phosphate buffer. The fecal inoculum was prepared by diluting the fecal samples with the buffer (0.5:10 w/v) followed by homogenization in a Stomacher® (IUL Instrument, Barcelona, Spain) for a period of 30 min. Subsequently, 1 mL of the intestinal fraction was added with 9 mL of fecal inoculum. The samples were incubated at 37 °C under anaerobic conditions with continuous shaking at 60 rpm (VWR, Bridgeport, NJ, USA). All samples were processed in duplicate, and 0.5 mL aliquots were taken at 0, 2, 8, 24, 48 and 72 h.

2.6 Short chain fatty acids (SCFA) quantification by gas chromatography (GC-FID)

SCFA were measured by gas chromatography with flame ionization detection (GC-FID) according to the method proposed by Wallace *et al*. (2015) with some modifications. Samples obtained from colonic fermentation were centrifuged at 10,000 xg for 5 min at 4 °C, subsequently the supernatant was collected and transferred to a 2.5 mL glass vial and capped. Analysis was performed on a gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and an Agilent HP-FFAP column (25-m length x 0.32-mm internal diameter x 0.50-µm film thickness). The carrier gas was helium with a total flow rate of 8.7 mL/min. The temperature program began at 120 °C and then elevated to 240 °C. Samples were injected (1 µL) in a splitless mode. SCFA levels were expressed as micromoles of SCFA per gram of wet fecal matter based on standard curves.

2.7 Biological evaluation of digested and fermented samples of polysaccharides-rich fraction and flour obtained from *Opuntia ficus-indica* (L.) Mill

2.7.1 Inhibition of nitric oxide (NO) production as an inflammation biomarker

Mouse macrophage cells (Raw 264.7) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in Dulbecco's Modified Eagle's Medium-F12 culture medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were cultured in 96-well plates at $5x10^5$ cells/mL and allowed to adhere for 24 hours. Next, 50 µL of the selected samples

were added and incubated for 24 hours. At the end of this period, half of the cells were stimulated with lipopolysaccharide obtained from *Salmonella enterica serotype typhimurium* (LPS, Sigma-Aldrich, St. Louis, MO, USA) at 10 µg/mL as final concentration, while the other half used as control. Nitric oxide (NO) production was determined with the Griess System Kit (Promega, Madison, WI). The kit uses sulfanilamide and N-1-naptylenediamine hydrochloride (NED) as reagents to produce a colored compound that absorbs at 545 nm, which was measured using a microplate reader (Synergy HT, Bio-Tek, Winooski, VM, USA). A nitrite standard was used for quantification, considering the cell viability. Results are expressed as the concentration of nitric oxide (µM) generated by each of the treatments compared to a control (without treatment).

2.7.2 Cellular antioxidant capacity (CAA)

The cellular antioxidant capacity (CAA) assay previously reported by (Pacheco-Ordaz et al. 2018) was employed to evaluate the effects of OFI polysaccharides-rich fraction and flour on oxidative stress. Human adenocarcinoma cells (Caco-2) were acquired from the ATCC. Cells were cultured in a black-walled, clear-bottom 96-well microplate at a density of $5x10^5$/mL. After 24 h, the medium was removed, and cells were washed with phosphate buffered saline (PBS). After that, cells were treated with 100 µL of digested or fermented samples containing 2′,7′-dichlorofluorescin diacetate (DCFH-DA) at 60 µM final concentration, and the cells were incubated at 37 °C for 20 min. After incubation, the treatment solutions were removed, and the cells were washed again with PBS. Finally, 100 µL of 2,2′-azobis-(2-methyl-propionamidine) dihydrochloride (AAPH) solution at 500 µM. Fluorescence was measured in the microplate reader at 538 and 485 nm of emission and excitation, respectively. Fluorescence was measured every 2 min for 90 min at 37 °C. The results were expressed as the production of reactive oxygen species (ROS), in relative fluorescence units (RFU's), generated by each of the treatments in comparison with a control that did not receive any treatment.

