DEGRADACIÓN BIOLÓGICA DE UNA PELÍCULA DE ALMIDÓN DE YUCA Y ÁCIDO POLILÁCTICO POR *Ulomoides dermestoides* (Chevrolat, 1878)

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Trabajo para optar al título de Doctor en Ciencias Agrarias y Agroindustriales

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Tesis realizada por Margarita del Rosario Salazar Sánchez, bajo la dirección y supervisión de los abajo firmantes, aprobada por los mismos y aceptada como requisito parcial para obtener el grado de Doctor en Ciencias Agrarias y Agroindustriales

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DEDICATORIA

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DATOS BIOGRAFICOS

Margarita del Rosario Salazar Sánchez, obtuvo el título de Bióloga en 2008, y de Magíster en Recursos Hidrobiológicos Continentales en 2013, en la Universidad del Cauca (Colombia). Se ha desempeñado como profesora en los programas de biología, ingeniería agropecuaria e ingeniería agroindustrial. Inició como investigadora en el Grupo de Investigación Ciencias Ambientales (GEA) de la Universidad del Cauca desde 2008 en las líneas de investigación de bioindicación y calidad del recurso hídrico, actualmente es parte del Grupo de Investigación en Ciencia y Tecnología de Biomoléculas de Interés Agroindustrial (CYTBIA), en la línea de investigación de biodegradación.

A Margarita por su dedicación le han dado reconocimientos como Joven Investigador de Colciencias en 2012, Becaria para sus estudios de Maestría por su alto promedio académico y Becaria para realizar su estudio doctoral en Ciencias Agrarias y Agroindustriales.

Entre otros muchos logros, es de resaltar, su empeño y colaboración en la formación de estudiantes investigadores como tutora de los Semilleros de investigación en Biomoléculas, Bioempaques y Aplicaciones de la microscopía en la investigación de ciencias biológicas, en los cuales ha formulado y ejecutado varios proyectos de investigación.

Su tema de interés como profesora e investigadora incluyen la ecotoxicidad, biodegradación de polímeros, ecología y conservación; temas en los que se ha publicado capítulos de libros y varios artículos en revistas nacionales e internacionales.

BIOLOGICAL DEGRADATION OF A CASSAVA STARCH AND POLYLACTIC ACID FILM BY *Ulomoides dermestoides* (Chevrolat, 1878)

Margarita del Rosario Salazar Sánchez
2019

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INTRODUCTION

On the planet the management of plastic waste has become an environmental problem, which has given rise to policies of rationalization of the use of plastic materials. Plastic recovery technologies use as raw material, recovered material, which is also used as an energy source, however these alternatives have proved inefficient and are far from being a definitive solution to this problem (E. Rudnik & Briassoulis, 2011).

The increase in the use of plastics is harmful to the environment, which has resulted in the world turning its gaze towards biodegradable polymers, especially those from agricultural raw materials, which has shown an opportunity to use renewable sources in the production of some of these polymers and reduce dependence on the use of petrochemical sources (Versino, López, & García, 2015), in this context at the University of Cauca, films obtained from thermoplastic cassava starch (TPS) and polylactic acid (PLA) have been proposed as biodegradable plastics (Villada et al., 2008 Acosta Zuleta et al., 2007;Navia & Villada Castillo, 2013; Palechor Tróchez et al., 2016; Velasco Mosquera et al., 2008; Villada Castillo et al., 2012; Villada et al., 2008) with potential industrial and commercial uses. However, under uncontrolled composting conditions, the biodegradability of this type of material may be of low efficiency, so it is necessary to develop alternative procedures to verify its biological degradation (Shah et al., 2008, Iovino et al., 2008; Smetana et al., 2016; Bassi, 2017). The above, due to the use of this type of materials in a range of multiple applications, such as food packaging products, containers, films, foams including agriculture (Glenn, Orts, Imam, Chiou, & Wood, 2014; Palechor Tróchez et al., 2016), has become a problem of global magnitude, due to the short time of use, and the final disposal of these materials, which is increasingly severe (Tumwesigye, Oliveira, & Gallagher, 2016).

On the other hand, the accumulation of plastics has become a difficult problem to control in many countries. Some of them have adopted policies to rationalize the use of

plastic material, especially in the case of bags. For example, China from June 2008, created a policy that prohibits the manufacture of bags larger than 25 µm thick, charging a tax for the use of bags with thicker thicknesses. In South America: Chile, Argentina, Brazil and Colombia are already addressing the issue in order to rationalize the use of plastics by adopting policies aimed at protecting the environment (AMBIENTE, 2016; Palechor Tróchez et al., 2016).

Considering the international normative requirements to consider a polymer as biodegradable, specific conditions must be met to achieve this biodegradation, such as ISO 14855-2 in the framework of ASTM EN-13432 on aerobic degradation of biodegradable polymers, which requires a compostability process to be carried out at 58±2 °C, which in the first instance limits that under natural or alternative landfill conditions, a material can be effectively biodegraded. The plastic film obtained from thermoplastic cassava starch (TPS) and polylactic acid (PLA) is biodegradable under composting conditions. However, it is necessary to find and verify its biodegradability mediated by a biological species under natural environmental conditions that allow to broaden the spectrum of degradation of the material and subsequent use, since it has been concluded that the biodegradable potential of any material depends on the nature of its components and the conditions under which the biodegradation process is performed (Massardier-Nageotte et al., 2006, Du et al., 2008).

Furthermore, the biological degradation processes of films obtained from TPS and PLA have not been approached from a perspective that integrates the disintegration, assimilation and mineralization phases associated with biological species of macroinvertebrates of potential use in the food industry. Research involving macroinvertebrates, such as the case of *Ulomoides dermestoides*, has focused on the field of animal and human food industry as a source of protein (Barroso et al., 2014; Sánchez-Muros, Barroso, & Manzano-Agugliaro, 2014; Xiaoming, Ying, Hong, & Zhiyong, 2010), and some studies report this beetle as the cause of spoilage in dry food (Smetana, Palanisamy, Mathys, & Heinz, 2016; Van Huis et al., 2014, Salomone et al., 2017).

In the field of polymers, research with insects of the Tenebrionidae family within the framework of biodegradation, the disintegration of polystyrene (polymer of petrochemical origin) is reported, as well as studies as a supplier of chitin as an input for biodegradable materials (Panini et al., 2017, Bassi, 2017, Murugan, Han, Gan, Maurer, & Sudesh, 2016; Y. Yang et al., 2015a, 2015b). However, there are few reports using these beetles in the degradation of polymers obtained from renewable sources, as TPS andPLA.

Noting the fact that biodegradable polymers, not having an adequate final disposition, can become major environmental pollutants, as can plastics obtained from petrochemical sources (Avérous & Halley, 2014). Therefore, it is proposed to carry out the biodegradation study of a biodegradable plastic film obtained from a mixture of TPS and PLA taking into account the three stages of the process: disintegration, assimilation and mineralization using a promising biological species for the problem of solid waste management as the insects of the family Tenebrionidae.

Since biodegradation processes for materials obtained from renewable sources such as TPS/PLA plastic film have not been approached from an integral perspective, where all phases of biodegradation are covered together (Ren, 2010; Yates & Barlow, 2013). Therefore, the need arises to evaluate the impact of biodegradable films on a biological species, contributing to science in the knowledge on the behavior of materials during their biodegradation, their effect on macroinvertebrates and demonstrating the use of insects in industry as natural degraders of biodegradable polymers.

The development of this work was carried out under a methodological proposal, which in some cases is constructivist and selective and structured observation (Angrosino & Mays de Pérez, 2000). Analyses were performed using mixed techniques (quantitative and qualitative) such as thermogravimetry (TG), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), among others. Through the biodegradation study, using macroinvertebrate species of the coleopteran family Tenebrionidae (under natural environmental conditions), a metabolic analysis of the coleopteran was performed, with the purpose of obtaining data to characterize the physical and chemical structure of the

films obtained from TPS/PLA during the coleopteran-mediated biodegradation process (Jiménez-Buedo & Vielba, 2009).

Approach the biodegradation of TPS/PLA films using a systemic perspective (Morin, 2001; Motta, 2014), allows to study and integrate intrinsic and extrinsic elements to the plastic film, considering the three phases of biodegradation: disintegration, assimilation and mineralization in a joint way and not separated as they are being done. With this study, elements are provided that provide clarity in the knowledge of degradation of these materials obtained from renewable sources using a biological species as a precursor of biodegradability. And with this, the development of systemic biodegradability techniques carried out as a research process makes it possible to account for the environmental impacts associated with biopackages and generate important advantages for end consumers by contributing to the solution of technical, economic and environmental problems in specific areas of science (Bastioli, 1998; Breuninger, Piyachomkwan, & Sriroth, 2009) uch as polymers, environmental and food.

The contribution to the solution of technical, economic and environmental problems responds to the global problem of guaranteeing the specific conditions and requirements that must be guaranteed to polymers obtained from renewable sources for biodegradation (Massardier-Nageotte et al., 2006; Bastioli, 1998; Baughan, 2015; Fellows, 2017), that until now are included in physicochemical parameters at laboratory level or large reactors that are difficult to implement in final waste disposal sites such as the case of Colombia that has open-pit fillers, where the *Ulomoides dermestoides*, due to its characteristics, can be an alternative for the management of biodegradable plastic waste and at the same time be useful in the food industry, obtaining knowledge about the *Ulomoides dermestoides* as a sustainable alternative in the degradation of biodegradable polymers, for the subsequent use to the final disposal of plastics and improvement of environmental quality by reincorporating biomass to be plastics from renewable sources, in addition, obtaining a potential raw material for the food industry.

This investigation has been developed in accordance with the studies of biodegradable packaging development of the University of Cauca, and in general as an alternative towards the sustainable and sustainable development of the investigations around biodegradable polymers. In order to achieve this study, three objectives were proposed that respond to the phases of biodegradation in a joint and integral manner, which allow elucidating the scope of the proposal for the sake of social transference: i) Evaluate the degree of disintegration of a plastic film of cassava starch and polylasctic acid by monitoring its structural changes caused by *Ulomoides dermestoides*, ii) Determine the degree of mineralization of a plastic film of cassava starch and polylactic acid by quantifying carbon dioxide (CO_2) produced by *Ulomoides dermestoides* and iii) Evaluate the effect of the assimilation of a plastic film of cassava starch and polylactic acid on *Ulomoides dermestoides*.

STRUCTURE OF THE THESIS

This memory, which summarizes the research process, is described in chapters and is considered in the following way:

Chapter 1 comprises the review and analysis of the relevant literature on biodegradation of polymers and general concepts of materials, using insects. The literature includes the vision and scope of the research from the state of the art, theoretical framework, conceptual, problem statement, justification and objectives.

Chapter 2 describes the methodology used in the research, which specifies the physical, thermal, chemical and optical techniques used to obtain the results of the proposed objectives.

In chapter 3, the results of the degradation evaluation of the film obtained from TPS/PLA in a conventional environment such as compost are presented, in order to evidence the change of its properties and to establish degradation comparison parameters with respect to the biological entity of the *Ulomoides dermestoides*.

Chapter 3 presents the evaluation of the degree of disintegration and the determination of the degree of mineralization of a TPS/PLA plastic film through the quantification of carbon dioxide (CO_2) produced by the *Ulomoides dermestoides* and the structural changes caused by the coleopteran.

In chapter 4, it includes the results of the evaluation of the effect of the assimilation of a TPS/PLA plastic film on the *Ulomoides dermestoides* coleopter.

In chapter 5, the conclusions and perspectives of future research in the framework of biodegradation are presented.

Finally, the bibliographical references that are part of the compilation of this document are presented.

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CHAPTER I

CHAPTER I

Contents

1. STATE OF ART

The annual increase in the consumption of plastics from fossil resources is currently a situation of global concern, where global plastic production exceeded 300 million metric tons per year in 2017 (Law, 2017), and since the final disposal of these products generates a negative impact on the environment, mainly due to their accumulation in landfills (De Azeredo et al., 2014). In these circumstances, several researchers have directed their efforts at studying and obtaining plastics from natural resources such as albumin, collagen, glucose, carboxymethylcellulose, chitosan, chitin and starch (Tharanathan, 2003; Bastioli, 1998, Velasco Mosquera et al., 2008, Villada Castillo et al., 2012; Avérous and Halley, 2014).

Colombia plans to reduce the use of petrochemical plastic bags by 80% by 2020 and eliminate their use by 2025 (Xanthos and Walker, 2017), with the introduction of biodegradable polymers (AMBIENTE, 2016), for which national researchers are making progress in studies on design and verification of biodegradability of polymers obtained from blends with starches (Velasco Mosquera et al., 2008; Villada Castillo et al., 2012, Villada Castillo et al., 2011, Chávez-Salazar et al., 2017, Alvarado et al., 2013, Acosta Zuleta et al., 2007; Palechor Tróchez et al., 2016).

At the local level, for approximately 10 years, as an advance towards the topic of biodegradable polymers, the physicochemical, thermal and microscopic characterization of native starch and sour cassava from Cauca was performed, which serve as raw material in the development of new products in the food industry (Acosta Zuleta et al., 2007), obtaining that the matrices of these products presented surfaces with plasticized regions with irregular spaces, with vitreous transitions between 18°C and 21°C due to the size and shape of the granule of the starch variety used (Acosta et al., 2006).

Subsequently, the Research Group on Science and Technology of Biomolecules of Agroindustrial Interest (CYTBIA) of the University of Cauca, led a program that was presented and approved in the Ministry of Environment and Rural Development in the

"National call for co-financing of research, technological development and innovation programs and projects for the agricultural sector through productive chains" in 2008 (Villada et al., 2008), he program was titled "Use of cassava products and by-products (*Manihot esculenta* Crantz) in the development of biodegradable packaging" and had the participation of the Regional Center for Cauca Productivity and Innovation (CREPIC). The projects that made up the program are described below (Navia and Villada Castillo, 2013):

The first project focused on the production and characterization of biodegradable flexible films by simple screw extrusion from cassava starch, plasticizer and PLA, which aimed at developing flexible plastics from cassava starch from seven varieties that were cultivated in the hillside area of the municipality of Mondomo (Cauca), in which the starch was gelatinized and then mixed with plasticizers and additives, coupling agents and other polymers such as polylactic acid to obtain flexible sheets using the single screw extrusion methodology, using an extrusion temperature range of 140°C, 142°C and 150°C. The applications of the flexible material obtained were oriented towards the protection of flowers or in the packaging of food products for export (Villada et al., 2008).

