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Surface and nutraceutical properties of edible films made from starchy sources with and without added blackberry pulp

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ABSTRACT

The surface and nutraceutical properties have been poorly studied on edible films. The aim of this study was to investigate the surface properties and potential health effects in terms of *in vitro* digestibility and anti-inflammatory activity. The materials were developed from native plantain starch and pre-gelatinized plantain flour with and without added blackberry pulp using casting methodology. Thermogravimetric analysis, contact angle, scanning electron microscopy, atomic force microscopy, resistant starch, *in vitro* digestibility, cell viability, reactive oxygen species, anti-inflammatory activity and sensory evaluation were the tests carried out in this study. Films containing blackberry pulp had more compact and smooth morphologies, which were related to the lower *in vitro* digestibility rate and the higher resistant starch content. In addition, these materials had higher anti-inflammatory activity, higher cell viability, and better acceptance by the panelists, thus suggesting potential health effects of consumers with special feeding regimes such as obese, diabetics and celiacs.

1. Introduction

Today, synthetic polymers are the main packaging materials since they offer versatile solutions for several needs of food packaging. Conventional packaging polymers are questioned due to increasing environmental concerns and their petroleum-based sources. Research on sustainable alternative materials for food packaging is a hot topic for over two decade. Starch is one of the renewable natural polymers, which can be widely used as an important industrial raw material (Gutiérrez, Tapia, Pérez, & Famá, 2015a). Furthermore, starch packaging materials are excellent vehicles for incorporating a wide variety of additives, such as antifungal agents, antioxidants, dyes, antimicrobials, among other nutrients (Han, 2003; Clarinval, & Halleux, 2005; Imran et al., 2010).

In the food packaging industry, the use of proper packaging materials and methods to minimize food losses and provide safe and wholesome food products have always been the main interests. Due to the improved performance in the properties of packaging materials such as 1) gas (oxygen, carbon dioxide) and water vapor barrier properties, 2) high mechanical strength, 3) thermal and chemical stability, 4) dimensional stability, 5) heat resistance, 6) recyclability, 7) biodegradability, 8) good optical clarity, as well as 9) developing active antimicrobial and antifungal surfaces, and 10) sensing and signaling microbiological and biochemical changes, food packaging has been one of the most concentrated composite technology development.

In our previous study, we have determined that the addition of a natural filler as blackberry pulp in starchy matrices, allows obtaining functional, active and intelligent films (Gutiérrez, 2016). Besides, the incorporation of blackberry pulp improved the physicochemical properties because the blackberry pulp provoked cross-linking reactions between starch chains due to citric acid that was found in pulp.

Intelligent packaging is intended to monitor and provide information about the quality of the packaged food or its surrounded environment to predict or decide the safe shelf life (Yam, Takhistov, & Miltz, 2005). The intelligent packaging may respond to environmental conditions, alert a consumer to contamination of pathogens, detect harmful chemicals or degradation products caused by food deterioration, indicate food quality, and initiate self-healing. In this sense, Gutiérrez (2016) has recently proposed the implementa-

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tion of intelligent films obtained from starchy matrices with the addition of blackberry pulp for fishery products.

On the other hand, the surface properties, nutritional aspects and sensory evaluation have been poorly studied so far in this type of materials. It can even be said that the nutritional aspects of these materials have been forgotten. At this regard, the nutritional aspects globally are having ever-increasing attention since these can improve the quality of life of people with special feeding regimes such as celiacs and diabetics.

Historically, the nutrition tips for celiac disease has focused on the foods to avoid in a gluten-free diet, and in the case of diabetic patients on the reduction of carbohydrates (Matos, & Rosell, 2011). Whereby, there are growing concerns about the nutritional adequacy of the gluten-free dietary pattern or carbohydrate-reduced diets because it is often characterized by an excessive consumption of proteins, and fats, and a reduced intake of dietary fiber, vitamins and minerals (Thompson, Dennis, Higgins, Lee, & Sharrett, 2005; Catassi, & Fasano, 2008). As a consequence, this population is suffering nutritional deficiencies that could lead to anemia, osteopenia or osteoporosis (Thompson et al., 2005). In this sense, food products made from plantain have been proposed in the diet of people with special dietary regimes because they are gluten-free and can also be an important source of dietary fiber (Zandonadi et al., 2012). This is one of the main reasons for the use of matrices containing starch from plantain.

Furthermore, higher starch digestibility rate have as a result higher glycemic index (GI). High GI foods such as white bread have been associated with increasing health risks related to obesity, diabetes and coronary heart disease (Livesey, Taylor, Hulshof, & Howlett, 2008). Thus, food products with low GI and high dietary fiber are of interest for consumers as well as for food manufacturers (Singh, Dartois, & Kaur, 2010; Reis, & Abu-Ghannam et al., 2014). Likewise, processing methods, feed material composition, microstructure and textural properties of food influences on starch digestibility (Al-Rabadi, Torley, Williams, Bryden, & Gidley, 2012; Singh, Kaur, & Singh, 2013). Thus, it is important to understand how the physic-chemical properties of starch edible films affect to starch digestibility (Englyst, Kingman, Hudson, & Cummings, 1996; Singh et al., 2013). *In vitro* starch digestibility indicates the rate of glucose release under simulated physiological conditions, which are also used to predict the GI of starchy foods (Englyst, Vinoy, Englyst, & Lang, 2003; Germaine et al., 2008).

According to the literature, cross-linked starches could be an alternative to conventional starches, since they decrease the digestibility rate (Liu, Ming, Li, & Zhao, 2012). Therefore, taking into consideration that Gutiérrez (2016), has reported that the blackberry pulp provokes cross-linking in starch chains, thus, it has been assumed that these films could be applied in order to obtain edible food packaging, which could also be consumed by people with special feeding regimens.

In this context, *in vitro* digestibility and resistant starch content has been recently studied by Gutiérrez and Álvarez (2016) for films based on native plantain flour/*aloe vera* gel blends. The term "resistant starch" (RS) was adopted by Hans Englyst, a British physiologist, in the early 1980s. RS is defined as dietary starch that does not digest in the small intestine (EURESTA, 1991; Saura-Calixto, Goñi, Bravo, & Mañas, 1993). RS, therefore, behaves like dietary fiber and may have potential as a health-related in gradient in foodstuffs. The primary difference between digestible and resistant starches is the accessibility of the starch to digestive processes and subsequently the ease with which the glycosidic bonds contained within the starch molecules can be severed (Stephen, 1995). Starch that is protected by cell walls or other barriers to the actions of the digestive enzymes and acidic conditions of the digestive tract is considered Type I RS. Examples of this type RS would be the starch in partially milled seeds and grains. Highly crystalline native starches such as those from raw bananas or potatoes cannot be reduced to degradation products that can be absorbed through the small intestine and are considered Type II RS. If the starch in a food system is gelatinized, subsequently cooled, and allowed to retrograde, a similarly resistant starch is created (Type III). Some foods containing retrograded starches (type of resistant starch) can be mentioned: corn flakes, cooked potatoes, and canned peas. Finally, it is also possible to make starch resistant by modifying it and altering its native structure (Type IV).