3. Results and discussion

3.1 Structural and morphological analysis of O. *ficus-indica* polysaccharide-rich fraction

A structural analysis of polysaccharide-rich fraction by FITR was made and results depicted in **Figure 1,** which indicates a characteristic band around 3200-3300 cm^{-1} corresponding to stress vibrations of the hydroxyl groups (OH). Another band observed in the region 950-1200 cm^{-1} showed the presence of tension vibrations of side groups (C-O-H) and vibrations of glycosidic bonds (C-O-C). The presence of signals around 1079 cm^{-1} suggested the presence of C-O bonds, while the bands around 1582 cm^{-1} were attributed to carboxylate groups -C (= O)-O-. The absorption bands at 1412 cm^{-1} were attributed to the presence of free carboxylic acid groups. These findings agree with previous reports that show the characteristic bands of the OFI polysaccharide-rich fraction (Chaouch *et al.*, 2018; Lira-Ortiz *et al.*, 2014).

The morphology of the OFI polysaccharide-rich fraction was viewed and analyzed using scanning electron microscopy or SEM (**Figure 2)**. In agreement with the reported by Mannai et al. (2018), a distribution of densely packed layers of differentiated flat and hexagonal cells was observed in this study.

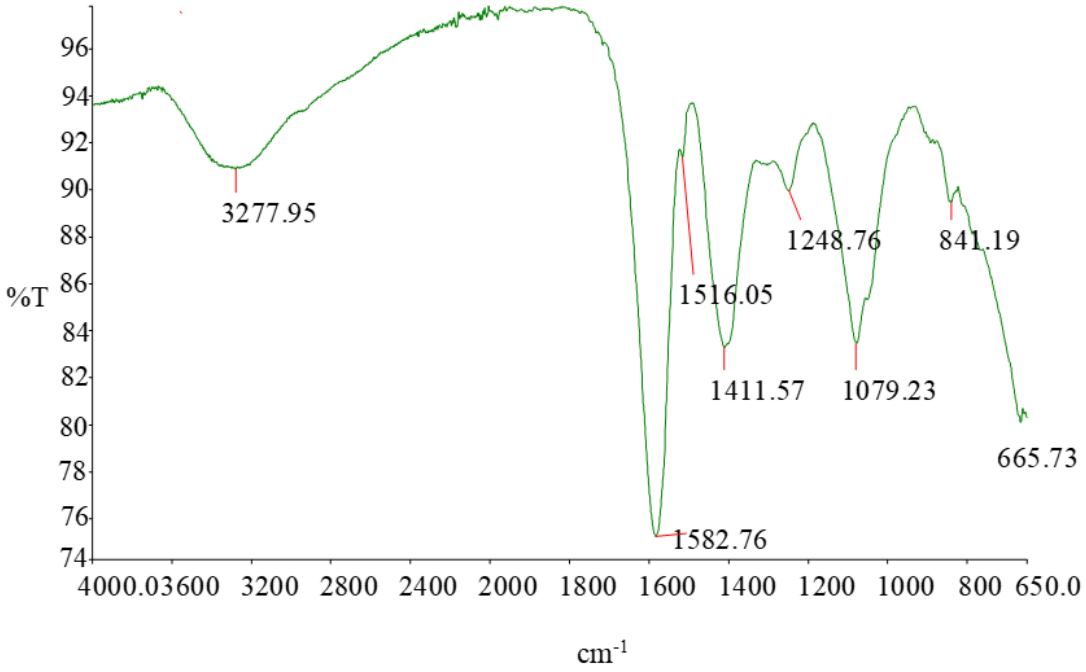

Figure 1. FTIR-ATR spectrum of the polysaccharide fraction extracted from *Opuntia ficus-indica* (L.) Mill