The second project was based on the production and characterization of biodegradable thermoformed packaging from cassava flour, fique fiber and plasticizer, in which flour from seven varieties of cassava cultivated in the northern flat zone of the department of Cauca was used. The flour was mixed with plasticizers and additives, and later the semi-rigid plastics were obtained by the hot compression molding technique. Product applications focused on food packaging

And the third project developed an active packaging for bananas from modified cassava starch and capsaicin by blown extrusion. This project used starch from six varieties of cassava grown in the hillside area of the municipality of Mondomo (Cauca). The objective of this project was to incorporate a component in the flexible plastic matrix and use the active bioplastic bags to cover banana bunches in the field; the films resisted the entire banana production stage until harvest, in which the qualitative disintegration of

the polymer residue was evaluated by burying it in the substrate of the crop, disappearing in approximately 3 months.

Currently, the CYTBIA Research Group is developing the project "Development of Biodegradable Packaging" funded by the General Royalty System, in which patent No. 11124719 "the process of obtaining flexible biodegradable films composed of cassava starch, polylactic acid and polycaprolactone" has been improved (Villada Castillo et al., 2011) and patent No. US9416275B2 "the biodegradable film obtained from cassava starch and its manufacturing process" (Villada Castillo et al., 2012), patents produced by the researchers who are the base of this study.

In the local context, most of the efforts were focused on synthesis and not much attention was dedicated to the identification of environmental requirements for biodegradable polymers and their verification (Bastioli, 1998, Baughan, 2015, Fellows, 2017) until 2016. Consequently, efforts have been made to understand the biodegradation processes of the polymers coming from cassava starch and polylactic acid obtained by the CYTBIA researchers. This film constitutes an alternative for the use of raw materials of Caucan vegetable origin and improvement of the environmental quality as it is a biodegradable polymer in composting conditions with a biodegradability percentage of 52.8% under ISO 14855-2 (Palechor Tróchez et al., 2016).

As for the use of *Ullomoides* in the food and non-food industry, humans have used insects for thousands of years, in some cases as emergency food, in other circumstances as staple food, and in other cases as delicacies. Estimates of the number of insect species consumed by humans vary, but at least 1400 species have been recorded as human food worldwide (Durst and Shono, 2010).

In the area of polymers the insects have been studied as precursor of bacterial polyhydroxyalkanoates (PHA), for example, it is reported the use of the larva of Tenebrio molitor that from the consumption of *Cupriavidus necator* (Murrugan et al, 2016), to retrieve PHA granules. In this study the purification was performed using water, detergent and heat and resulted in almost 100% pure PHA granules. Scanning electron microscopy

measurements and dynamic light scattering revealed that biologically recovered PHA granules retained their native spherical morphology. This study demonstrated the possibility of using flour worms as a biological agent to partially purify PHA granules, taking into account that PHA is a raw material for obtaining biopolymers

Research on Tenebrionides has been based on the life cycle of the insect since 1978 when it was described by Linneaus, later by Chevrolat in 1978. The evaluation of the properties of Tenebrionidae as a protein source are widely reported worldwide, however most publications focused on use as a human food source, reporting for a protein content in the larval stage of 47.2%, in pupa 54.6% and 66.3% in adult (Sánchez et al., 2014).

As for the evaluation of enzymatic content of the *Tenebrionidae*, there are studies on the purification, characterization, cloning and sequencing of ß-glycosidases of larvae, using electrophoresis techniques and chromatography of ionic exchange and hydrophobic interaction, in which it was found that four ß-glycosidases (denominated 1, 2, 3A and 3B) that are not present in animal feed are present in the lumen of the middle intestine of larvae. These enzymes have four subsites for glucose binding and can hydrolyze oligosaccharides, glycosides and alkylglycosides. Their role in the biodegradation of polymers obtained from renewable sources due to the intermediate digestion of hemicellulose and cellulose (Ferreira, 2001).

Studies conducted by Yang et al., (2015a, 2015b) of Beijing University reported, biodegradation of polymers using *Tenebrinidae* coleopters, for the disintegration and mineralization of Polystyrene (PS) obtained from petrochemical sources. These researchers found that PS degraded efficiently in the larval intestine within a retention time of less than 24 h. Fed PS foam as a single diet, the larvae lived as well as those fed a normal diet (bran) for a period of 1 month. Starch analysis of Styrofoam larvae was performed using gel permeation chromatography (GPC), 13C cross polarization nuclear magnetic resonance spectroscopy (CP / MAS NMR) and magical angle magnetic resonance spectroscopy (NMR) and Fourier transformed infrared thermogravimetric spectroscopy (TG-FTIR), it was found that excision/despolymerization of long-chain PS molecules and the formation of depolymerized metabolites occurred in the larval gut. In a trial period of 16 days, 47.7%

of the ingested PS carbon was converted to CO_2 and the residue (approximately 49.2%) was considered starch with a limited fraction incorporated into the biomass (approximately 0.5%). Tests with PS were marked with alpha 13C or beta 13C confirmed that PS marked with 13C was mineralized to $13CO_2$ and incorporated into lipids. From this research it is also concluded that the discovery of the rapid biodegradation of PS in the larval gut reveals a new destination for plastic residues in the environment.

Another report on the evaluation of degradation of polystyrene and polyethylene, the researchers divided into 4 groups of 11 larvae, with an approximate mass of 13 g and assigned a sample of 25g polymer as food, which they called polystyrene, unicel, diapers and garbage bag. This resulted in a percentage of biodegradability of 96% for the polystyrene sample, 84% for unicel, 65% for diapers and 64% for the garbage bag (Hermosillo et al., 2016).

1.1. THEORETICAL AND CONCEPTUAL CONTEXT

1.2 Cassava starch

Cassava (*Manihot esculenta* Crantz) is an important widely cultivated food crop due to its wide adaptability to soil and drought, and is the most important root crop in the tropical regions of the world. The main use of cassava is for human consumption, animal feed and as a raw material for industry, in applications such as adhesives, textiles and paper, among others. Starch is the most carbohydrate-rich component of cassava and is widely used in the pharmaceutical and food industries due to its unique thickening properties, high purity, low cost and its ability to form clear viscous pastes (Tan *et al.*, *2017*).

The starch molecule is a carbohydrate composed of hundreds or thousands of D-glucose units, which are linked together by glycosidic bonds α-D(1-4) forming linear polymer (amylose) chains with α-D(1-6) large glycosidic chains forming branched chains (amylopectin). Both molecules are integrated into a semi-crystalline granule (Buléon et al., 1998, Ai and Jane, 2016). The short part of the amylopectin chains takes the form of a "double helix" (Avérous and Halley, 2014), these chains associate in groups or clusters forming ordered regions or crystalline sheets while the branched parts of the molecule constitute amorphous sheets, this structure forms cassava starch granules comprising a granule diameter of between 1 - 5 µm, have a size, shape, and composition that depends on their botanical source (Bergthaller and Hollmann, 2007). Cassava starch contains 17% amylose compared to 21% maize and 22% potato starch (Bates *et al.*, 1943). The processing and properties of these semi-crystalline materials are closely linked to the genetics of starch and various levels of granule structure to macromolecular structure and crystalline macrostructures (Bergthaller and Hollmann, 2014).

However, the hydrophilic nature of starch plays an important role in initiating biodegradation processes, but also limits its technical applications. Therefore, attempts

have been made to overcome this limitation by modifying the starch structure, formulation, and processing techniques (Su and Sun, 2017), considering that the biodegradation or incineration of starch does not result in any net CO_2 gain, the manufacture of starch-based plastics as alternatives to common petrochemical derived polymers becomes attractive and is a factor that can significantly influence the overall performance and biodegradability of starch-based polymers or in mixture with renewable sources such as lactic acid, polycaprolactone, among others (Gattin et al., 2002).

1.3 Thermoplastic starch

Plastification of the native starch granule is obtained by structural disruption resulting from a decrease in crystals during the extrusion process and the action of the plasticizer, a new type of material known as thermoplastic starch (TPS) emerges (Villada et al., 2008). TPS has several attributes, in addition to biodegradability, it is a renewable, low-cost, flexible material that can be easily conditioned to different thermoplastification processes using standard equipment used in the manufacture of synthetic polymers (Acosta et al., 2015).

1.4 Polylactic Acid

Polylactic acid (PLA) is a linear aliphatic polyester produced by polycondensation of lactic acid produced naturally or by the opening of the catalytic ring of the lactic group. Lactic acid is produced (through starch fermentation) as a co-product of wet milling of corn (Shah et al., 2008, Castro-Aguirre et al., 2016).

The PLA used in the plastic film of this study is PLA 4032D (NaturalWorks, LLD, USA), which is characterized by being obtained from a combination of fusion with 2,5% by weight (% microcrystalline cellulose (MCC) and cellulose microfibres (CMF) treated or untreated) using a two roller laboratory mill, where the nature of the dispersed phase of PLA presents in MCC an average particle size of 20 µm and CMF with diameters of 5 to 15 µm and has a melting point of 160 °C. PLA compounds are very promising materials, as they can show better performance (high strength and stiffness, improved thermal stability,

higher HDT and better processing (crystallization capacity), low flammability, antistatic to conductive electrical characteristics, barrier properties etc.). Maintaining the specific biodegradability properties of the polymeric matrix (Murariu & Dubois, 2016).

The PLA is generally completely biodegradable when composted at temperatures of 60 °C and above. The first stage of degradation of polylactic acid is through hydrolysis to water-soluble compounds and lactic acid by the rapid metabolization of these products into CO_2, water and biomass by a variety of microorganisms (Shah et al., 2008). Some reports on the degradation of PLA oligomers (molecular weight ~ 1000) include species such as *Fusarium moniliforme* and *Penicillium sp.*, (Rudnik and Briassoulis, 2011, Gattin et al., 2002), the degradation of PLA by *Amycolatopsis sp.* (Iovino et al., 2008, Fukushima et al., 2009) and by *Bacillus brevis* (Castro-Aguirre et al., 2016). In addition, the enzymatic degradation of low molecular weight (molecular weight ~ 2000) PLA has been demonstrated using esterase type enzymes such as *Rhizopus* delemer lipase (Hironobu et al., 1989), the biodegradation of PLA mediated by macroinvertebrates studies have few scientific reports.

1.5 *Ulomoides dermestoides*

It is a species of coleoptera of the family Tenebrionidae, better known as flour worm (in its larval stage), is a promising source of alternative proteins and is already being produced on an industrial scale (Murugan et al., 2016, Panini et al., 2017). This insect is commonly produced in mixed grain diets, although it can also consume meat or feathers, among other alternatives, due to its omnivorous nature (van Broekhoven et al., 2015, Rojas et al., 2016).

Contains in its dry base high amounts of crude protein (47-60%) and lipids (31-43%), a relatively low ash content (5%) and fresh larvae contain approximately 60% water, as well as a good source of vitamins and minerals (Makkar et al., 2014; Salomone et al., 2017). In poultry diets, the meal worm is a potential alternative feed source, particularly to replace soybean meal or fish meal (De Marco et al., 2015, Piccolo et al., 2017).

As for the life cycle, a female *Ulomoides* ovipone about 580 eggs; the oviposition period is variable between 25 and 140 days, depending on the conditions of the medium and food. The newly hatched larvae are active, consume food and move freely; they acquire their maximum development between 89 and 100 days, after molting between 9 and 18 times; in this state they remain active consuming substrate until they reach the pupal stage (which lasts between 12 and 16 days), then emerge as adults. The complete cycle from egg to egg, takes between 300 and 350 days depending on environmental conditions, but in hatchery the complete cycle lasts approximately 10 to 12 weeks (Artigas, 1994). The larvae of *Ulomoides* are elateriformes, cylindrical and elongated body, with hard exoskeleton with short legs and well developed head and the urogomphi is at the end of the abdomen, the pupa is exarada, the appendages are not attached to the body, but exposed externally (Vinokurov *et al.*, 2006).

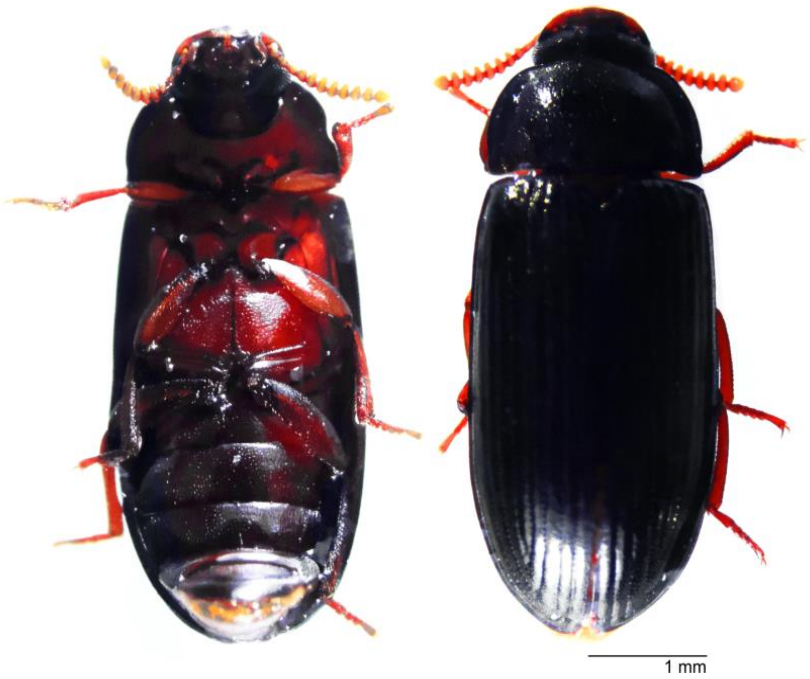

Figure 1. *Ulomoides dermestoides.*

The use of Tenebrionides in the field of plastics, Wei-Min Wu of Stanford University and his colleagues have discovered that microorganisms in the intestine of the worm insects of the Tenebrionidade family can biodegrade one of the most polluting plastics such as polystyrene, which they evaluated through analysis of respirometry and

surface loss, finding that some Tenebrionidae can degrade up to 40% of polystyrene (Yang et al., 2015a, Yang et al., 2015b), which he suggests this discovery has "opened a new door to solving the global problem of contamination by non-biodegradable materials" (Murugan et al., 2016, Bradley, 2016), because biodegradable plastics or plastics from renewable sources require specific conditions to achieve biodegradation, otherwise they will be just as pollutant as those of petrochemical origin (Ren, 2010).