Likewise, Gutiérrez (2016) has also reported on the anti-radical capacity (DPPH•) of films containing blackberry pulp. Taking this fact into account, we also decided to investigate the antioxidant activity in the cells. In this study the anti-inflammatory activity of the films was also investigated, since this test is known to be a marker of anti-cancer activity, which may be associated with the polyphenolic compounds found in the blackberry pulp (Gutiérrez, 2016).

The aim of this research work was to investigate the surface properties and the beneficial effect on health in the developed films.

2. Experimental

2.1. Materials

Native plantain starch, pre-gelatinized plantain flour and blackberry pulp were obtained and characterized previously by Gutiérrez (2016). According to Gutiérrez (2016) a total amylose content about 35.0% for the native plantain starch and 10.0% for pre-gelatinized plantain flour were obtained from DSC results. Food grade glycerol (Aldrich, product code: G7893) was employed as plasticizer in the formation of the films.

2.2. Film formation

All films were prepared by solvent casting according to a method described previously (Gutiérrez, 2016). Film-forming solutions (FFS) containing 2% w/v of native plantain starch or pre-gelatinized plantain flour, 1.9% w/v glycerol and 500 mL distilled water were prepared. Particularly, films with addition of 4% of blackberry pulp were obtained incorporating the pulp 2 min before the end of process of obtaining the FFS. This was done in order to avoid oxidative damages in the pulp. The mixtures were heated above gelatinization temperature and maintained with continuous magnetic stirring near 30 min. After, the viscous gels were poured into stainless steel trays 40 × 30 cm, and dried in a Mitchell dehydrator (Model 645 159) for 24 h at 45 °C. The resulting films were then carefully removed from the casting molds. Then, films were stored at 57% relative humidity (RH) (saturated solution of NaBr) for one week at 25 °C before the characterizations. During this period the containers were protected of light at a dark room in order to avoid the photodegradation of the antioxidant compounds and pigments. Samples of thermoplastic starch (*TPS*) and thermoplastic flour (*TPF*) were named as follows: native plantain starch (*TPS-NPS*), native plantain starch with incorporation of blackberry pulp (*TPS-NPSB*), pre-gelatinized plantain flour (*TPF-PPF*) and pre-gelatinized plantain flour with incorporation of blackberry pulp (*TPF-PPFB*).

2.3. Film characterization

2.3.1. Determination of degree of substitution (DS) in the starch films esterified with citric acid from added blackberry pulp

The amount of citric acid esterified in films with added blackberry pulp was determined by the method described by Klaushofer, Berghofer, and Steyrer (1978). This is based on the reaction of citric acid and Cu^{2+} which forms a stable complex during titration with a solution of copper sulfate. DS was calculated based on the average number of substituent groups per anhydroglucose unit, as follows (Mei, Zhou, Jin, Xu, & Chen, 2015).

$$DS = (162xW)/(100xM) - (M - 1)xW \quad (1)$$

where W (% by weight of substituent) = [bound citrate (g)/sample (g) bound citrate (g)] x 100, and M = molecular weight of the citric acid substituent (175.1 g/mol). Each sample was analyzed in triplicate.

2.3.2. *Thermogravimetric analysis (TGA)*

A simultaneous Thermogravimetric/Differential Thermal Analyzers (TGA/DTA DTG-60 Shimadzu instrument) was used to study the thermal resistance of the edible films, as well as to analyze the behavior of the different degradation phases of these materials. Samples between 5 and 10 mg were heated from room temperature up to 400 °C at a rate of 10 °C/min and a nitrogen flow 30 mL/min. The weight loss of the materials was recalculated on dry basis in order to avoid distortions as a result of the different moisture contents of the films. Analyses were performed in triplicate to ensure repeatability.

2.3.3. *Contact angle*

Contact angles (θ) were determined at room temperature using a USB Digital Microscope (model DIGMIC200X, China) equipped with Image Analysis Software 220 × 2.0MP video, with 0.0001° precision. A drop of distilled water (2 μL) was placed on the surface exposed to drying air during film preparation. Measurements were taken just at the moment when the drop of water came into contact with the film surface. This was done in order to avoid false results caused by phenomena such as dehydration, swelling and dissolution (Vogler et al., 1995). The contact angles were calculated by analysing the images to determine the angles formed by the intersection of the liquid-solid interface (drop of water-surface of the film) and the liquid-vapor interface (tangent to the boundary of the drop) (Karbowiak, Debeaufort, Champion, & Voilley, 2006). At least 12 contact angles were measured per film. Results were reported as mean ± SD.

2.3.4. *Scanning electron microscopy (SEM)*

The morphology of surface of each film exposed to drying air were investigated using a JEOL JSM-6460 LV instrument. For analysis of surface, the film samples were mounted on aluminium stubs with double-sided adhesive tapes and by sputter were coated with a thin layer of gold for 35 s.

2.3.5. *Atomic force microscopy (AFM)*

The three-dimensional images of the surface topography of the films exposed to drying air were obtained using an Agilent 5500 in tapping mode with silicon nitride tips and having a PicoView image software (Gutiérrez, Suniaga, Monsalve, & García, 2016; Gutiérrez, & González, 2016a).

2.3.6. *Determination of resistant starch (RS)*

The resistant starch content was determined as starch remnants in dietary fiber residues from the methodology described by Gutiérrez and Álvarez (2016). Absorbance was measured at 500 nm. Resistant starch was calculated as glucose (mg) x 0.9. Analyses were performed in triplicate to ensure repeatability. Results were reported as mean ± SD.

2.3.7. *in vitro digestibility tests – starch hydrolysis index*

The *in vitro* rate of starch hydrolysis was evaluated using the methodology described by Hernández, Emaldi, and Tovar (2008) and Zamora-Gasga, Bello-Pérez, Ortíz-Basurto, Tovar, and Sáyago-Ayerdi (2014) and using some modifications proposed by Gutiérrez and Álvarez (2016), e.g. plantain starch was used as reference. Data were plotted as degree of hydrolysis versus time curves.

2.3.8. *Cell viability*

Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, according to Di Nunzio, Toselli, Verardo, Caboni, and Bordoni (2013). Results were expressed as percentage of the value obtained in non-stressed control cells.

2.3.9. *Reactive oxygen species (ROS)*

ROS was determinate following the method described by Valli et al. (2012). The intracellular ROS levels were stated as percent of the value found in unsupplemented control cells (US). Samples were analyzed in triplicates and values were reported as mean ± standard deviation.