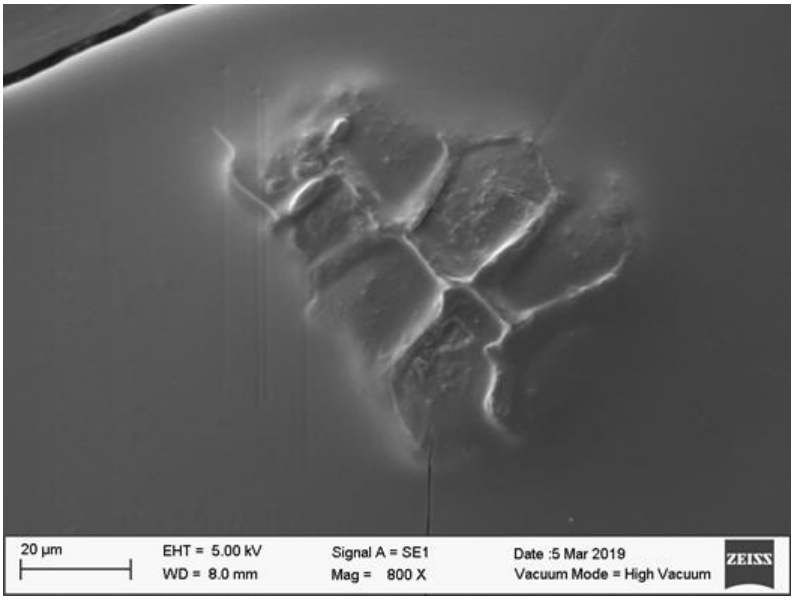

Figure 2. Morphology observed of the polysaccharide fraction extracted from *Opuntia ficus-indica* viewed by scanning electron microscopy (SEM)

3.2 Production of short chain fatty acids

SCFA generated from the colonic fermentation of the OFI polysaccharides-rich fraction or flour (**Figure 3A**) indicated that the fraction mainly generated acetic, propionic, and butyric acids at all times of fermentation. After 72 h, the highest concentrations of propionic, butyric, isovaleric and valeric acids were observed. At this time, the concentration of propionic acid increased 4.21 and 1.67 times compared to concentrations attained at 8 h and 24 h, respectively. On the other hand, acetic acid showed its maximum level at 24 h and this level remained constant until 72 h. Regarding the OFI flour, the same SCFA trends were observed when fermenting the polysaccharide-rich fraction (**Figure 3B)**. Acetic, propionic, and butyric acids were the most abundant acids at all times of fermentation. After 72 h, the concentration of propionic acid increased by 5.50 and 1.44 times with respect to concentrations observed at 8 h and 24 h, respectively. Acetic acid showed its maximum level at 24 h and this level remained constant until 72 h. On the other hand, **Figure 3C** shows that OFI flour produced the highest amount of propionic acid, even above the levels observed for inulin. These results agree with data reported by Reyes-Reyes *et al*. (2019) supporting the idea of *O. ficus-indica* as a prebiotic. Acetic acid is involved in reducing plasma cholesterol levels and preventing the development of brain and cardiovascular diseases, as well as being a source of energy for peripheral intestinal cells. Butyric acid participates in the satiety signaling mechanism, decreases inflammation, strengthens the immune system, and prevents colon cancer (Wang *et al.*, 2019). Similarly, propionic plays an essential role by inhibiting one or more metabolic pathways of 3-hidroxi-3-metil-glutaril-CoA (HMG-CoA) reductase and inhibiting acetyl-CoA reductase in the liver, which causes a significant decrease in cholesterol levels (Wang *et al*., 2019). Yang *et al.* (2019) evaluated the effect of the administration of water-soluble polysaccharide of *Opuntia humifusa* stems in mice fed with a high-fat diet (HFD) and results clearly indicated that the water-soluble polysaccharide improved lipid and hormone profiles. Other polysaccharides from wheat bran, cereal fiber, barley β-glucans and

mango fiber have also shown their effectiveness to lowering lipid levels through SFCA production along with their antioxidant effects (Nie *et al.*, 2017; Han *et al.*, 2019; Tong *et al.*, 2015; Hernández-Maldonado *et al.*, 2019).