1.6 Biodegradation

According to (Eubeler et al., 2010), biodegradation is a process that generally occurs in the final disposal of plastics as waste until their return into the natural carbon cycle. In this sense by definition, biodegradable polymers are those that can be degraded to carbon dioxide, water and biomass (Tharanathan, 2003). Biodegradability is also defined as the propensity of a material to break down into its constituent molecules by natural processes (Singh, 2008).

Concerning the methods to monitor and evaluate the biodegradability of starch polymer mixtures, the following are reported in the literature: respirometry, morphology, microscopy, gravimetry, physics and morphology, spectroscopy, chromatography, microbiological and enzymatic techniques (Karim et al., 2000, Bastioli, 1998).

The respirometric method consists in the biodegradation of the test material by environmental microorganisms under aerobic or anaerobic conditions, the carbon dioxide produced is measured (Gattin et al., 2002, Tadasa and Takeda, 1986), the oxygen consumed, or biogas produced (Massardier-Nageotte et al., 2006). The standards on which biodegradation research under aerobic conditions is based are: ASTM D5338-92, ISO 14855 for composting (Palechor et al., 2016), ASTM D5988 (Camacho et al., 2016), ISO 17556 for soil, ASTM D5209-92 for aerobic sludge, ASTM D5210-92 for anaerobic sludge, ASTM D5511-11 for anaerobic digesters, ASTM D6691-01 for aerobic marine inoculum, ISO 9408 and ISO 14852 for aerobic aquatic inoculum, and ISO 14852 for anaerobic inoculum (Yang et al., 2005, Kijchavengkul et al., 2006, Krzan et al., 2006, Ehrenstein and Pongratz, 2013, Philp et al., 2013).

In morphological studies is observed the physical appearance of polymers (color, shape, size, any breakage / holes visible on the surface of the polymer, and / or microbial growth) is recorded before and after biodegradation (Muthukumar et al., 2010). The colour change can also be used as an indication of the biodegradation of starches through the use of a colourimeter (Schwartz and Whistler, 2009).

Similarly, the scanning electron microscopy (SEM) technique is commonly used to evaluate the surface morphology of polymers and to detect the growth of microorganisms in polymers during biodegradation (Robyt, 2009, Muthukumar et al., 2010, Singh and Sharma, 2008).

Gravimetry studies are not necessarily the most effective method for measuring the biodegradation of starches as they normally contain a high percentage of water. This water content can significantly increase biodegradation, which is shown as an increase in weight. It consists of weighing before and after biodegradation the polymer samples and determining the weight loss in percent (Gattin et al., 2002, Singh et al., 2016).

As for the physical changes in which tensile tests (tensile strength and elongation at break) are performed, which are used to determine the changes in mechanical properties during biodegradation (Acosta et al., 2015, Cerruti et al., 2011, Al-Hassan and Norziah, 2012, Arık Kibar and Us, 2013, Atichokudomchai et al., 2004, Biliaderis, 2009, Buléon et al., 1998). The X-ray diffraction technique is often used to measure the degree of crystallinity in starches. The differential scanning calorimetry technique and thermogravimetric analysis are also used to investigate the thermal transitions of polymers by measuring their transition temperatures, melting point and also crystallinity; all these properties are affected during the biodegradation process (Biliaderis, 2009). Figueroa et al., (2016), consider that to evaluate the biodegradation of starch-polymer mixtures in soil, thermal analysis is preferred over conventional weight loss method as it overcomes errors in test results due to adhering soil or microbial growth.

Fourier Transformed Infrared Spectroscopy (FTIR) techniques are used to obtain qualitative information on changes in chemical structure or formation of functional groups in starches during biodegradation (Jayasekara et al., 2003, Muthukumar et al., 2010).

Gel permeability chromatography is used to determine changes in the mean molecular weights (Mn and Mw) and the polyspersity index (relationship between molecular weight and mean weight Mw/Mn) of polymer samples during biodegradation (Mischnick and Momcilovic, 2010, Zhang et al., 2010). And high-performance liquid chromatography (Kitahara et al., 2002, Mischnick and Momcilovic, 2010) is used to detect the presence of monomers and oligomers formed in aqueous or gas phases during biodegradation

Microbial degradation of materials is assessed by subjecting test materials to either a wide range of environmental microorganisms found in compost, soil, seawater and continental water, and activated sludge samples (Gattin et al., 2002, Shah et al., 2008) o for selected pure cultures of bacteria and fungi known to have biodegradation potential (Singh and Sharma, 2008).

One of the following microbiological techniques used to evaluate the biodegradation of starch mixtures into polymers by microorganisms or enzymes produced by microorganisms is microbial growth/clear area, where the test sample is disinfected, dried and placed in solid or liquid media that do not contain additional carbon source, and sprayed with environmental samples (soil / compost / seawater) or pure cultures of bacteria and fungi. After incubation to specific test conditions, the test material is visualized for any microbial growth and/or clear zone formation (in solid media). Some studies have reported on the susceptibility or resistance of starch polymer mixtures to microbial degradation using these techniques (Tosungnoen et al., 2014, Chookietwattana, 2014).

The enzymatic degradation method involves the incubation of sterile film samples in a buffer solution broth or medium containing microbial or synthetic enzymes. Enzyme activity is measured spectrophotometrically in a liquid medium and the percentage of weight loss over the time period is calculated. Some studies have reported the enzymatic

degradation of starch polymer is mixed by microbial agents and commercially available enzymes (Copinet et al., 2001, Gattin et al., 2002, Biliaderis, 2009; Vikman et al., 1995; Uthumporn et al., 2010, Tomasik and Horton, 2012, Sangwan et al., 2014, Rajan et al., 2008, Höfer, 2015, Ali Razavi and Amini, 2016).

There are also some academic reports of eco toxicity of polymers obtained from starches or mixtures in which the percentage of survival of the plant or animal species is evaluated to evaluate the toxicity of the residue of the disintegrated polymer (Oke, 1978, Jayasekara et al., 2003, Brimer, 2015), and this is the step to advance in the formulation of biopolymers and their biodegradation.

1.7 Phases of biodegradation

According with Greene (2014), the biodegradation of polymers is framed in the phases disintegration, assimilation and mineralization. The following is a brief description of each phase using *Ulomoides dermestoides* (Fig. 2):

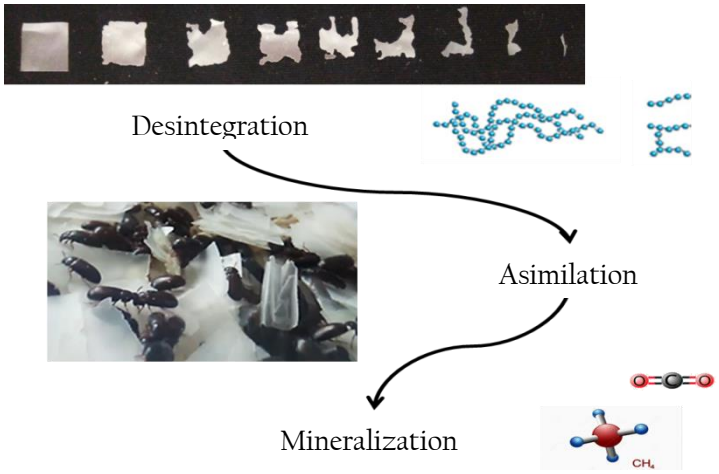

Figure 2. Phases of biodegradation.

1.7.1 Desintegration

Disintegration is one of the consequences of polymer degradation and may be due to the action of chemical or biological factors, which simultaneously degrade the polymer

by breaking the bonds that form it, generating new compounds of low molecular weight or reducing the degree of polymerization, increasing the crystallinity and therefore the breakage of the polymer into smaller particles (Bitinis et al., 2014).

The *Ulomoides dermestoides* coleoptera, being a chewing insect, can through its buccal apparatus deteriorate materials of organic origin, and taking into account that the spectrum of digestive proteinases in larval stage in the middle intestine, the pH of the content of the middle intestine increases from 5.2-5.6 to 7.8-8.2 from the anterior to the posterior, this pH gradient reflects in the optimal pH of the activity. These data support a complex protein digestion system, and the correlation of proteinase activity and pH indicates a physiological mechanism of enzymatic regulation in the intestine (Vinokurov *et al.*, 2006) giving way to the process of assimilation.

1.7.2 Assimilation

The fragments produced by disintegration or the polymer itself can be incorporated by the organism that consumes them through metabolic processes for their use and development (Rudnik, 2008).

1.7.3 Mineralization

Biodegradation is a biochemical transformation of compounds into mineralization by organisms. Mineralization of organic compounds under aerobic conditions produces carbon dioxide and water and under anaerobic conditions methane and carbon dioxide (Bastioli, 1998; Shah, 2008; Greene, 2014).

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CHAPTER II

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Contents

2. METODOLOGY

In order to meet the specific objectives proposed within the framework of biodegradation, a film obtained from cassava starch and polylactic acid with *Ulomoides dermestoides* (Chevrolat, 1978), the following methodology was proposed.

2.1 Sample preparation

The plastic film object of this research is produced in the laboratory of Rheology of the University of Cauca. The film is a polymer obtained from renewable sources such as cassava starch (*Manihot esculenta* Crantz), and polylactic acid. Three films were used: a film of thermoplastic starch from Cassava (TPS), a film of polylactic acid (PLA Ingeo 4032D, M_n=88500 g mol^{-1} and M_w/M_n=1.8) and the film of mixture of the two compounds at a ratio of 72:28 (TPS-PLA). The polymers were obtained in a double screw extruder (HaakePolylab OS model, ThermoScientific, Germany). Prior to the elaboration of the film, a pellet cord was obtained (Inmagraf, Colombia) each a pellet was 0.5 cm long and 0.5 cm in diameter. PLA maleated with benzoyl peroxide (initiating agent) was extruded in a simple screw (HaakePolylab OS, ThermoScientific, Germany). The pellets are then obtained from the extruded PLA cord. Then the mixture of TPS and PLA pellets are extruded in a simple screw to obtain the film, by means of a blow die.

2.2 Biodegradation process under controlled composting conditions

For the identification of film deterioration and morphological changes, 1 x 1 cm frames were cut from the material to facilitate the visualization of structural changes, and aerobic biodegradation under controlled composting conditions described in ISO 14855-2:2007 with a Mycroxymax respirometer equipped with a high precision CO_2 sensor, with a measuring range of 0-3% (Columbus, USA) was performed. The samples were incubated

in compost bins at a constant temperature (58°C ± 2 °C), using IF260plus incubators (Memmert, Germany), for a period of 1 month, with weekly measurements taken and as control was used microcrystalline cellulose.

2.3 Insects recollection

Adults of *Ulomoides dermestoides* were collected in Piendamó (Cauca - Colombia) (North latitude 2.645602 and West longitude 76.537918) (SIRGAS), in the litter fall zone, some adults were placed in the Biological Collection of the Natural History Museum- University of Tolima, the remaining individuals were transported to the laboratory, where they were reproduced in vials at 28°C using an incubator (Memmert, Germany). Insects were fed using an oat-based diet.

2.4 Diets for insects

When a considerable number of insects were obtained, they were divide in three groups where one group was fed with one of three diets: Oats (control-Diet 1), and two plastic films: thermoplastic cassava starch (*Manihot esculenta* Crantz) (TPS) (Diet 2) and a film of TPS and polylactic acid (PLA) (Diet 3), these last two polymers were obtained in a double screw extruder (HaakePolylab OS model, ThermoScientific, Germany). Prior to the elaboration of the film, a pellet cord was obtained (Inmagraf, Colombia) each a pellet was 0.5 cm long and 0.5 cm in diameter. PLA maleate with benzoyl peroxide (initiating agent) was extruded in a simple screw (HaakePolylab OS, ThermoScientific, Germany). The pellets are then obtained from the extruded PLA cord. Then, mixture of TPS and PLA pellets is extruded in a simple screw to obtain the film, by means of a blow die (Castillo et al. 2016).

2.5 Structural change analysis of the film

For the disintegration of flim under controlled conditions in compots, the tests were performed within the framework of ASTM E-1252-98, using Fourier Transform Infrared Spectroscopy (FT-IR) (IRAffinity-1S, Shimadzu brand, Japan). Weighed 10g sample, frozen with liquid nitrogen for pulverization in an analytical mill (PX-MFC90D Lab Brance, Germany). Using the ATR method, 3 mg of sample and 97 mg of Potassium Bromide (KBr) were weighed into a powdered film sample tablet (‹2 µm) and KBr at a ratio of 1:50, using an interval of 500 - 4000 cm^{-1}, 3 scans and a spectral resolution of 1 cm^{-1}.

And the charaterizations for structural changes of the films by biological degradation, the FTIR analysis was performed using the ASTM E-1252-98, using an FTIR spectrometer Spectrum 100 (Perkin Elmer, USA) with an ATR device for the film at a 45° angle and a screening of 100 scans. The spectrum worked was in the range of 4000 - 500 cm^{-1}.