2.3.10. *Anti-inflammatory activity – cytokines secretion in the cell media*

The anti-inflammatory activity from HepG2 cells (a human hepatoma cell considered as a good model to study *in vitro* cytotoxic agents) (Mersch-Sundermann, Knasmüller, Wu, Darroudi, & Kassie, 2004) was determined according to a method described elsewhere with slight changes (Valli et al., 2015). To induce an inflammatory stimulus some cells also received 100 ng/mL of lipopolysaccharides (LPS), known to be a strong stimulator of inflammatory response (Hong, Seo, Lee, & Choi, 2004). After 18 h the medium was removed and maintained at −20 °C until cytokines were quantified.

The level of the pro-inflammatory interleukin-8 (IL-8) and the anti-inflammatory and interleukin-10 (IL-10) was estimated in the media in both basal condition and after cell treatment with LPS using the Multi-Analyte ELISArray Kit (Qiagen; Hilden, Germany) quantitative sandwich immune assay. Results were normalized for cell protein content (Bradford, 1976), and expressed as percentage of non-stressed unsupplemented control cells.

2.3.11. *Sensory evaluation*

The sensory qualities of film pieces (2.5 × 2.5 cm) was evaluated at 7 days of storage. Analyses were performed in a sensory analysis laboratory with individual booths for each panelist in accordance with ISO standard 8589:2007. For the hedonic tests, the coded (3 digit) samples were presented at random order to a panel comprised of thirty judges between 20 and 35 years of age (50% females, 50% males). The panelists were recruited among students and personnel of Department of Analytical Chemistry (Mention Food Science and Technology) of the Faculty of Pharmacy at the Central University of Venezuela. The panelists were asked about the different quality attributes of the films using a scale with anchors at 0 and 10, where 0 indicated dislike extremely and 10 indicated like extremely. The attributes evaluated were: color by visual observation under white lightning, texture by biting with front teeth, taste by masticating, oral dissolution by masticating, and overall acceptability. The panelists' average response was calculated for each attribute.

2.4. *Statistical analysis*

Experimental data was analyzed using Excel (Microsoft Inc.) and SPSS software (SPSS Inc.). The one way ANOVA procedure followed by LSD test was used to determine the significant difference ($p < 0.05$) between treatment means.

Table 1
Degree of substitution (*DS*) of the different films.

Parameter	TPS-NPS	TPS-NPSB	TPF-PPF	TPF-PPFB
DS	–	0.028 ± 0.001^b	–	0.017 ± 0.001^a

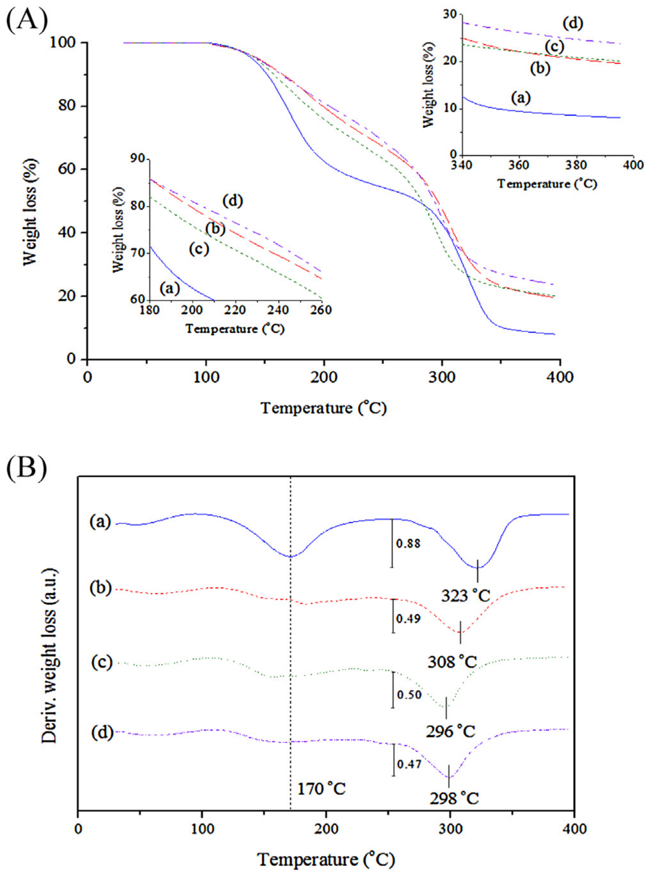

Fig. 1. (A) TGA and (B) DTGA curves of the different films studied: (a) native plantain starch (TPS-NPS), (b) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (c) pre-gelatinized plantain flour (TPF-PPF) and (d) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB).

3. Results and discussion

3.1. Degree of substitution (DS) by the titration method

Table 1 shows the degree of substitution in films with added blackberry pulp. The citric acid found in the blackberry pulp produced the cross-linking of the starch matrices. Similar results were reported by Kapelko-Żeberska, Buksa, Szumny, Zięba, and Gryszkin (2016) for other starches cross-linked with citric acid.

The degree of substitution found for the TPS-NPSB film was higher than that of the TPF-PPFB film, suggesting that the systems based on starch sources with a higher amylose content were more susceptible to cross-linking with the blackberry pulp citric acid.

Equal letters in the same row indicate no statistically significant differences ($p \leq 0.05$). Thermoplastic starch (TPS) and thermoplastic flour (TPF) films: Native plantain starch (TPS-NPS), native plantain starch with incorporation of blackberry pulp (TPS-NPSB), pre-gelatinized plantain flour (TPF-PPF) and pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB).

3.2. Thermogravimetric analysis (TGA)

Fig. 1 shows the TGA and DTGA (derivative TGA) curves of the developed films. Curves obtained from TGA analysis were used to examine the changes in thermal stability of evaluated systems (Fig. 1A). The degradation pattern of glycerol-plasticized TPS films under nitrogen atmosphere is generally accepted to involve three main weight-loss steps (González, Retegi, González, Eceiza, & Gabilondo, 2015; Gutiérrez, Morales, Pérez, Tapia, & Famá, 2015), i.e. 1) the loss of humidity (25–100 °C), 2) the decomposition of the glycerol-rich phase (100–200 °C) and 3) the degradation of the partially decomposed starch (~300–320 °C). From Fig. 1A, the first stage of thermal degradation was not observed, since weight loss of materials was recalculated on dry basis in order to avoid distortions as a result of the different moisture contents of the films, which were previously reported by our research group elsewhere (Gutiérrez, 2016). An obvious phase separation in the TPS-NPS film was observed, which was evident by the double weight loss in the material itself. DTGA curves confirms that indicated (Fig. 1B). Similar results have been reported by Sanyang, Sapuan, Jawaid, Ishak, and Sahari (2015) for films based on sugar palm (*Arenga pinnata*) starch.

On the other hand, lower thermal resistance in the TPF-PPF film than in the TPS-NPS film was observed. Considering to Pelissari, Andrade-Mahecha, do Amaral Sobral, and Menegalli (2013), this behavior could be attributed to the greater H-binding interactions between proteins that are found in plantain flour (Gutiérrez, 2016) and glycerol, which is associated with a plasticizing effect of proteins.