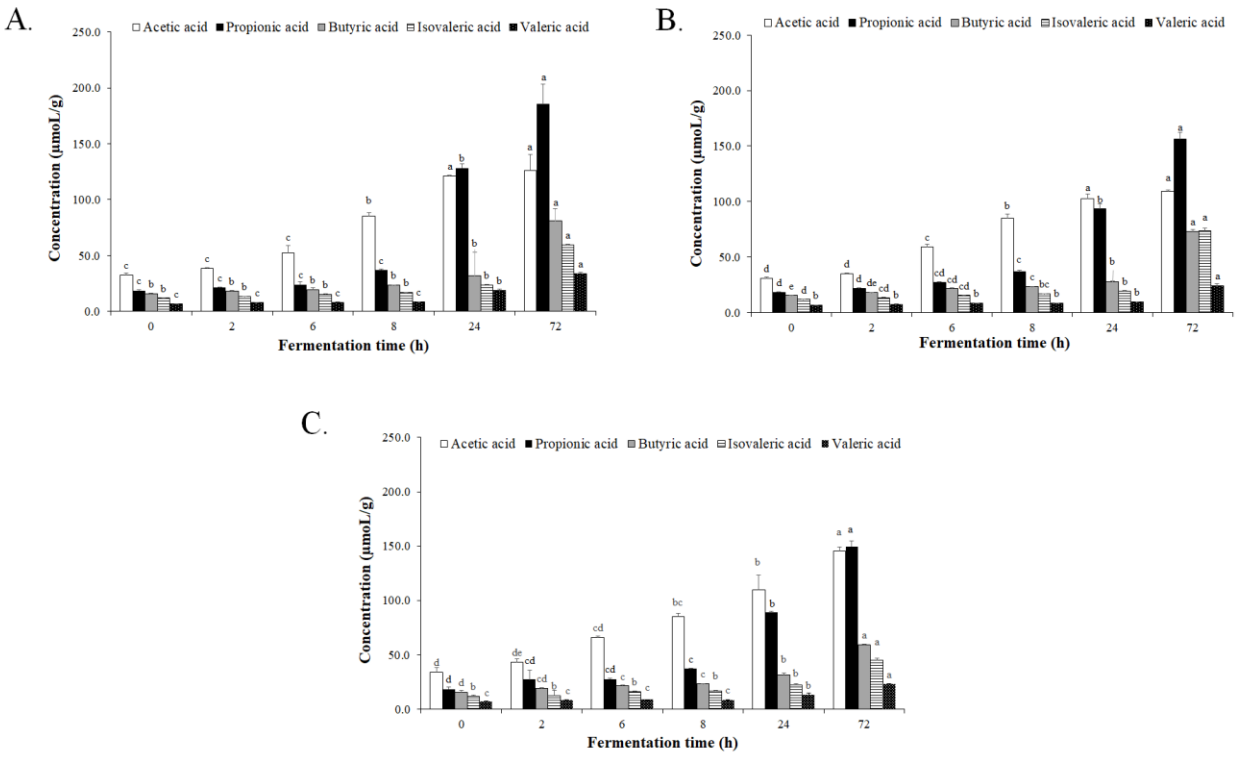

Figure 3. Concentration of short chain fatty acids (SCFA) generated during *in vitro* colonic fermentation of (A) *Opuntia ficus-indica* polysaccharide-rich fraction, (B) *Opuntia ficus-indica* flour and (C) inulin.

3.3 Measurement of nitric oxide production as inflammatory biomarker

All treatments showed a significant reduction in the levels of nitric oxide produced by macrophage cells compared to the control (**Figure 4)**. The fermented polysaccharide-rich fraction reduced the nitric oxide concentration by 5.7 times with respect to the same fraction obtained at the end of the gastrointestinal digestion. Similarly, the OFI flour fraction obtained at the end of the colonic fermentation phase reduced the nitric oxide concentration by 3.2 times with respect to the same fraction obtained after the gastrointestinal digestion. These results suggest that during the colonic

fermentation a biotransformation process occurred producing more active molecules with anti-inflammatory effects. On the other hand, the higher anti-inflammatory effect observed in the treatment with polysaccharides-rich fraction could be related to the high availability of soluble fiber which enhanced the production of SCFA, mainly butyric and acetic acids *(Parada-Venegas et al.*, 2019). A similar behavior was reported for polysaccharides extracted from the brown alga *Sargassum horneri* that could inhibit the production of nitric oxide in RAW cells with a mean inhibitory concentration of (IC_{50} value: 95.7 µg/mL). In addition, these polysaccharides demonstrated the ability to modulate the production of nitric oxide synthase (*i*NOS) and cyclooxygenase 2 (COX-2), both enzymes closely related to the inflammatory response (Sanjeewa *et al.* 2017). Likewise, the anti-inflammatory effects of OFI flour through the reduction of nitric oxide production, along with other inflammatory biomarkers, have been previously reported (Antunes-Ricardo *et al*., 2015).