2.6 Thermal characterization

A thermogravimetric analysis (TGA) and a differential sweep analysis (DSC) were performed using a TGA/SDTA 851e (Mettler Toledo, USA) and a DSC Q2000 (TA instruments, USA) respectively. The method used for reading the thermometer was a first stage of equilibrium at 25 °C, an exothermic ramp of 10 °C min^{-1} at 110 °C, an isothermal ramp at 60 °C, an endothermic ramp of 5 °C/min at -40 °C, an isothermal ramp of 2 min, and a final ramp of 10 °C min^{-1} at 250 °C. The method used for reading the thermometer was a first stage of equilibrium at 25 °C, an exothermic ramp of 10 °C min^{-1} at 110 °C, an isothermal ramp at 60 °C, an endothermal ramp of 5 °C min^{-1} at -40 °C, an isothermal ramp of 2 min, and a final ramp of 10 °C min^{-1} at 250 °C. A sample of 3, 5 to 4 mg of the material obtained from the disintegration and fragmentation test is available.

2.7 Gel Permeation Chromatography analyses (GPC)

For this test, pieces of film (~ 1.0g) in a circular shape (1.75 cm in diameter) with 20 adult individuals of *Ulomoides dermestoides* were available in breeding boxes with continuous ventilation and controlled temperature in an incubator (Memmert, Germany). During 5 days, (to: initial film, t1 to t5 films corresponding to each evaluation day), the sample of the fragmented film was extracted and weighed on an (OHAUS Galaxy TM 160D scale, Delta Scientifica SAS, Venezzia - Italy). From this material 300 mg of the film was diluted in 20 mL of Chloroform with agitation for 3 hours and then centrifuged and filtered to remove the soluble part in order to perform the reading of resilient PLA using a Viscotek TDA 305 Gel Permeation Chromatograph (GPC) (Malvern Company, United Kingdom).

2.7 Microscopic characterization

The characterization of the samples was performed by scanning electron microscopy (SEM) using QUANTA 200F (FEI, The Netherlands). Each SEM sample was coated with Gold-Palladium (0.8 nm thick coating) in an EmiTech K575X Peltier Cooled (QuorumTech, United Kingdom). Samples were observed at 10.0 KV at different magnifications from 50 X to 2000X, and photos of the samples were taken using a stereoscope SMZ 800 (Nikon, Japan), and some photomicrographs were taken with an optical microscope (Nikon Eclipse 80i, Japan), and with a scanning electron microscope (SEM) (Jeol JSM6490LV with a probe for chemical microanalysis INCAPenta FETx3), the samples were coated with palladium-gold, the equipment was operated at 20kV of accelerating voltage. The images were analyzed using Image-Pro Analyzer software (Media Cybernetics, Inc, USA).

2.8 Mineralization test

200 g of film were placed in fractions of approximately 1 cm^2 with 40 adults of *Ulomoides dermestoides*; in glass reactors where the temperature was controlled at 25 °C, relative humidity of 60-80% with an incubator (Memmert, Germany) and the constant flow of oxygen with the Microoxymax respirometer (Columbus, USA). A fraction of the film was removed every week and the disintegration of the film was qualitatively evaluated

every 7 days for 45 days during the process. For this case the conversion of CO_2 produced to degraded polymer was estimated as part of the biodegradation evaluation by establishing the growth of biomass (larvae of *Ulomoides dermestoides* coleoptera) vs. the production of CO_2, applying the concept of linearization and using the following equation of kinetics (Eq.1), for the growth of biomass.

$$\mu = \mu_{max} \frac{S}{k_s + S} \qquad \text{(Eq.1)}$$

Where, μ is the specific growth rate of the microorganisms, μ_{max} is the maximum specific growth rate of the microorganisms, S is the concentration of the limiting substrate for growth, K_S is the "half-velocity constant" the value of S when $\mu/\mu_{max} = 0.5$.

The initial biomass corresponded to 40 adult coleoptera (1.2968 mg), the rate of CO_2 generated in the disintegration process was determined with the Mycrooxymax Respirometer (Columbus, USA), until obtaining through equation 1 the grams of non-biodegradable polymer biomass, by CO_2 emission.

2.9 Digestive tract extraction

For extraction of digestive tracts, dissections were made in cold lethargic coleopterans in metal chambers for 15 min at 5°C. Dissection was performed using the detachment of the elytra, wings and dorsolateral cuts of thorax and abdomen. Then, cuticle of rostrum and head was fractured and cuticle fragments were removed to facilitate cleaning and release of intestinal tract.

2.10 Identification of insect specie

2.10.1 DNA isolation

DNA from insects was isolated as follow: The insect intestines were placed in 100 µL of TE1X. Then 100 µL of lysozyme (25 mg mL^{-1}) was added and left to stand for 5

minutes at room temperature. After that, 100 µL of proteinase K (20 mg mL^{-1}) were added and samples were left incubating for 20 minutes at 42°C. Subsequently, 50 µL of 20% SDS were added and left to stand for 5 minutes at room temperature and samples were heated for 10 minutes at 42°C. Then, 400 µL of CATB 1X were added mixing gently, after, samples were incubated for 10 minutes at 65°C. After that, 700 µL isoamyl phenol-chloroform-OH (24:24:1) were added mixing gently, then samples were centrifuged for 15 minutes at 13300 g. The supernatant was transferred to a new tube. Repeating the previous step. Subsequently, 600 µL isoamyl chloroform-OH (24:1) were added mixing gently, Samples were centrifuge at 13300 g for 10 minutes, supernatant was transferred to a new tube, then 30 µL sodium acetate (3M, pH = 5.0) were added to a volume of isopropanol, mixing gently and left to stand for 24 hours at -20°C. After that, samples were centrifuged at 13300 g for 30 minutes and supernatant was discarded. Then, 500 µL EtOH at 70% were added and samples were centrifuged for 5 min to 13300 g. Subsequently, supernatant was discarded and left to dry for 3 hours, tubes were uncovered and inverted, then, 100 µL of milli-Q water was added. Samples were left to re-suspend for 24 hours at 4°C (Truett 2000). DNA integrity was determined using agarose (1%) gel electrophoresis.

2.10.2 DNA amplification by PCR.

Cytochrome oxidase subunit 1 gene was amplified using the primer pair C1-J-1718 (5'-GGG GGA TTT GGA AAT TGA TGA TTA GTT TCC-3') and C1-N-2191 (5'-CCG GGG GTA AAA TTA AAA TAT AAT ATA AAC TTC-3') (Fratini, Cannicci, and Schubart 2018; Simons et al. 1994). Each PCR reaction mix was as follow: 14.5 µL of Milli-Q water, 3.5 µL of 10X buffer (10 mM $MgCl_2$); 0.5 µL dNTPs (10 mM); 2 µL of each primer (10 mM); 0.5 µl of Taq DNA polymerase (50/µL) and 2 µL DNA (14.31 ng/µL). The PCR protocol was: 10 minutes at 94 °C, followed by 35 cycles (1 minute at 94 °C; 1 minute at 55 °C; 1 minute at 72 °C) and five-minute at 72 °C, PCR reaction was performed using a thermocycler (Axygen MaxyGene TM, FisherBiotec, Australia). Amplified fragments were separated using an agarose (1.5 %) gel electrophoresis (100 V/40 min). Gels were observed on Spectroline Ultraviolet Transiluminator Longliffet ® (New York, USA) using the UVP Mini Darkroom GDS-800 System Labswork 4.5 software (Upland, CA, USA).

2.10.3 Metagenomics analysis of coleopteran digestive tract microbiome

DNA from one gut per sample was amplified using the following pair of specific primers WBACC1^{GC} (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCC CCC GGT CGT CAG CTC GTG TCG TGA GA-3') and WBAC2 (5'-CCC GGG AAC GTA TTC ACC GCG-3') (Bester et al. 2010). The final PCR reaction volume was 50 uL, containing: 29 µL of sterile deionized water, 7 µL of 10X/MgCl2 50 $mmol^{-1}$ buffer, 1 µL of dNTP's 10 $mmol^{-1}$, 4 µL primers WBAC1CG and WBAC2 10 $mmol^{-1}$, 1 µL of Taq polymerase paq-5000 (Aligent Technologies) and 4 µL of DNA 100 ng uL^{-1}. The PCR was performed in an Axygen Maxygen Termocycle (Foste City, CA, USA) using the following amplification program: One denaturation step at 94 °C for 5 minutes, then, 35 cycles (1 minute to 94 °C, 1 minute at 57.5°C and 1 minute at 72°C) and an elongation step at 72°C for 5 minutes and final temperature at 4°C. The PCR was performed in an Axygen Maxygen Termocycle (Foste City, CA, USA).

Amplified PCR products were analysed by denaturing gradient gel electrophoresis (DGGE) using a Dcode TM Universal Mutation Detection System (Bio-Rad, USA). The PCR products were loaded into a 8% polyacrylamide gel (Acrylamide/N,N-methyl-enebisacrylamide, 37.5/L) in TAE 1X buffer (40 mm/mol I-Tris-HCl, pH 7.4, sodium acetate 20 $mmol^{-1}$, 1.0 mm mol^{-1} Na_2-EDTA) (Muyzer, Waal, and Uitierlinden 1993). Electrophoresis was performed at 60°C with a denaturation gradient of 30-60% first at 20V/10 min. and after 80V/12 h. At the end of the vertical electrophoresis, the gels were washed with distilled water and stained with ethidium bromide (0.1 uL^{-1} of the 0.5 mg mL^{-1} stock solution) (Tatsadjieu et al. 2010). The gels were then processed in the longliffet Spectroline Ultraviolet Transilluminator using the UVP MiniDarkroom GDS-800 System Labswork 4.5 software. The bands in the vertical gel were cut, purified and re-amplified using primer sets for bacteria, following the conditions described above for PCR (Doyle and Doyle 1987; Ye et al. 2018).

The amplified products were sequenced using the fluorescence-based sequencing method Taq FS dye terminator cycle in an automatic sequencer (Perkin Elmer, Applied Biosystem; model 3730, Foster City, CA, USA), the obtained sequences were aligned with

the BLAST tool (Basic Logical Alignment Search Tool) and analysed in Genbank's NCBI Website database, highly similar sequences.

2.11 Statistical analyses

Statistical analysis for the biodegradation process under controlled composting conditions was performed using SPSS for Windows version 20.0. For the statistical evaluation of the results derived from the determinations of the biodegradation process of all the studied samples, a univariate analysis of variance was used (ANOVA and Tukey test for 95% reliability) (minimum significant difference, SMD, test). The differences were considered significant when $p<0.05$.

For the intestinal microbioma, the analysis of the categorical data was performed using SxR tables, in this case, the chi-square test was employed with the objective to detect an association between diet and the number of different bacterial species found in the intestinal microbioma of *Ulomoides dermestoides.* The banding patterns generated during the DGGE assay were interpreted as binary code in this case, 0 and 1 are interpreted as allele absence and presence, respectively. The analyses of principal component, cluster analysis, minimum spanning tree, and biodiversity indexes were performed using Info-Gen software (version 2011).

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CHAPTER III

CHAPTER III

Contents

3. BIODEGRADATION PROCESS UNDER CONTROLLED COMPOSTING CONDITIONS

Abstract

This chapter it's about structural changes of cassava starch and polylactic acid films submitted to biodegradation process, where Polylactic acid (PLA) and starch are compounds used in the manufacture of packaging to replace petroleum products as biodegradable and environmentally friendly materials. This study evaluated the structure and surface of a film manufactured by extrusion from cassava starch and PLA, which underwent a biodegradation process under compost conditions following the guidelines of ISO 4855-2:2007. Samples were taken every week for one month to perform Fourier Transform Infrared Spectroscopy (FT-IR) tests to identify functional groups on film, and High-Resolution Optical Microscopy (HROM) and Scanning Electron Microscopy (SEM) tests, from these techniques Structural changes in the film were evidenced. The addition of PLA increases the carbonyl index. The introduction of anhydrous malic acid (MA) in PLA/TPS mixtures may lead to an increase in the carbonyl index, The TPS/PLA composite film was framed in the three phases of biodegradation: disintegration, fragmentation, and mineralization. In week 4 a reduction in film size was observed with a thinning of the film with fractures that produced fragmentation and disintegration. These results are published in International Journal of biological Macromolecules.

Keywords: Biopolymers, Starch, Polylactic Acid, Cassava.

3.1 Introduction

Biodegradation is a biochemical transformation in the mineralization of compounds by microorganisms. Mineralization of organic compounds under aerobic conditions produces carbon dioxide and water, and under anaerobic conditions, methane and carbon dioxide. The biodegradation of polymers can be improved by increasing their surface area or reducing their molecular weight through abiotic hydrolysis, photooxidation and physical disintegration (Buléon, Colonna, Planchot, & Ball, 1998; Palmisano & Pettigrew, 2014), producing changes in surface properties or loss of mechanical strength (Avérous & Pollet, 2012; Nayak, 2007), assimilation by microorganisms (Luyt & Malik, 2019; Singh & Sharma, 2008), enzymatic degradation (Emadian, Onay, & Demirel, 2017), main chain breakage and subsequent reduction in the mean molecular weight of polymers (Hergenrother, Wabers, & Cooper, 1992; Mittal, Halley, & Avérous, 2011; Ratner, Gladhill, & Horbett, 1988). Degradation can occur by any of the above mechanisms, alone or in combination with each other.

The "biodegradation process" is defined as the decomposition of materials into CO_2, CH_3, H_2O, inorganic compounds or biomass. The tracking of the enzymatic action of microorganisms, the predominant mechanism in this process, is measured over a specific period of time under standard conditions (ASTM/D-5488-94d). Biodegradability is also defined as the propensity of a material to break down its constituent molecules by natural processes (often microbial digestion). The metabolites released by degradation, if not toxic to the environment, are expected to be redistributed through the carbon, nitrogen and sulphur cycles. Microorganisms provide chemicals that attack materials, causing biological degradation (Singh & Sharma, 2008; Yatigala, Bajwa, & Bajwa, 2018). This biodegradation converts them into intermediate materials or final products by solubilization, simple hydrolysis or by enzymatic action. The polymer molecules, but not necessarily broken down to produce fragments, but the integrity of the material decreases in this type of process (Kale et al., 2007).