Furthermore, the onset of the second stage of thermal degradation (decomposition of the glycerol) in the blackberry pulp-containing films was slightly higher than that of the samples without the addition of blackberry pulp, thus leading us to conclude that the addition of blackberry pulp allows to increase thermal resistance. A similar behavior have been reported by Gutiérrez, Tapia, Pérez, and Famá (2015b) for films based on corn starch chemically modified by cross-linking with sodium trimetaphosphate (STMP). These results confirm that reported by Gutiérrez (2016) where blackberry pulp caused crosslinking reactions in starch chains.

Lastly, in the third stage of TGA curves were observed stable curves from 340 °C to 400 °C. In addition, a most profound decomposition was detected in the plantain starch-based film compared to the TPF-PPF film. This, could be explained due to a higher ash content that is found in this matrix (Gutiérrez, 2016). Besides, a less weight loss was registered in blackberry pulp-containing films, thus suggesting a greater contribution to degradation products from the organic compounds such as cross-linked starch structure or aromatic ring formed by thermal decomposition (Ruiz, 2006).

3.3. Contact angle

Fig. 2 shows the measurements of the contact angles of the films studied. According to Ojagh, Rezaei, Razavi, and Hashem (2010) it is well known that the contact angle of water increases with an increase in surface hydrophobicity. Other authors have suggested that an increase in the water contact angle of biopolymer films could be due to strong inter-molecular hydrogen bonding under the film surface (Karbowiak et al., 2006). In this sense, the TPS-NPS film had a lower contact angle compared to the TPF-PPF film. Therefore, the phase separation observed from TGA results of the TPS-NPS film, allowed to infer that glycerol is found free, thus increasing the hydrophilicity of the material. Similar results were reported by Gutiérrez and González (2016a) for taro starch-based films.

On the other hand, films containing blackberry pulp (TPS-NPSB and TPF-PPFB) showed higher contact angle values. Vogler (1998)

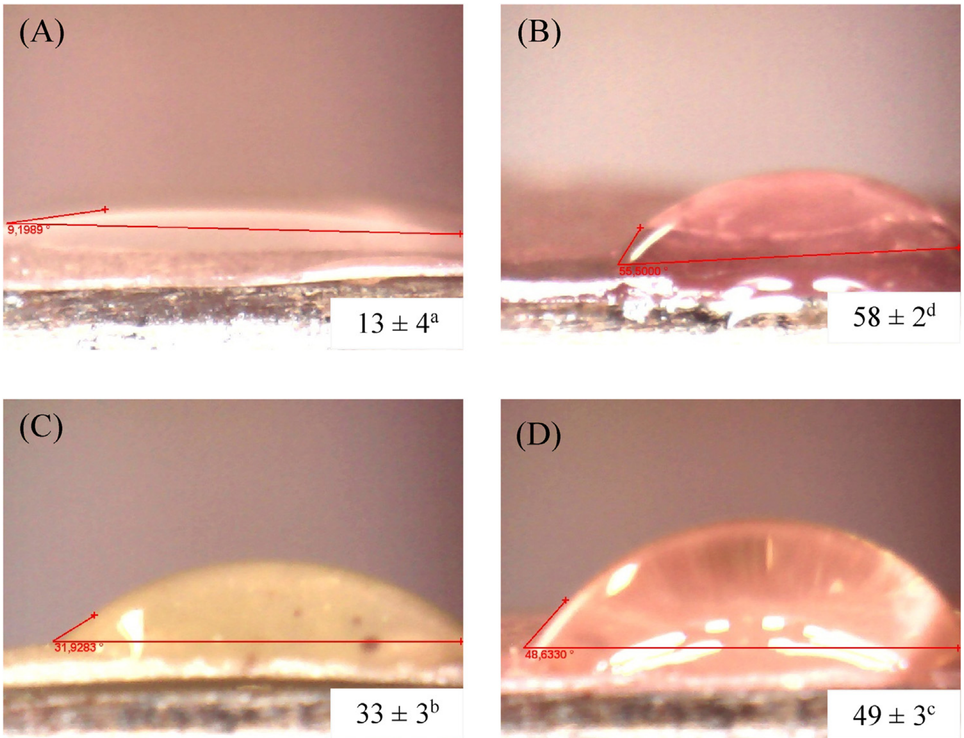

Fig. 2. Contact angle of the films: (A) native plantain starch (TPS-NPS), (B) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (C) pre-gelatinized plantain flour (TPF-PPF) and (D) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB). Different letters (a, b, c, d) in the contact angle values indicate statistically significant differences ($p \leq 0.05$).

indicates that a hydrophobic surface requires more polar sites (Lewis sites) in order for the humidity to increase. This means that the Lewis sites would be affected, thus generating a reduction in the surface polarity of blackberry pulp-containing biopolymer films. According to Gutiérrez and González (2016b) an increase in hydrophobicity in films based on native plantain flour/*aloe vera* gel blends occurred because of the higher surface energy in these materials, as a result of the crosslinking between the citric acid found in the *aloe vera* gel and starch chains. This would be in line with the results set out above. Therefore, it can be said that water structure requires more energy to break the hydrogen-bond network between the matrices and glycerol. Otherwise, occurs in films with lower contact angle values, where water structure does not require so much energy to wet the surface. In this way, water can break the hydrogen-bond network between the matrices and glycerol, when blackberry pulp was not incorporated.

3.4. *Scanning electron microscopy (SEM)*

Fig. 3 shows the SEM images of the surface exposed to air drying of the different films. It can be seen that the films produced are non-porous. Specifically, the TPS-NPS film shows granular particles (Fig. 3A), which may be associated to retrograded starch. It is worth noted that in a previous work of our research group determined the greater tendency towards retrogradation of the native plantain starch compared to the pre-gelatinized plantain flour (Gutiérrez, 2016). Based on this fact, the morphology obtained for TPS-NPS film can be justified due to the tendency to retrogradation of the native plantain starch.

Moreover, all the systems presented a compact structure, but this was far more marked in the films made from native plantain starch (Fig. 3A and B, TPS-NPS and TPS-NPSB, respectively). Gutiérrez et al. (2015a) obtained similar structures in their study of cassava-glycerol films. This was probably produced by the high amount of amylose present in cassava starch systems (Noel, Ring, & Whittman, 1992).

Additionally, more compact and closed microstructures were observed in films containing of blackberry pulp (Fig. 3B and D, TPS-NPSB and TPF-PPFB, respectively). Following to Gutiérrez et al. (2016) a more compact structure leads to lower water adsorption as it makes interactions between the starch-glycerol and water less likely, leading to a decrease in the polar glycerol-starch character of the films (García-Tejeda et al., 2013). This would agree with results from contact angle values reported for these systems, since in films with adding blackberry pulp the hydrophilic character decreased.

Vogler (1998) has also indicates that a hydrophobic surface requires Lewis sites in order for the humidity to increase. Therefore, the more compact and closed structures in films containing blackberry pulp (TPS-NPSB and TPF-PPFB) compared to the films without blackberry pulp (TPS-NPS and TPF-PPF) gives indication of a reduction of Lewis sites, possibly because this kind of structure acts as a physical barrier to water.