3.4 Cellular antioxidant activity of digested and fermented samples obtained from *O ficus-indica*

Intestinal and colonic fermented digests showed a significant reduction in the reactive oxygen species (ROS) production compared to the untreated control. **Figure 5** shows that both the OFI polysaccharide-rich fraction and flour significantly reduced the cellular oxidation mechanisms from 36% to 40%, without differences between sources. *O. ficus-indica* has been identified as an adequate source of phenolic compounds with antioxidant activity such as isorhamnetin, quercetin and kaempferol (Antunes-Ricardo *et al.* 2014).

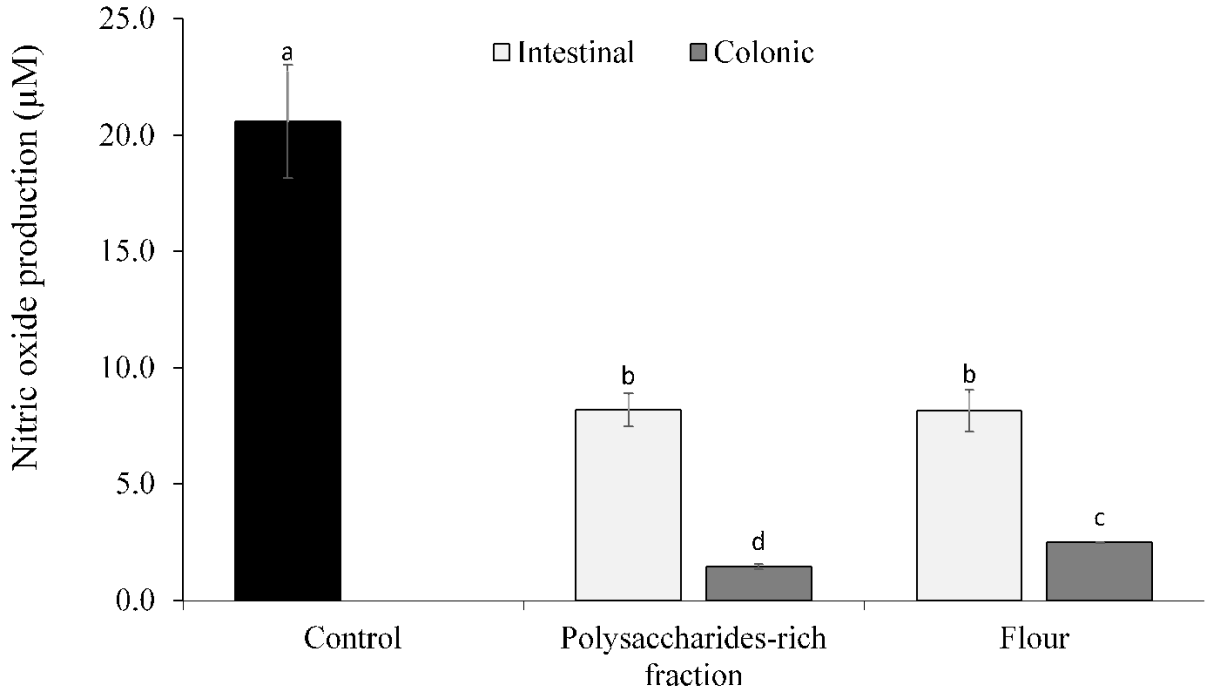

Figure 4. Comparison of intestinal and colonic samples of flour and polysaccharides-rich fraction obtained from *Opuntia ficus-indica* on the production of nitric oxide.