From this perspective, cassava starch is an economical and renewable biopolymer that can be a substitute for plastics made from petroleum-derived materials (Bras & Saini,

2017; Mittal et al., 2011). Thermoplastic starch (TPS) is an amorphous or semi-crystalline composite material obtained by mixing starch with glycerol (Mittal, Akhtar, Luckachan, & Matsko, 2014). PLA is obtained from the polymerization of lactic monomers (Carvalho, 2008; Natamai, n.d.). The result of the addition of TPS to PLA is degradable materials because some microorganisms secrete enzymes that consume the polymer, using starch as a source of nutrients (Sikorska, Musioł, Zawidlak-Węgrzyńska, & Rydz, 2017).

The objective of this part was to determine the possible structural changes at the molecular and surface level of the film made up of cassava starch and PLA, subjected to an aerobic biodegradation process under composting conditions, using Fourier Transform Infrared Spectroscopy (FT-IR), High Resolution Optical Microscopy (HROM) and Scanning Electron Microscopy (SEM) techniques.

3.2 Results and discussion

3.2.2 Biodegradation process

For microcrystalline cellulose and film, a biodegradation percentage of more than 50% was obtained in approximately 4 weeks under the parameters of ISO 14855-2:2007, indicating that the biodegradation process under controlled composting conditions for the film is positive (Du et al., 2008; Pradhan, Misra, Erickson, & Mohanty, 2010). The accumulated production of carbon dioxide (CO_2) resulting from the biodegradation of the samples during 32 days is shown in Figure 3. The results obtained in the respirometric tests show that both materials experienced an aerobic biodegradation in compost, indicating a greater mineralization, that is, generation of CO_2. The biodegradability of starch and TPS has been widely reported (de Campos et al., 2013; Li, Sarazin, Orts, Imam, & Favis, 2011)(Li et al., 2011)(Li et al., 2011)(Li et al. 2011)(Li ‹i›et al.‹/i›, 2011)(Li et al. 2011)‹sup›21‹/sup›(Li et al. 2011)(Li et al. 2011)(Li et al. 2011)(Li et al. 2011)(Li et al. 2011). Biodegradation occurs in the amorphous domains and at the ends of the starch chain, where the chains are more flexible and mobile than crystalline regions.

The differences in the respirometric profiles were observed in the first seven days: the initial breathing rate of the cellulose, indicated by the slope of the curve, was higher than the TPS/PLA film. Consequently, considering the film, this is attributed to the presence of low molecular weight sugars: fructose, glucose and sucrose, mainly (Alasalvar, Grigor, Zhang, & Quantick, 2001). These compounds are easily consumed by microorganisms than starch, due to the latter's macromolecular characteristic.

The lower initial rate of TPS/PLA film compared to control (cellulose) results from the plasticizing action of glycerol molecules on starch, increases the accessibility of anhydroglucose units at active sites for catalytic enzymes (Otoni et al., 2018).

After 4 days, the CO_2 evolution of the film was equal to the cellulose, the latter being higher from day 5 onwards. The depletion of monosaccharides and disaccharides present may be the cause of this behavior. On the other hand, the presence of wet inoculum and microbial enzymes may be the cause of the gradual decomposition of water-soluble starch granules.

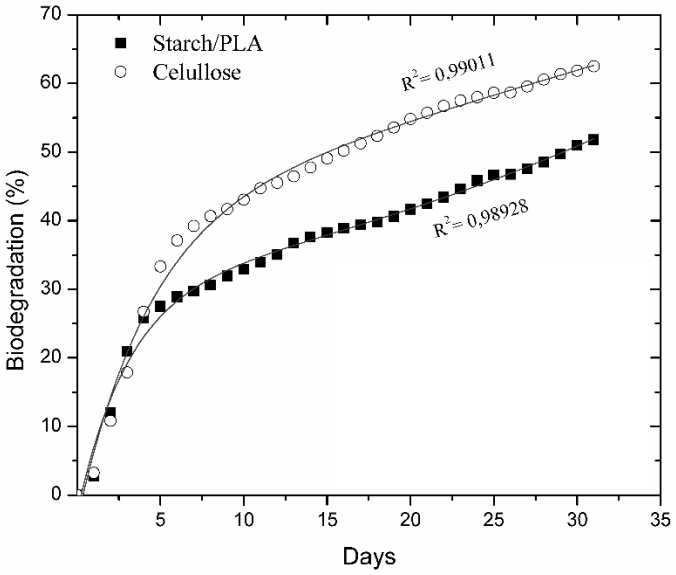

Figure 3. Biodegradation curves for TPS-PLA film. percentage of biodegradation, although the proportional rate of CO_2 decreases

The experimental points of both film and control (cellulose) were adjusted to an exponential decay equation. The percentage of biodegradation of TPS/PLA film is adjusted to this model with an adjusted determination coefficient R^2 of 0.9893 in 32 days, it is assumed that the control mineralization also behaves linearly for R^2 = 0.9901 estimating that the mineralization of the film would reach approximately a value greater than 50%. This amount is subject to an exponential decrease in the percentage of biodegradation, although the proportional rate of CO_2 decreases (Fig. 3).

However, the mineralization of cellulose could cease when its linear behavior changes to a horizontal plateau, before the film, due to the presence of organic matter. Moreover, CO_2 is not the only product of the aerobic biodegradation of a polymer. Otoni et al. reported that most of the organic carbon is biodegraded into CO_2 (Otoni et al., 2018), but part of it is incorporated into the soil as humus and biomass and another fraction remains as dissolved organic carbon (Li et al., 2011).

3.2.3 Structural change analysis

Changes in the chemical structure of pure components (PLA, TPS and Glycerol) and their mixtures in the 4000-550 cm^{-1} region were monitored. The carbonyl index was determined to monitor the behavior during the PLA biodegradation process, which is the relationship between the height of the spectrum of the carbonyl group in the 1840 - 1700 cm^{-1} region and the height of the spectrum related to hydrocarbon bonds in the 800 - 700 cm^{-1} region of the PLA (Figure 4).

The TPS FTIR spectra show three separate bands in the 1100 - 900 cm^{-1} region located at 1079 cm^{-1}, 1041 cm^{-1} and 926 cm^{-1}, that vibrations arise from the C-O-C stretch corresponding to α 1-4 glucosidic bonds and bending vibrations of C-OH (Table 1). van Soest et al., reported that these absorbances are related to starch crystallinity and water content in the TPS (van Soest, De Wit, Tournois, & Vliegenthart, 1994). These changes were observed in TPS absorbance in the 1100 - 900 cm^{-1} region where the TPS crystal band between 1041 cm^{-1} and 926 cm^{-1} presents a shoulder band in the IR PLA/TPS spectrum. These changes can be attributed to the alteration of the specific conformation, the long-

range order and the crystallinity of the starch. The high temperatures and cutting speeds in the twin screw extruder could disturb the crystallinity of starch in TPS (which is given by hydrogen bonds between starch and glycerol), by the mixture in the molten state with PLA (Mittal et al., 2014).

Table 1. Band assignments for FTIR spectral features (cm^{-1}) of TPS/PLA films in this experiment per week.

W1		W2		W3		W4	
cm^{-1}	Band assignments	cm^{-1}	Band assignments	cm^{-1}	Band assignments	cm^{-1}	Band assignments
709	C=O	707	C=O	704	C=O	702	C=O
759	C=O	757	C=O	757	C=O	759	C=O
863	-CH	870	-CH	870	-CH	865	-CH
927	-COC	1045	-CO	1045	-CO	1038	-CO
1026	-CO	1090	-CO	1090	-CO	1086	-CO
1080	-CO	1187	-COC	1187	-COC	1186	-COC
1150	-COC	1386	$-CH_3$ flexion	1386	$-CH_3$ flexion	1386	$-CH_3$ flexion
1383	$-CH_3$ flexion	1457	CH_3	1457	$-CH_3$ flexion	1457	CH_3
1460	$-CH_3$ flexion	1655	-OH flexion	1655	-OH flexion	1658	-OH flexion
1656	-OH flexion	1758	C=O	1758	C=O	1754	C=O
1757	C=O	2947	CH_3 symmetrical	2947	CH_3 symmetrical	2929	CH_3 symmetrical
2942	CH_3 symmetrical	2999	CH_3 symmetrical	2999	CH_3 symmetrical	3000	CH_3 symmetrical
3401	-OH Stretch	3400	-OH Stretch	3508	-OH	3429	-OH

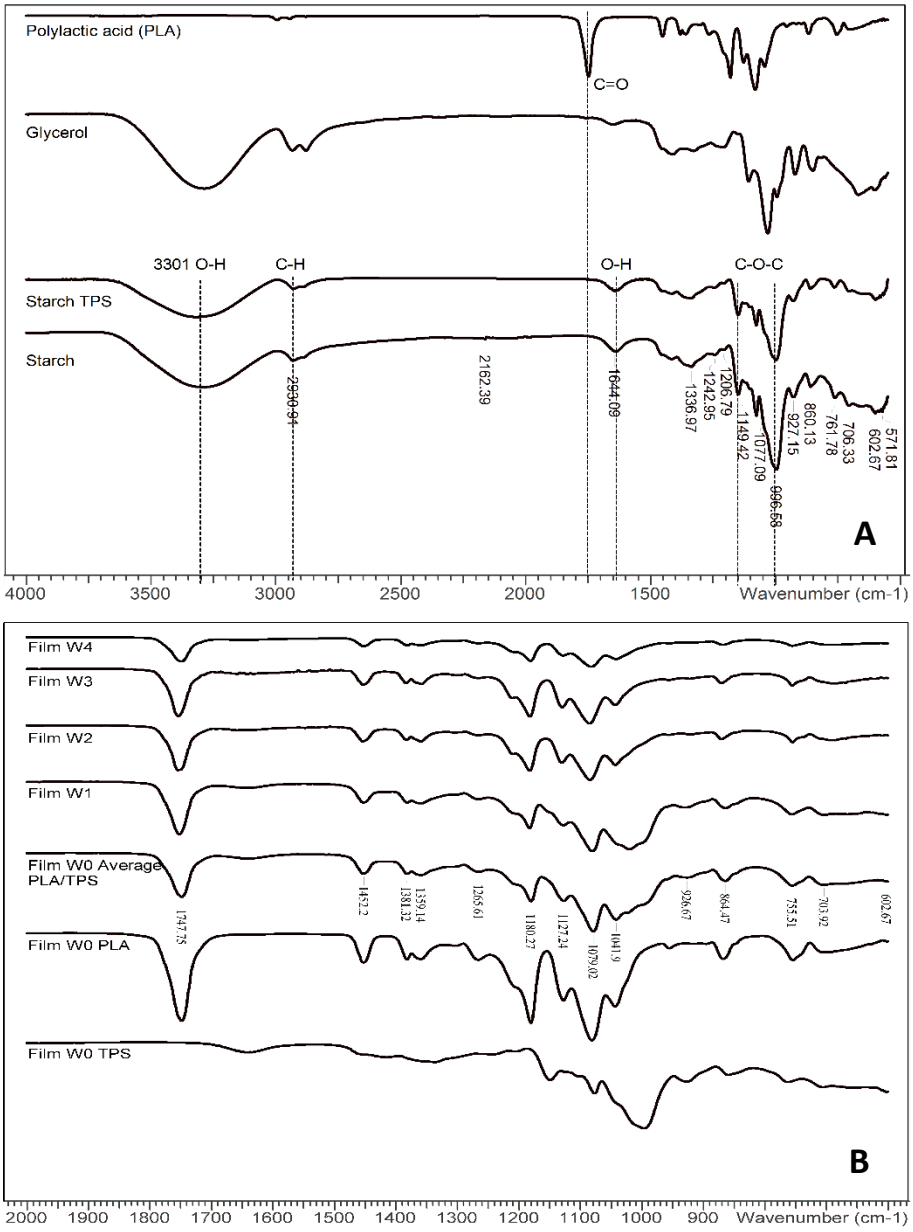

Figure 4. Fourier-transform infrared spectroscopy: (a) spectra pure components and (b) structural changes of pure components and films under to composting as a function of time (weeks).

The figure 4 (b) shows the structural change of the films subjected to the biodegradation process as a function of time (in weeks). In the spectra it is observed that the stretch C=O appears at 1747 cm^{-1} characteristic of the PLA. Similarly, the amorphous

zone of the PLA was observed at 869 cm^{-1} (Kale et al., 2007; Vargas-Villagran, Romo-Uribe, Teran-Salgado, Dominguez-Diaz, & Flores, 2014). The stretches of the CH_3 group show asymmetric flexion bands at 1452 cm^{-1} (Chieng, Ibrahim, Yunus, & Hussein, 2014; Muller, González-Martínez, & Chiralt, 2017). In this study it was also observed that the peak of the carbonyl group located at 1747 cm^{-1} decrease as the biodegradation process advances. These kinetic and dynamic effects of the mineralization process (total degradation of the polymer) depend on the nature and chemical characteristics of the materials. However, compost seems to provide an adequate means for the biodegradation of certain materials. In fact, temperature (22 °C ± 2) and relative humidity (60 to 70 %) facilitate the degradation of PLA, which induces significant microstructural changes (Iovino, Zullo, Rao, Cassar, & Gianfreda, 2008). Some authors have reported that there are no differences in the degradation mechanism with absence or presence of microorganisms, for the PLA film, being the hydrolysis of the ester bond the predominant mechanism for the degradation of the PLA film and not the microbial action (Torres, Li, Roussos, & Vert, 1996). Sedničková et al., indicates that amida I (-NH-CO) type bonds appear as a result of the decomposition of the -CH- and -C=O bonds (Sedničková et al., 2018), which obviously does not belong to the degradation products but is probably due to the existence of a microbial biofilm layer on the surface of the plastic sample during bacterial growth.