Another point to be highlighted is that the TPS-NPSB film (Fig. 3B) contains minor granular material compared to the TPS-NPS film (Fig. 3A). This suggests that the blackberry pulp interacts through molecular interactions with starch, thus reducing the retrogradation rate.

3.5. *Atomic force microscopy (AFM)*

AFM images of the edible films assessed are shown in Fig. 4. Surface roughness is another phenomenon that can be explained by an increase in the contact area and is strongly related to the molecular re-organization of the polymer matrix (Karbowiak et al., 2006). In this context, films containing blackberry pulp showed lower surface roughness (Fig. 4B and D, TPS-NPSB and TPF-PPFB, respectively). Similar results were reported by Gutiérrez and González (2016b) for films based on native plantain flour with added *aloe vera*

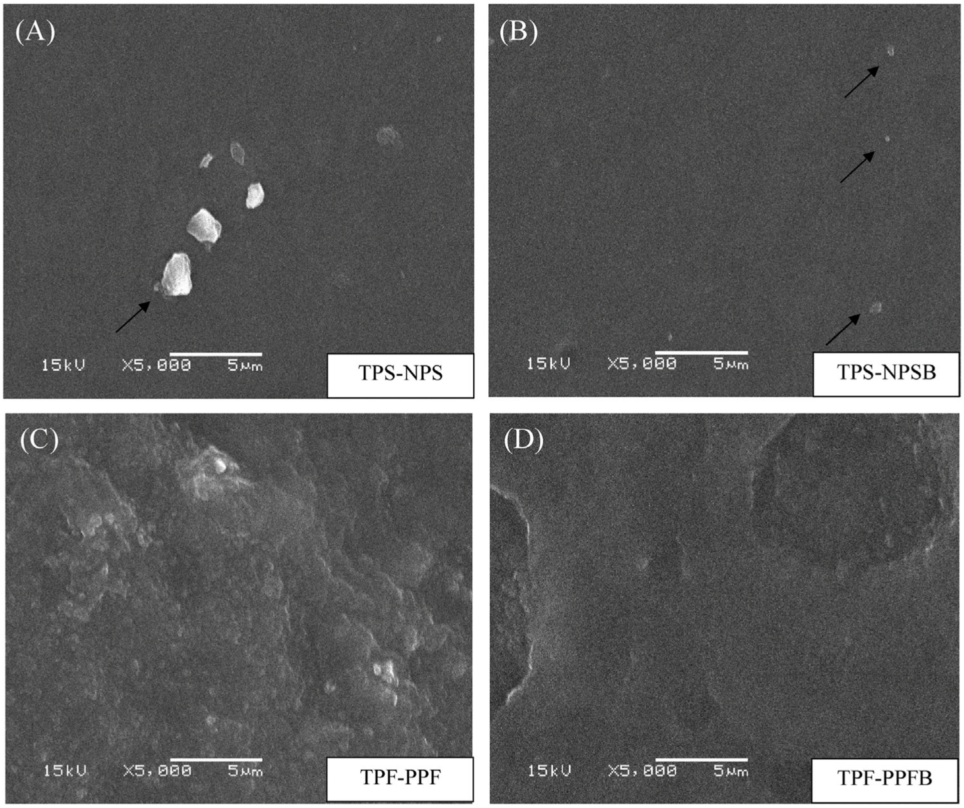

Fig. 3. SEM micrographs of the surface of the films based on: (A) native plantain starch (TPS-NPS), (B) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (C) pre-gelatinized plantain flour (TPF-PPF) and (D) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB). At 5kX of magnification. Black lines indicate granular particles.

gel. According to the authors this phenomenon occurs as a result of the best hydrogen-bond interactions between the matrix and glycerol, which are given below the surface of the films. In the same way, the TPS-NPSB film (Fig. 4B) had a less surface roughness than the TPF-PPFB film (Fig. 4D). By contrast, twisting forces of starch during the retrogradation process cause a greater surface roughness in these materials (Gutiérrez et al., 2016; Gutiérrez, & González, 2016a, 2016b). The latter would justify the highest roughness presented by the film based on native plantain starch (TPS-NPS, Fig. 4A). Similarly, the microstructure observed by SEM for TPS-NPS sample (Fig. 3A) matches with the morphology observed by AFM for the same material (Fig. 4A), which would indicate that the granular material observed provokes an increase in surface roughness.

3.6. *Resistant starch (RS) and* in vitro *digestibility*

Fig. 5 shows the results for resistant starch content and *in vitro* digestibility of the developed films. All values of resistant starch content for films obtained (Fig. 5A) were higher than those reported by Gutiérrez and Álvarez (2016) for films made from native plantain flour with added *aloe vera* gel. This could increase the potential benefit effect of resistant starch content in these materials, since it is well known that the resistant starch can be fermented by a wide variety of bacteria that are found in the colon, producing short chain fatty acids which play an important role in human nutrition and wellbeing (Björck, & Asp, 1994; García-Alonso & Goñi, 2000).

It is worth remembering one important aspect from the DTGA results (see the DTGA results, Section 3.1) and is that a more marked phase separation was observed in the TPS-NPS film with respect to the other systems. Thereby, a high anisotropy can be presented for TPS-NPS film, making it difficult to say that the value of resistant starch obtained for this material can be taken as "true", since this would vary at each position of the film. This would justify the higher standard deviation associated with this determination compared to other systems.

Despite this the added blackberry pulp increased the resistant starch content in films (TPS-NPSB and TPF-PPFB). Although no significant differences were observed between the use of the different starchy matrices ($p \geq 0.05$) (plantain starch and pre-gelatinized plantain flour). This was possibly related to the crosslinking effect caused by citric acid found in blackberry pulp (Gutiérrez, 2016). Similar results were reported by Gutiérrez and Álvarez (2016) for cross-linked films based on native plantain flour with added *aloe vera* gel.

Moreover, *in vitro* digestibility was decreased after adding blackberry pulp independently of the matrix used (Fig. 5B). According to Gutiérrez and Álvarez (2016) these results can be associated with a higher resistant starch content. Therefore, adding blackberry pulp in this kind of food can be beneficial for consumers with special regimes such as diabetics, obese and celiacs, since according to Björck, Granfeldt, Liljeberg, Tovar, and Asp (1994) a lower starch digestion rate can promote a moderated *in vivo* glycemic response.

Another aspect to highlight is that a more closed and compact morphology in the TPS-NPSB and TPF-PPFB films (Fig. 3B and D) could be related to the lower *in vitro* digestibility rate, since this could be a physical barrier to enzymatic action in these systems. From the works previously carried out by our research group we came to similar results in films derived from native plantain flour with added *aloe vera* gel (Gutiérrez and Álvarez, 2016; Gutiérrez and González, 2016b). In this regard, deeper studies to determine the effect of microstructure of solid foods with respect to their nutritional properties should be carried out.