These compounds are found in nature in the form of glycosides, which may limit their cellular permeability and therefore their participation in oxidation-reduction reactions. However, when these compounds are hydrolyzed in the gastrointestinal tract, they commonly show higher antioxidant potential (Avila-Nava *et al.* 2014).

Isorhamnetin glycosides have the ability to modulate ROS production at the cellular level, either to produce an antioxidant effect or to produce cell death due to oxidative stress (Antunes-Ricardo *et al.*, 2019). On the other hand, Li *et al.* (2016) reported that polysaccharides from cactus *O. dillenii* showed scavenging abilities showing a dose-response behavior with values of up to 46% antioxidant activity.

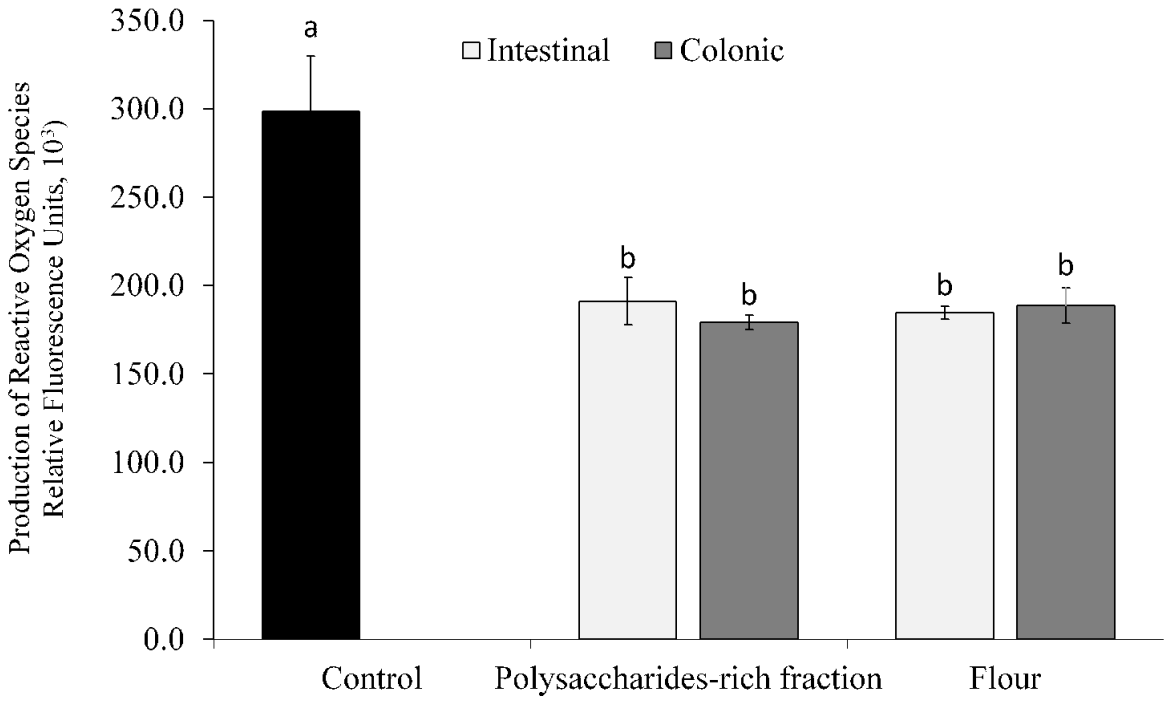

Figure 5. Cellular antioxidant capacity exhibited by intestinal and colonic samples of flour and polysaccharide-rich fraction obtained from *Opuntia ficus-indica* using an *in vitro* model of human colon cells (Caco-2).

4. Conclusions

These results showed that the polysaccharides present in the *O. ficus-indica* positively modulated inflammatory processes and oxidative stress, which are key markers of chronic diseases such as hypercholesterolemia. Likewise, these beneficial effects are closely linked to the high production of short chain fatty acids (SCFA) derived from the greater polysaccharide availability to be fermented by the colon microbiota, generating a biotransformation process that gives rise to new compounds. Both, the *O. ficus-indica* flour and the extracted polysaccharides-rich fraction, exhibited potential to be used as functional ingredients in the prevention of chronic diseases, which involve inflammation and oxidative stress.

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