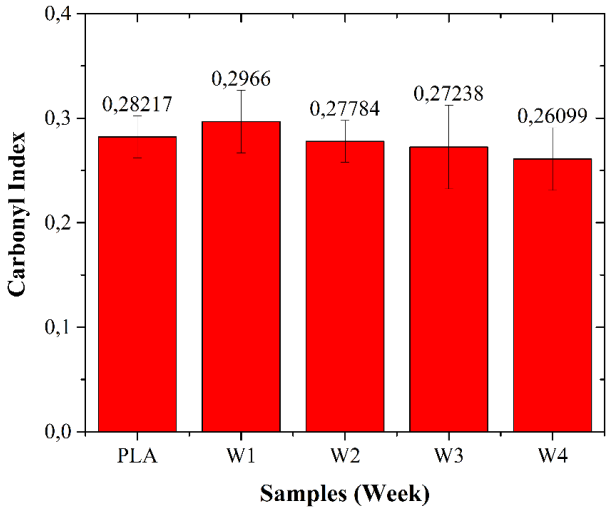

Figure 5. Carbonyl Index (IC): calculated by measuring the 1,747 cm^{-1} area of the carbonyl bond elongation vibration of C=O, with respect to the total areas measured in the sample.

The proportion of structures possessing carbonyl groups (C=O: carboxylic acids, ketones and amides) was calculated by measuring the 1,747 cm^{-1} area of the carbonyl bond elongation vibration of C=O, with respect to the total areas measured in the sample (carbonyl index) (Figure 5).

Figure 4 (A and B) together with the results from Figure 5 show that the addition of PLA increases the carbonyl index. The introduction of anhydrous malic acid (MA) in PLA/TPS mixtures may lead to an increase in the carbonyl index, as shown in Figure 5 at week zero. However, a decrease in the carbonyl index is observed in the second week and continues to decrease until the fourth week of the biodegradation process. Some authors have explained this behavior by suggesting that the mixture of PLA and MA in the first step of extrusion, the higher diffusion of MA in the PLA chains causes a higher carbonyl index. (Ali, Ahmadi & Taromi, 2017; Bergel, da Luz, & Santana, 2018). The decrease in the carbonyl index (CI) observed in week one suggests that the microorganisms preferentially used more oxidized molecules. However, the increase in the carbonyl index (IC) in the films at 58 °C indicates a microbial oxidation on the shorter chains. The above suggests the coexistence of two chemical mechanisms of biodegradation (Zain, Ab Wahab, & Ismail, 2018).

3.2.4 Morphological analysis

Figure 6 shows the degradation of polymers that is associated with changes in shape, color, surface morphology and rheological properties. As PLA is a material used as a control for its permanence in time, which is due to factors such as its low hygroscopic activity, a phenomenon that is attributed to a slow rate of hydrolysis at low temperatures (Pathak & Navneet, 2017) and to the low degradation capacity of PLA microorganisms in compost (Lu et al., 2014). However, it has been reported that fungi such as the *Tritirachium album* ATCC 22563 (Jarerat & Tokiwa, 2001) are capable of degrading and synthesizing this class of polymers (Torres et al., 1996).

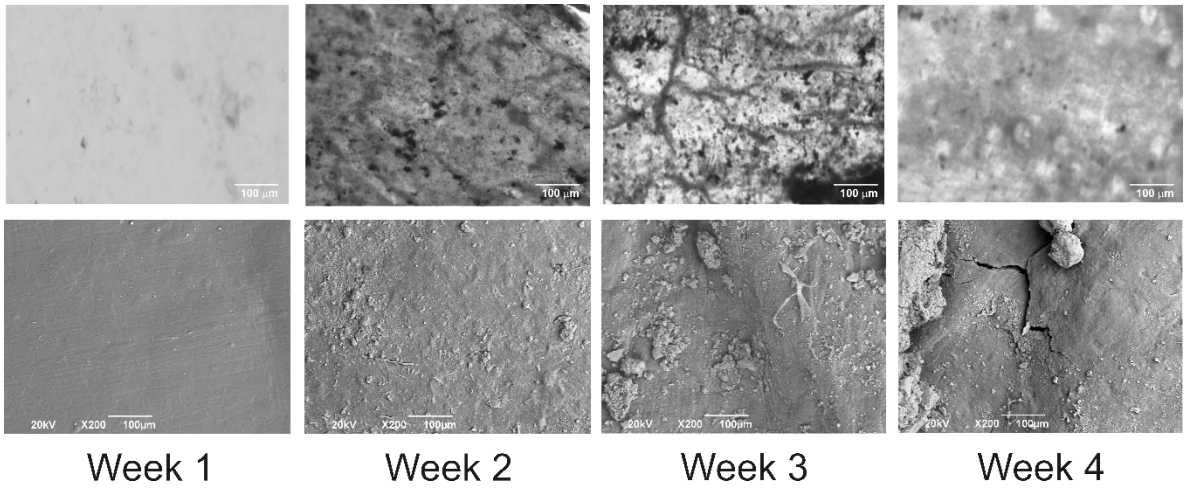

Figure 6. Morphological changes of the films under to composting as a function of time (weeks).

The morphology of the PLA/TPS film was examined by SEM and is shown in Figure 4, as time passes, the morphology of the surface changes. In week 1 the film is more homogeneous, the miscibility of the film components is denoted as reported by (Sedničková et al., 2018), in week 2 a roughness on the film surface begins to be evident and it is greater in week 3 with a detachment of the most superficial layer and the growth of microbiot on the film and some holes is denoted, (Przybytek, Sienkiewicz, Kucińska-Lipka, & Janik, 2018) reported small holes in the films that could not be associated with unplasticized starch granules and were most likely formed by water or steam formed during the molding process. In week 4 a reduction in film size was observed with a thinning of the film with fractures that produced fragmentation and disintegration.

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CHAPTER IV

CHAPTER IV

Contents

4. EVALUATION OF THE DEGREE OF DISINTEGRATION AND DETERMINATION OF THE DEGREE OF MINERALIZATION OF A TPS/PLA PLASTIC FILM BY *Ulomoides dermestoides*

Abstract

Biodegradable plastics and awareness of the potential problem of waste and its impact on the environment have raised new interest in the area of degradable polymers, so it is important to consider the biodegradation of polymers obtained from renewable sources. The results of the disintegration carried out by *Ulomoides dermestoides* in a film obtained from thermoplastic cassava starch and polylactic acid, show that this coleopter has the ability to physically change the polymer with the reduction of its molecular weight, giving a perspective for the management of solid waste.

Keywords: Biodegradation, Biopolymer, Ulomoides, Cassava.

4.1 Introduction

The accumulation of plastic waste is a threat to the environment, taking into account the mechanical properties of plastics, synthetic materials that are still used in large quantities by the food and non-food industry and the general public. The strategy that has been adopted in this situation is the development of plastic materials that can be easily degraded in the environment after use. Microorganisms and macroinvertebrates are humanity's natural allies in this struggle. An alternative to this are plastics produced from a mixture of renewable sources such as thermoplastic cassava starch and polylactic acid, in which the rate of degradation of polymer mixtures is initially controlled by the degradation of starch, which is the most readily biodegradable component (A. A. Shah, Hasan, Hameed, & Ahmed, 2008; A. Shah, Masoodi, Gani, & Ashwar, 2017).

Global biodegradation research has focused on studying the environmental impact of biodegradable plastics, test materials exposed to natural environments such as soil, compost, seawater and wastewater (Breslin, Senturk, & Berndt, 1998; Singh & Sharma, 2008, Cho, Moon, Kim, Nam, & Kim, 2011) or simulated natural laboratory environments using accelerated test conditions (Abd El-Rehim, Hegazy, Ali, & Rabie, 2004; Cerruti et. al, 2011 Cerruti et al., 2011), tests are performed at regular time intervals and monitored for changes in various properties such as gravimetric or molecular weight, tensile strength, chemical structure, thermal properties, and microbial growth.

Disintegration is one of the consequences of polymer degradation and may be due to the action of chemical or biological factors, which simultaneously degrade the polymer by breaking the bonds that form it, generating new compounds of low molecular weight or reducing the degree of polymerization, increasing the crystallinity and therefore the breakage of the polymer into smaller particles (Bitinis et al., 2014), and determines the degree of fragmentation of the material after a certain time that should generally reach a size less than 2 mm. The purpose of this study was to determine the capacity of an *Ulomoides* coleptera in proportionality with a bioreactor used in biodegradation tests (ISO,

2007, 2013), and also to track the molecular and enzymatic weight responsible for the disintegration and fragmentation of the polymer by the beetle.

Ulomoides dermestoides is a specie of coleoptera of the family Tenebrionidae, better known as flour worm (in its larval phase), is a promising source of alternative proteins and is already being produced on an industrial scale (Murugan, Han, Gan, Maurer, & Sudesh, 2016, Panini et al., 2017). This insect is commonly produced in mixed grain diets, although it can also consume meat or feathers, among other alternatives, due to its omnivorous nature (van Broekhoven, Oonincx, van Huis, & van Loon, 2015, Rojas, Morales-Ramos, & Riddick, 2016), it is proposed as an alternative for the disintegration of plastic materials and as an ecotoxicity testing agent for polymer residues.

4.2 Results and discussion

4.2.1 Thermal characterization: DSC and TGA

The thermal analysis for the desintegration of TPS/PLA films is showed in Fig. 7 and Table 2. The neat PLA showed semi-crystalline nature with a peak melting points (Tg) of 57.274 °C, respectively, Mano et al., (2003) reported Tg around 54 °C for pure amorphous PLA, considering that the amount of D-lactide profoundly influences the crystallinity of polylactides, as the percentage of D-lactides increases, the crystallization kinetics becomes slower and the maximum achievable crystalline content is reduced and as a consequence (Gorrasi and Pantani 2013), the D-lactide content for the PLA 4032D used in the blend is around 2 % with a maximum degree of crystallinity of approximately 45 % (Gorrasi and Pantani 2013). The PLA film present a very weak crystallization peak (Tc) presents at and 149.818 °C (Kong et al. 2019), while a broad crystallization exotherm appears at 104.702 °C, according with some other reports of PLA and TPS blends (Frone et al. 2013; Sheng et al. 2008). TPS exhibited a very broad transition, indicating its amorphous morphology (Mittal, Akhtar, and Matsko 2015), this coincides with (Jacobsen et al. 1999; Jacobsen and Fritz 1996; Mano et al. 2003).

The TPS/PLA blend film in t0 had a glass transition temperature (Tg) of 57.752 °C, and present a crystallization temperature of 153.254 °C and a single melting peak; and its melting temperature is 163.867 °C (Jian-feng Zhang and Sun 2004), for each day of treatment the TPS/PLA blend showed a Tg of 57.752 °C in t0 increasing to 57.4695 °C in t5 and a Tc reduce the temperature during the experimental time with the coleoptera raw and digestion from 153.254 in t0 to 150.65 °C in t5, presenting at the final of the study a Tm of 162.8552 °C.

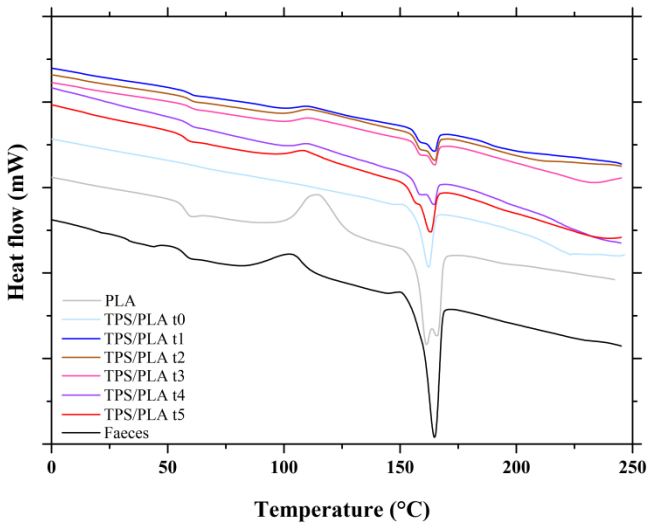

Figure 7. DSC melting thermograms of disintegration process of the film. a: Pure components. b film in disintegration.

The thermal stability of TPS, PLA and TPS/PLA samples determined by thermogravimetric analysis in nitrogen is shown in Fig. 8, where the blend thermal degradation curve is evident, where the thermal degradation of all film samples experiences a three-stage weight loss, one of weight loss refers to the volatile or interstitial water content of the film component, in itself the first stage of degradation refers to the degradation of the Cassava TPS content and the second stage of degradation is the PLA content, and at the end it is the decomposition products include carbon monoxide and carbonaceous residue (Pimentel et al. 2007). The starting temperature of thermal degradation of the pure PLA film is about 329 °C, and the degradation is completed at about 371 °C, and for the TPS film the starting temperature of thermal degradation is about 246 °C and the end temperature at 337 °C. The effect of Ulomoides dermestoides on the

TPS/PLA blend is evidenced in the initial degradation temperature change for all at 297.67 °C in t0 and for t5 at 293.67 °C, the final degradation temperature for t0 is at 85.56 °C and for t5 at 84.28 °C, this is evident in the changes of weights of degradation (Table 2).

Table 2. DSC parameters for TPS, PLA and TPS/PLA in different times of disintegration by *Ulomoides dermestoides*.