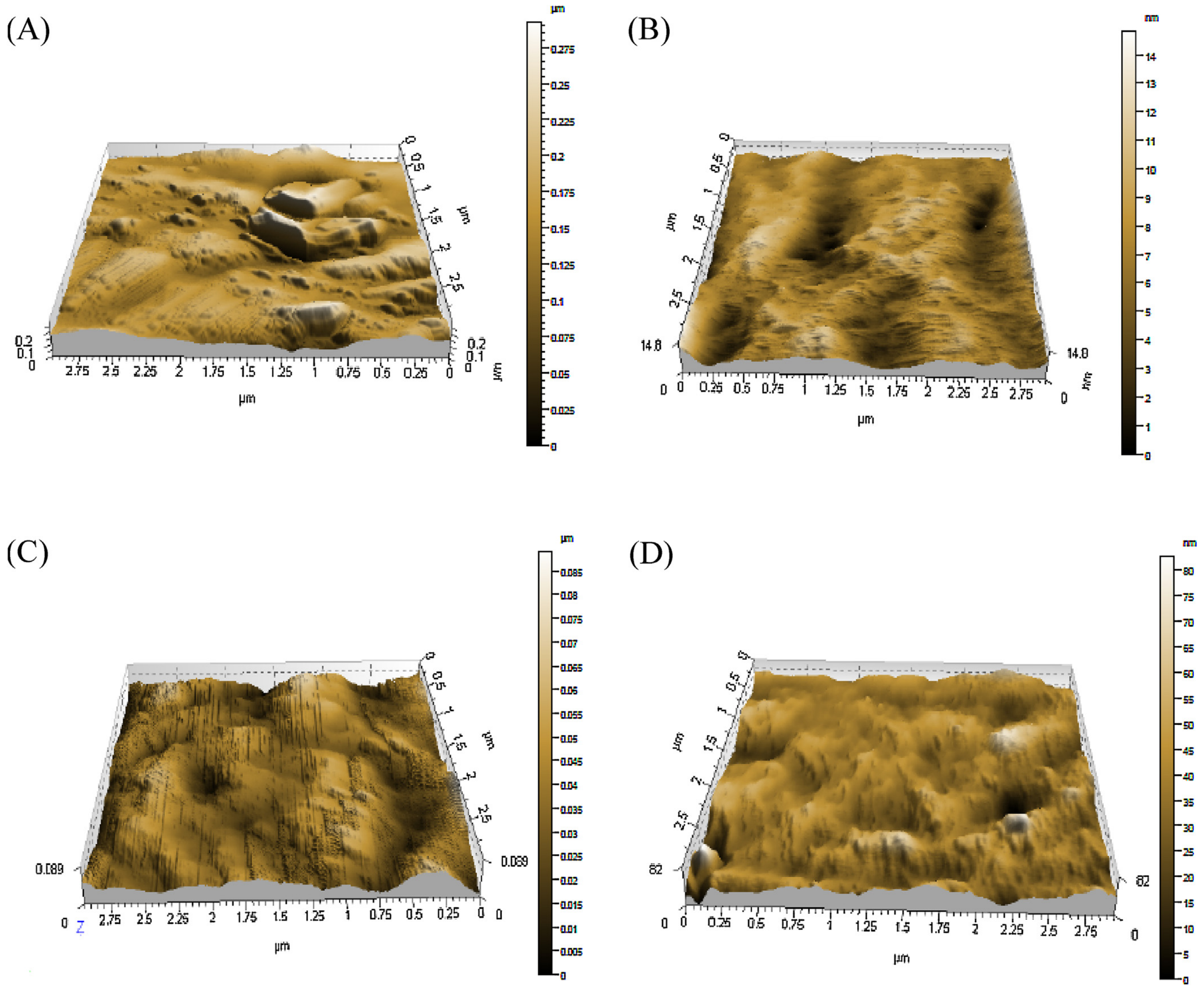

Fig. 4. AFM images of the films: (A) native plantain starch (TPS-NPS), (B) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (C) pre-gelatinized plantain flour (TPF-PPF) and (D) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB).

3.7. Anti-oxidative activity – cell viability and reactive oxygen species (ROS)

Fig. 6 shows the results related to the anti-oxidative activity of the developed films. The exposure to an oxidative stress (H_2O_2) caused a reduction in cell viability in all cells except those supplemented with films containing blackberry pulp (TPS-NPSB and TPF-PPFB) (Fig. 6A). Probably the above described behavior is related to the content of polyphenolic compounds reported by Gutiérrez (2016) for these same systems. To the authors' knowledge, there are no reports to date in this type of study on edible films that allow comparison with other results reported in the literature. Therefore, this is the first work that demonstrates the beneficial effect of edible films based on starch or flour on cell viability when in such matrices is added a natural filler. However, no significant differences ($p \geq 0.05$) on cell viability were observed using different matrices. Another fact to note is that films without added blackberry pulp showed no pro-oxidant effect, i.e. no significant differences ($p \geq 0.05$) were observed regard to control. Therefore, apparently these materials are electrochemically stable without the addition of blackberry pulp. This makes us think in future works related to the chemical potential of these materials and other applications within the food industry.

On the other hand, upon the H_2O_2 treatment the ROS concentration is remained unaltered ($p \leq 0.05$) in films without added blackberry pulp (TPS-NPS and TPF-PPF) compared to control (Fig. 6B). This confirms that films without blackberry pulp could not prevent oxidative damage to the cells in an oxidative environment. In contrast, films containing blackberry pulp decreased the concentration of the oxidizing agent (H_2O_2), suggesting the anti-oxidative activity of TPS-NPSB and TPF-PPFB films on cells.

In this sense, it can be concluded that a mechanism proposed for the antioxidant activity of films developed on healthy cells, is related to the capture of free radicals (H_2O_2), thus avoiding oxidative damage on healthy cells.