Samples	Tg (ºC)	Tc (ºC)	Hc (J/g)	Tm (ºC)	Hm (J/g)	Tmc (ºC)	Hmc (J/g)	Xc (%)
PLA	57.2749	149.8189	1.447	161.3321	52.9807	104.7021	33.8871	37.3950554
TPS	69.0856	122.3403	32.318	160.262	21.5921	162.2621	31.5921	25.7036925
TPS/PLA-t0	57.7528	153.2544	1.906	163.8674	37.1264	107.8804	15.4879	12.4429587
TPS/PLA-t1	59.0675	154.5302	1.3695	164.4688	34.1178	110.7737	16.3343	18.7807565
TPS/PLA-t2	59.0409	153.6349	3.81584	164.7319	29.3281	111.2186	17.5729	17.6199099
TPS/PLA-t3	59.4606	153.6769	3.49384	164.7453	21.2267	111.1562	12.0772	12.6719717
TPS/PLA-t4	58.5959	152.2228	4.5262	164.4172	42.0659	110.3736	14.947	17.5000468
TPS/PLA-t5	57.4695	150.65	4.5758	162.8552	37.6484	108.6024	13.5455	14.6118116
Faeces	33.1166	150.625	2.3861	164.8226	60.1419	103.1239	25.9448	30.192245

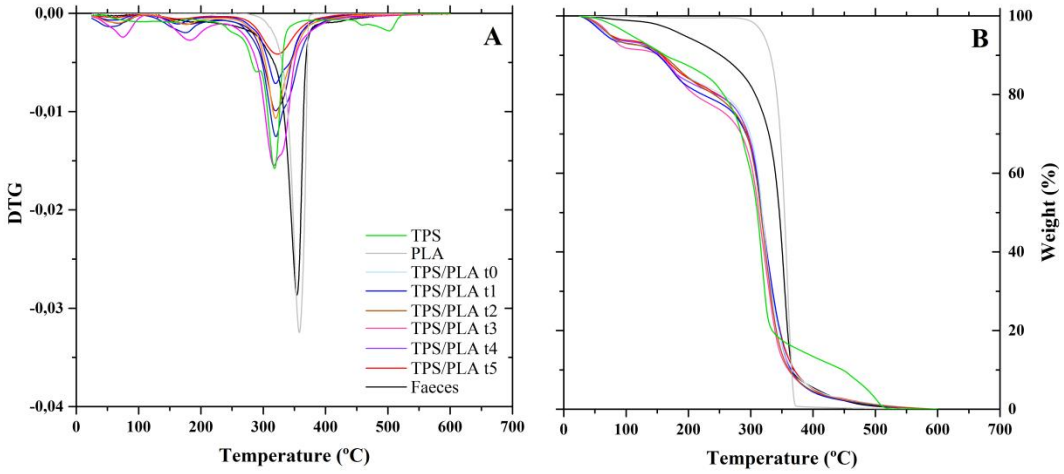

Figure 8. Termogravimetric (TG) curves (a) and derivative thermogravimetric (DTG) curves (b) of neat polymers and polymer blends in disintegration effect of *Ulomoides dermestoides*.

Table 3. TGA data determinate for TPS, PLA and TPS/PLA in different times of disintegration.

Sample	$T_{0.05}$ (°C)	$W_{0.05}$ (%)	$T_{0.5}$ (°C)	$W_{0.5}$ (%)	$T_{0.9}$ (°C)	$W_{0.5}$ (%)
	Initial		Maximun		End	
PLA	329.67	8.69	357.7	51.74	371	98.81
TPS	246.33	17.809	318.33	37.13	337	80.53
TPS/PLA-t0	297.67	29.26	320.33	47.06	354.33	85.56
TPS/PLA-t1	298.33	32.59	319.67	47.6	356.33	86.41
TPS/PLA-t2	297.67	32.26	319.67	45.48	347.67	84.57
TPS/PLA-t3	293.67	33.62	317	45.03	347	85.62
TPS/PLA-t4	297	30.47	319.67	48.31	355	86.31
TPS/PLA-t5	293.67	29.42	323	43.63	354.33	84.28
Faeces	331	31.45	353.67	32.72	365	88.22

4.2.2 FTIR analysis

The FTIR analysis (Fig. 9) show that the alcohols, hydroperoxides or carboxylic acids can be attributed to hydroxyl groups (-OH) situated at 3506 cm^{-1} (Madera-santana et al. 2016). The bands observed in 2995, 2945 and 2875 cm^{-1} were associated with the saturated CH_3 stretching bands and the asymmetrical symmetrical -CH symmetrical deformation bands (Kodal, Wis, and Ozkoc 2018). The spectrum region between 2000 and 700 cm^{-1} shows strong peaks of polyesters structure at about 1160 cm^{-1} belonging to the C-O-C stretch and 1746 cm^{-1} band indicates the presence of the ester group, that is the typical strong carbonyl stretching of PLA (C=O)(Arrieta et al. 2013; Chen et al. 2003; Lv, Zhang, and Tan 2019; Madera-santana et al. 2016; Wang, Yu, and Ma 2007). The 1402 and 1382 cm^{-1} bands are attributed to the deformation of the symmetrical and asymmetrical vibrations of the saturated stretch of C-H, and can be attributed to 1257 and 1060 cm^{-1} interval the vibrations of the stretch C-C, C-O-C and C-O (Chen et al. 2003; Torres Huerta et al. 2019; Zou, Yi, and Wang 2009), and the signals in 956, 867 cm^{-1} bands are attributed to the deformation vibration of groups =CH (Kodal, Wis, and Ozkoc 2018), this is related to the decrease in the crystallinity degree (Hc) of the PLA in the evaluated blend from 37.395 % neat PLA to 30.19% in faeces, according to FTIR spectra we find some changes in the PLA crystallization as it was degraded by coleoptera, although it is more evident that insects consume TPS first.

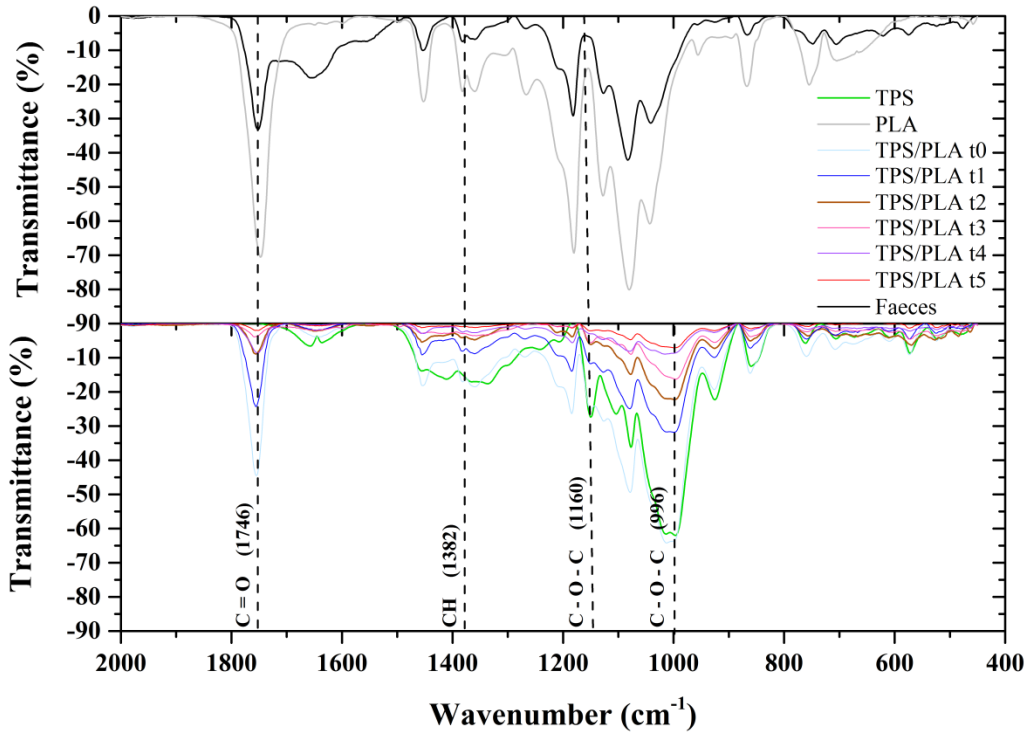

Figure 9. FTIR spectra of PLA, TPS and disintegration of TPS/PLA blend by *Ulomoides dermestoides*.

4.2.3 GPC analysis

The principal constitutional unit of PLA is lactic acid (Jianming Zhang et al. 2007). Lactic acid is an α-hydroxy acid with an asymmetric carbon atom, with two optical isomers: L-lactic acid (PLLA) and D-lactic acid (PDLA) (Oladapo, Zahedi, and Adeoye 2019). PDLA exhibited an Mw of 4.1 x 104 g mol^{-1} and a polydispersity of 1.92 (Zhao et al. 2014). The L-isomer is produced in humans and other mammals, while the D and L enantiomers are produced in bacterial systems (Hokmabad, Davaran, and Ramazani 2017).

Combinations of lactic acid based polymers and different low or high molar mass compounds have been found to affect the degradation behaviour as presented in the film on day 0. The presence of lactic acid and lacto(l-lactic) acid was demonstrated to increase the

biotic degradation of poly(l-lactide) (Hakkarainen, Karlsson, and Albertsson 1999), for the film, increasing the time of exposure to beetles, there is a diminution in Molecular weight Mn (for t0 was 39.172 Da and t5 was 56.378 Da) and an increase in Mw (for t0 was 76.222 Da and t5 was 115.022 Da) (Table 4).

Table 4. Number-average molecular weight (Mn), weight- average molecular weight (Mw), and dispersity (Đ) for TPS, PLA and TPS/PLA blends in different times of disintegration by *Ulomoides dermestoides*.

Sample	Mn (Da)	Mw (Da)	Đ	Mz (Da)	Mp (Da)	IV (dl/g)	Mark-Houwink Slope (a)	Mark-Houwink intercept (Log K)
TPS/PLA-t0	39.172	76.222	1.946	124.032	70.298	1.302	0.713	-3.330
TPS/PLA-t1	42.009	82.837	1.972	149.147	73.854	1.2275	0.737	-3.495
TPS/PLA-t2	55.378	94.718	1.710	129.394	94.397	1.2293	0.716	-3.458
TPS/PLA-t3	40.528	81.266	2.005	147.956	74.618	1.1965	0.729	-3.461
TPS/PLA-t4	16.387	110.113	6.720	203.599	101.486	1.1626	0.679	-3.343
TPS/PLA-t5	56.378	115.022	2.040	183.554	100.991	0.9709	0.711	-3.586

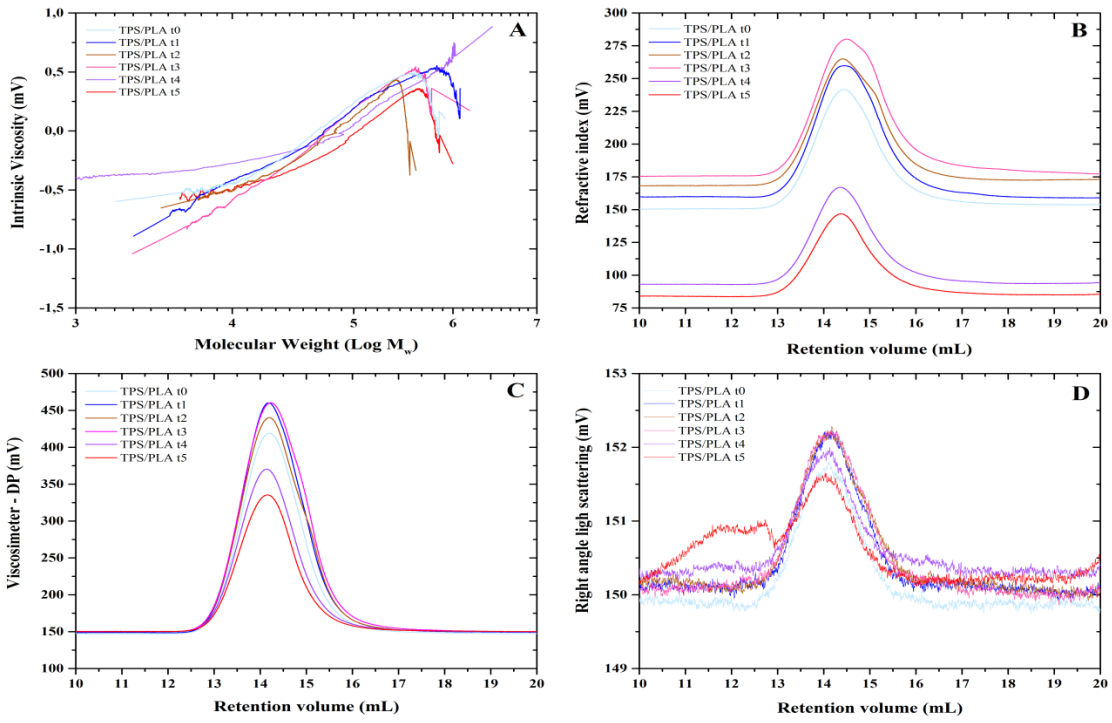

Figure 10. GPC chromatograph of polymers films: PLA, TPS and TPS/PLA in different degradation times by *Ulomoides dermestoides*.

4.2.4 Microscopic characterization

The disintegration of the film was achieved in 5 days (120 hours), obtaining a powder that can be used as a source of fertilizer for the soil, returning to the environment without causing pollution. The disintegration of the film samples is evidenced in the loss of area given by the chewing capacity of the coleoptera.

The chewing effect of the coleoptera on the film is evident in the fragments the rupture of polymer sheets, with ruptures at the edges of the film where the consumption of the polymer by insects begins and then in the central part of the polymer, which may be due to the consumption in the first place of the part composed by TPS and then by PLA. This chewing process is evident in the pieces torn from the edges of the film (Fig. 11).

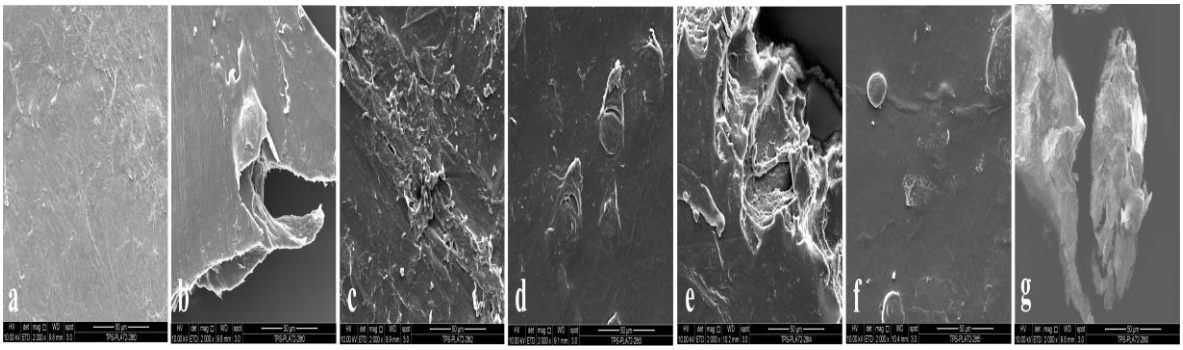

Figure 11. SEM micrographs of the film during the fragmentation and disintegration process by *Ulomoides dermestoides*. Each letter (a, b, c, d, e, e ,f) indicates the time in which the film was submitted to degradation of coleoptera until the faeces (g) taken every 24 hours.