3.8. Anti-inflammatory activity – cytokines secretion in the cell media

In this study, the anti-inflammatory effects of the different edible films were evaluated in cultured cells. The production of anti-inflammatory cytokines represents the cellular physiological response to an inflammatory stimulus (Schottelius, Mayo, Sartor, &

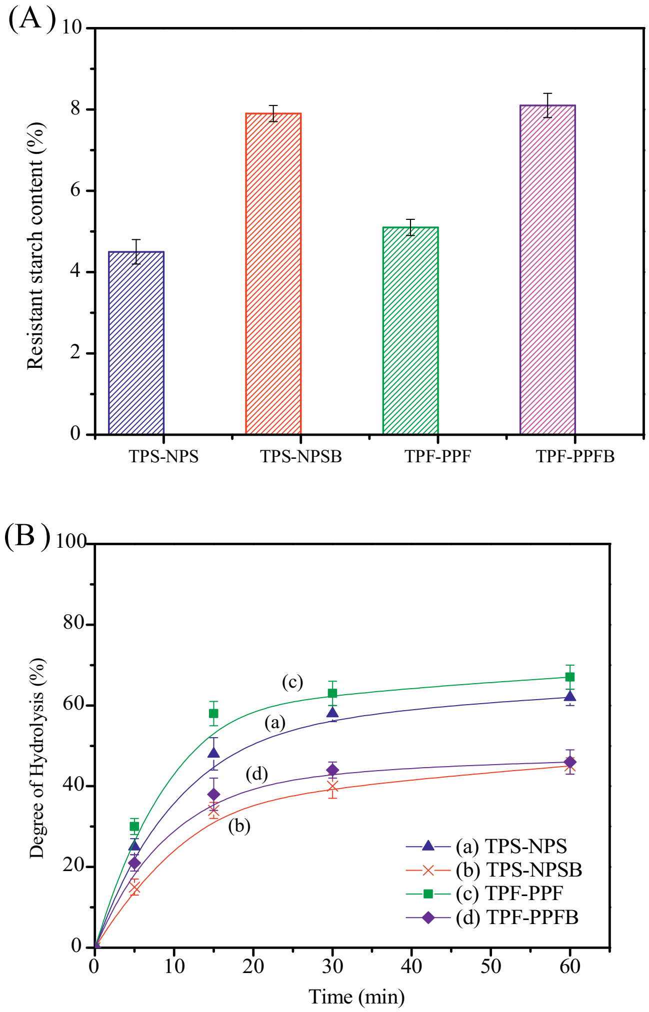

Fig. 5. (A) Resistant starch content and (B) *in vitro* α-amylolysis curves of the different films studied: (a) native plantain starch (TPS-NPS), (b) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (c) pre-gelatinized plantain flour (TPF-PPF) and (d) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB).

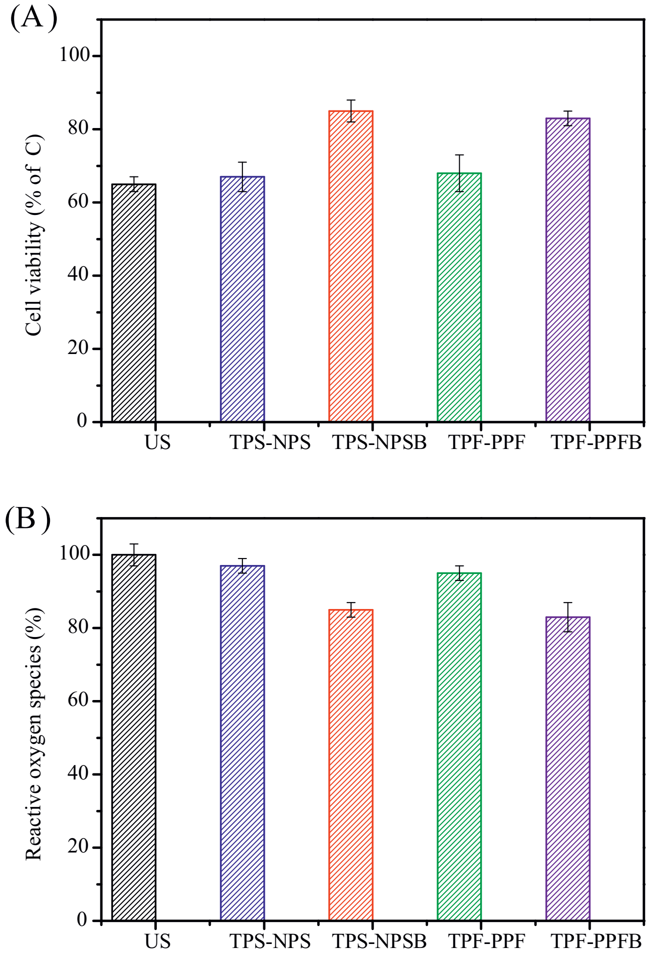

Fig. 6. (A) Cell viability and (B) ROS concentration in control (US) and supplemented cells after the oxidative damage. Thermoplastic starch (TPS) and thermoplastic flour (TPF): (a) native plantain starch (TPS-NPS), (b) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (c) pre-gelatinized plantain flour (TPF-PPF) and (d) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB). Data are means ± SD of at least 6 samples derived from 3 independent cell cultures.

Baldwin, 1999). To evaluate the possible effect of the different films on cell cytokine secretion, interleukin-8 (IL-8) and interleukin-10 (IL-10) were chosen as markers, since HepG2 cells are capable of producing a response to specific stimulation. Likewise, IL-10 is a prototypical regulatory cytokine, which exerts several immune-modulatory effects (Yamazaki, Murray, & Kita 2008), whereas IL-8 is a pro-inflammatory molecule, which also induces cytotoxic effects (Makni et al., 2011).

In basal condition, the blackberry pulp-containing films induced a significant decrease ($p \leq 0.05$) in the production of pro-inflammatory IL-8 (Fig. 7A). In a complementary way, the secretion of the anti-inflammatory IL-10 increased in all supplemented cells with blackberry pulp-containing films compared to the unsupplemented control cells (Fig. 7B), thus suggesting an anti-inflammatory action. According to the literature, the blackberry has anti-inflammatory properties, which are mainly attributed to their phenolic compounds (Bowen-Forbes, Zhang, & Nair, 2010). Therefore, the addition of the blackberry pulp in the materials developed demonstrated that these films are unique materials, since not only have active and intelligent properties, but also have beneficial properties on the health of consumers, which could be associated to the phytochemical compounds that are found in these films (Gutiérrez, 2016).

A significant increase ($p \leq 0.05$) of the pro-inflammatory IL-8 secretion was observed, upon the exposure to lipopolysaccharides (LPS) (Fig. 8A), thus suggesting a lower anti-inflammatory activity in blackberry pulp-containing films in conditions in which the cells are stressed.

3.9. *Sensory evaluation*

Fig. 9 shows the results of the sensory evaluation of the developed films. Overall, the attributes of the TPS-NPS, TPF-PPF and TPF-PPFB films were not acceptable to consumers. Similar results were reported by Gutiérrez and Álvarez (2016). Nonetheless, the TPS-NPSB film had a great overall acceptability, which was positively related to attributes of color and taste imparted by the blackberry pulp. However, although the TPF-PPFB film contained blackberry pulp this did not have appreciable overall acceptance, probably due to the flavor imparted by the matrix (pre-gelatinized plantain flour). It is worth remembering that starch has among its advantages, that it is a polymeric matrix that does not impart flavor (Gutiérrez Morales, Tapia, Pérez, & Famá 2015), this would explain what was described previously. Taking into account all the results obtained in this work, and highlighting the high acceptance of the TPS-NPSB film, this type of edible films would have a large potential market in a population that is increasingly con-

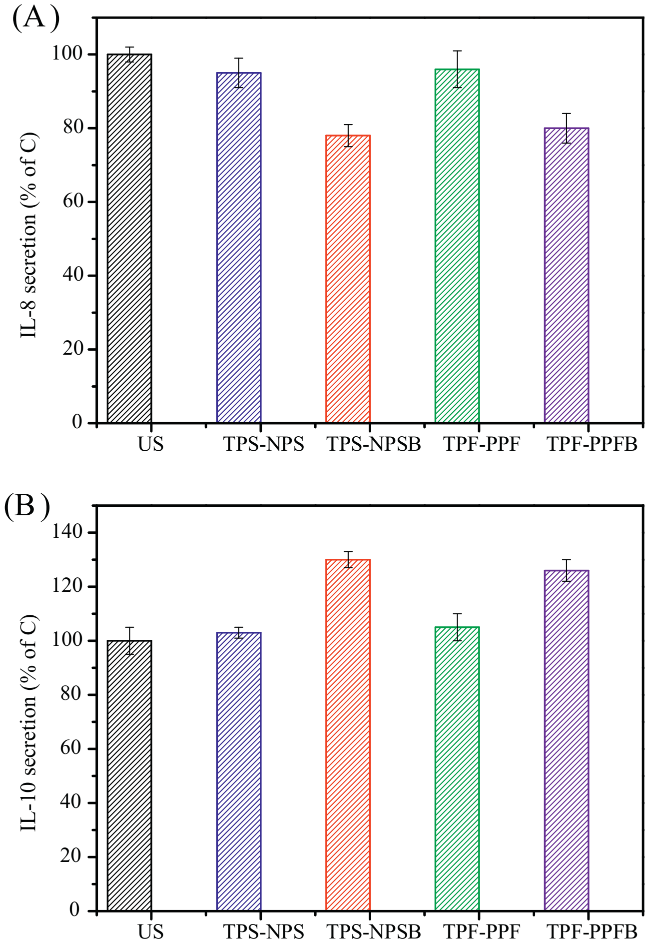

Fig. 7. (A) IL8 and (B) IL-10 secretion in basal conditions of the different films studied: (a) native plantain starch (TPS-NPS), (b) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (c) pre-gelatinized plantain flour (TPF-PPF) and (d) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB). Data are means ± SD of at least 6 samples derived from 3 independent cell cultures.

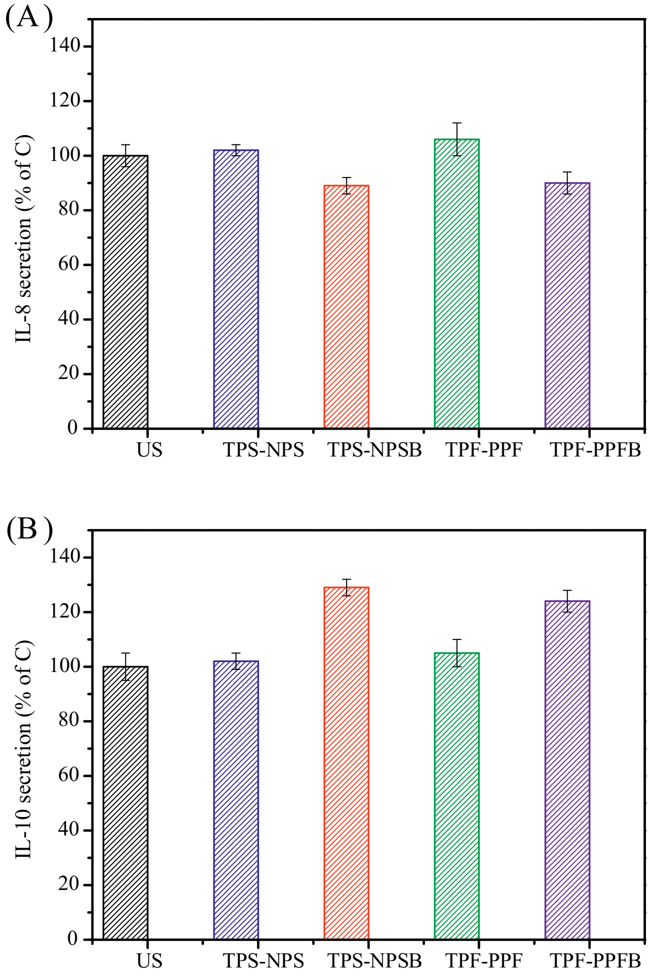

Fig. 8. (A) IL-8 and (B) IL-10 secretion after the pro-inflammatory stimulus of the different films studied: (a) native plantain starch (TPS-NPS), (b) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (c) pre-gelatinized plantain flour (TPF-PPF) and (d) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB). Data are means ± SD of at least 6 samples derived from 3 independent cell cultures.

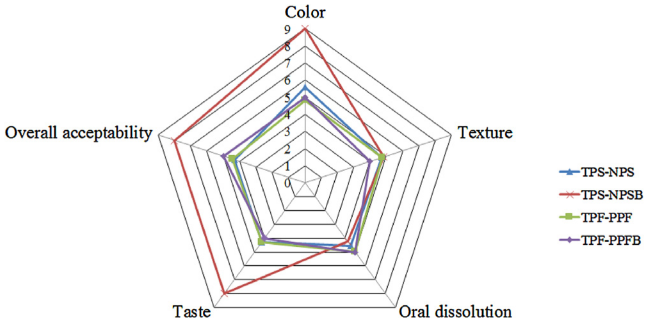

Fig. 9. Sensory evaluation of the different films studied: (a) native plantain starch (TPS-NPS), (b) native plantain starch with incorporation of blackberry pulp (TPS-NPSB), (c) pre-gelatinized plantain flour (TPF-PPF) and (d) pre-gelatinized plantain flour with incorporation of blackberry pulp (TPF-PPFB).

cerned about the benefits aspects of food. Finally, these results are of great importance, since in previous studies we determined a potential beneficial effect on health in similar materials, however, the sensory properties were not acceptable (Gutiérrez & Álvarez, 2016).

4. Conclusions

In this work, the films developed with added blackberry pulp had lower *in vitro* digestibility rate, which was related to a more compact and hydrophobic surface. A more compact and closed surface was related to the best hydrogen bond interactions established between the components of blackberry pulp and starchy matrices. Probably, citric acid in particular, and in general, organic acids contained in the blackberry pulp could cause crosslinking reactions, thus allowing the increase of resistant starch content in systems containing blackberry pulp. Similarly, films containing of blackberry pulp showed anti-inflammatory activity and higher cell viability, which is encouraging in the development of edible films with nutraceutical properties. In particular, the film based on plantain starch with added blackberry pulp had the best overall acceptability between the panelists. Therefore, films based on plantain matrices could be consumed by celiac, since these matrices are considered suitable for this population. Additionally, a lower *in vitro* digestibility rate in this system indicates its potential as a target food for obese and diabetic. Finally, it is worth noting there are still many challenges ahead, since the nutritional properties of edible films are just beginning.

Conflicts of interest

The author declares no conflict of interest.

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