It is observed that the film is not a homogeneous material and that the blown extrusion process results in a film with a rough surface with the presence of non-gelatinized starch granules arranged undifferentiated over the entire surface, the TPS is the continuous phase at large TPS and small PLA content in which PLA is dispersed in the form of droplets of a few micron size, the PLA part in the film could be the smoothest due to the viscosity properties of the polymer themselves, and by the same blown extrusion process (Müller et al. 2016). It is evident that after time the coleoptera bite the film

apparently where the amorphous part of the film corresponding to the thermoplastic starch is available, can be caused by the immissibility that characterizes the two components and the development of weak interactions between the phases, because the weak compatibilization between polyesters and hydrophilic molecules is caused by the process of grafting polar groups into the PLA molecule (Ortega-toro and González 2018).

Another reason why disintegration occurs is because of an intrinsic cause to the coleoptera, in the intestine naturally have three main parts: small intestine, middle intestine and hindgut (Marco et al. 2015). A posterior part of the digestive tract, the proventricle, has a muscular wall, grinds food, and regulates the passage of food into the midgut. Also, a number of glands that produce saliva may be associated with the digestive tract. The middle intestine (ventricle) is a very important part of the intestine, and enzyme digestion occurs predominantly in this part. Digestive enzymes such as proteases, lipases and amylases are produced by epithelial cells and secreted in the midgut, resulting in the breakdown of dietary proteins, lipids and polymeric carbohydrates (e.g. starch) into small molecules: peptides and amino acids, fatty acids and glucose, fructose and other monomeric sugars, respectively (Halloran et al. 2018), this is the motive for which in the FT-IR spectra was found the carboxyl group indicator of PLA in the time of study.

As for the faeces resulting from the feeding of the coleoptera, it presented a porous constitution, with some portions apparently laminated maybe some portions of the films no digested, and possible haemolymph (Ramsay 1964) perinephric fluid.

4.2.5 Mineralization test

The biomass growth curve shows the different stages of growth from adaptation to the substrate (film) to the stationary stage which coincides very well with the decrease in CO_2 production (Fig. 12).

To which the conversion of CO_2 produced to degraded polymer corresponds to 176.80 g/h of CO_2 with a final biomass generated of 1.8804 mg, remaining from the initial 200 g of experimentation 46.93 g of non-biodegradable polymer, given that the trial was only developed during 45 days (based on the lifecycle of the Coleoptera), the CO_2 emissions related to energy are influenced by the conversion efficiency of insects and the density of

the biomass in question (Halloran et al. 2018), and the polymer not degraded (present in the faeces) by the *Ulomoides dermestoides* will be used by microorganisms present in soil or in composting processes given the characteristics of the sources from which the film was obtained, taking into account that microorganisms in the intestine of larvae and adults Tenebrionidae can biodegrade one of the most polluting plastics such as polystyrene (Yang et al. 2015a, 2015b), in which he suggests that this discovery has "opened a new door to solve the global problem of plastic contamination" (Bradley, 2016); Murugan et al., 2016), not only petrochemical polymers but also Polymers obtained from renewable sources.

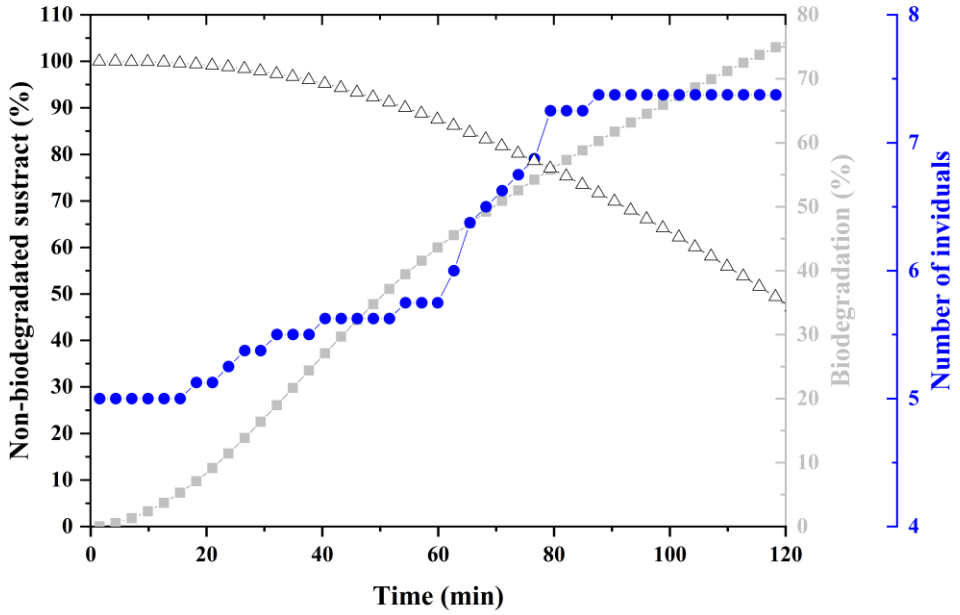

Figure 12. Mineralization process of the TPS/PLA blend, biomass growth curve of coleoptera vs non-biodegraded polymer by *Ulomoides dermestoides*.

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CHAPTER V

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Contents

6. EFFECT OF THE ASSIMILATION OF A TPS/PLA FILM IN THE COLEOPTERA *Ulomoides dermestoides*

Abstract

Because of their multiple applications, polymers became one of the most used materials and cause of pollution. One way of biodegradation of this type of material is using insect species, especially those from Coleoptera family. The objective of this study was to determinate changes in the intestinal microbiome of *Ulomoides dermestoides* Coleoptera undergoing three polymeric diets based on (1) oats, (2) thermoplastic cassava starch (TPS) and (3) an extruded matrix composed of TPS and polylactic acid (PLA). There were evidenced changes in the intestinal microbiota based on diet in this study: microorganisms identified in insects fed with diet 1 were: *Enterobacter* sp., *Enterococcus durans*, Enterococcus *faecalis*, *Escherichia* sp., and *Pantoea vagans*; intestinal microbiota of insects fed with diet 2 was composed by bacteria similar to *Bacillus maritimus*, no-cultivable *Enterobacter* sp., *Enterococcus alcedines*, *Lactobacillus oeni*, *Enterococcus faecalis*, and *Escherichia* sp. While, individuals fed with film based on TPS/PLA (Diet 3) showed the best adaptability to the polymeric diet, possibly evidencing a degradation and assimilation of the TPS/PLA film, due to changes on their intestinal microbiome with respect to those showed by insects with the other diets, identifying in insects with the diet 3, the presence of *Enterobacter* sp, *Enterococcus aquimarinus*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Escherichia* sp., and *Lactobacillus oligofermentans*. Polymeric diet changed the microbiome of the coleopteran digestive tract.

Keywords: Oats, Thermoplastic cassava starch, Polylactic acid, DGGE, CO1.

6.1 Introduction

Accumulation of plastic waste is a serious threat to the environment; given the properties of plastics, they will continue to be used in large quantities by industries and the general public. The strategy that has been adopted in this situation is the development of plastic materials that can be easily degraded in the environment after their use; microorganisms are the natural allies of humanity in this field. From all the methods proposed for the handling of plastic waste, use of biodegradable plastic materials is the most satisfactory solution (Emadian, Onay, and Demirel 2017), bearing in mind that for production of bioplastics, renewable raw materials are used (Nafchi et al. 2013).

The recently developed polymers with thermoplastic starch contents (TPS) and polyesters such as polymers obtained from renewable sources such as cassava starch and polylactic acid (PLA), represent an alternative for the plastics industry, however, PLA is biodegraded under specific temperature (Salazar-Sánchez et al. 2019) and microbiological conditions (Hammiche et al. 2019), on the contrary, their degradation is very slow, which is why it is necessary to evaluate the associated impact on the environment of these polymers considered biodegradable (Przybytek et al. 2018), which can be found in environments different from those intended for their correct final.

Yang et al., (2015a, 2015b) reported biodegradation of polymers using *Tenebrio molitor* where the final product was polystyrene (PS) disintegration and mineralization. The insects were fed with PS foam as a single diet and as control insects fed with a normal diet based on bran were used, for a period of 1 month. The study showed that PS was efficiently degraded in the larval intestine in a retention time of less than 24 h, in addition, it was determined that excision/depolymerisation of long-chain PS molecules and formation of depolymerized metabolites occurred in the larval intestine. During 16 days of follow-up, it was observed that 47.7% of the ingested PS carbon was converted to CO_2 and the residue (approximately 49.2%) was considered starch. From this study, it is concluded that the discovery of the rapid biodegradation of PS in the larval intestine provides new alternatives for management of plastic waste in the environment.

Other studies that have evaluated PS and polyethylene (PE) degradation reported that when insects were divided in 4 groups of 11 larvae each one, with an approximate mass of 13 g and supplying 25 g of the polymer as food called PS, Unicel, diapers and garbage bag; it was observed that biodegradability percentage was close to 96% for the sample of PS, 84% for Unicel, 65% for diapers and 64% for garbage bag (Adel et al. 2015; Brandon et al. 2018; S. Yang et al. 2018).

Coleoptera of Tenebrionidae family has been reported as a source of protein, which could be used as a human food source, because of its high protein contents during the different insect life stages,... in the larval stage, protein content is 47.2%, in pupa 54.6% and 66.3% in adult step (Sánch.ez-Muros et al., 2014). Enzymes of protein origin from this insect has been focus of research, especially for purification, characterization, cloning and sequencing of ß-glycosidases fro.m larvae, and four ß-glycosidases (denominated 1, 2, 3A and 3B) were found, which are not present in animal feed, but are present in the lumen of middle intestine of *Tenebrio* larvae. These enzymes have four sub-sites for glucose binding and can hydrolyse oligosaccharides, glucosides and alkyl glucosides. This activity is important for intermediate digestion of hemicellulose and cellulose, during biodegradation of polymers obtained from renewable sources (Ferreira-Villadiego et al., 2018).

There are not many reports using Coleoptera of the Tenebrionidae family as *Ulomoides dermestoides* to degrade polymers, and there is a lack of recognition of the Ulomoid microbiome and its changes when these insects are fed with different diets, which could be a potential alternative for degradation of polymers. The subunit 1 gene of the mitochondrial cytochrome c oxidase (CO1) from *Ulomoides dermestoides* has been sequenced. This gene is frequently referred as CO1 in the barcode approach and serves as the nucleus of the global bio-identification gene for insects and other animals (Tsang et al. 2018). This study was performed in order to determine the intestinal microbioma changes of *Ulomoides dermestoides* (Chevrolat, 1878) after be fed with different polymeric diets.

6.2 Results and discussion

6.2.1 Molecular identification of insect and species description

Sequencing of CO1 gene allowed molecular identification of the insect species used in this study. In this case, with 97 % of confidence the coleopteran used in this study was *Ulomoides dermestoides* according to the database NCBI:txid1552300. It is a specie of Coleoptera belonging to the Tenebrionidae family, known as flour worm (in its larval phase), which has been mentioned as an alternative source of proteins (Murugan et al. 2016; Panini et al. 2017).

This insect is commonly found fed on mixed grain diets, although it may also consume meat or feathers, among other food sources, due to its omnivorous nature (Broekhoven et al. 2015; Rojas, Morales-ramos, and Riddick 2016). It contains 47 - 60 % crude protein on a dry basis, 31 - 43 % lipids and 5 % ash. On the other hand, fresh larvae contain approximately 60% water, as well as a good source of vitamins and minerals (Makkar et al. 2014; Salomone et al. 2016). In poultry diets, the mealworm is a potential food source, particularly to replace soybean meal or fishmeal (Loponte et al. 2018; Marco et al. 2015; Raamsdonk and Jong 2017; Ushakova et al. 2015).

In terms of the life cycle, a female *Ulomoides dermestoides* ovipone about 580 eggs; the oviposition period is variable between 25 and 140 days, depending on the conditions of the medium and food. The newly hatched larvae are active, consume food and move freely; they acquire their maximum development between 89 and 100 days, after molting between 9 and 18 times. In this state, they remain active consuming substrate until reaching the pupal stage (between 12 and 16 days), then emerge as adults. The complete cycle from egg to egg takes between 300 and 350 days according to environmental conditions; but in a hatchery, the complete cycle lasts approximately from 10 to 12 weeks (Marvaldi, Reales, and Fern 2018) (Artigas 1994). *Ulomoides* larvae have an elateriform, cylindrical and elongated body, with hard exoskeleton with short legs and well-developed head and the urogomphi are at

the end of the abdomen (Figure 1), the pupa is exarada, the appendages are not attached to the body, but exposed externally (Costa 2014; Vinokurov et al. 2006). Morphologicaly *Ulomoides dermestoides* has been meticulously described by (Черней 2015). In taxonomic terms, the entomological classification for Coleoptera is based in Tronquet, (2014).

6.2.2 Molecular determination of coleopteran microbiome

Intestinal microbes of insects are composed of a wide variety of species, but their habitat in the insect's body is generally restricted to one region, commonly related to the hindgut (Bourtzis and Miller 2003). Classical identification of enterococci by phenotypic methods often gives ambiguous results; there are several molecular methods as alternative tools for the identification of Enterococcus species (Domig, Mayer, and Kneifel 2003).

Table 1. Taxonomy of *Ullomoides dermestoides*

Domain	Biota
King	Animalia (Linnaeus, 1758)
Sub-King	Eumetazoa (Bütschli, 1910)
Cladus	Bilateria (Haeckel, 1874)
Infra-Region	Protostomia (Grobben, 1908)
Cladus	Ecdysozoa (Aguinaldo, Turbeville, Linford, Rivera, Garey, Raff & Lake, 1997)
Phylum	Arthropoda (Latreille, 1829)
Sub-Phylum	Pancrustacea (Zrzavý & Štys, 1997)
Infra-Phylum	Altocrustacea (Regier, Schultz, Zwick, Hussey, Ball, Wetzer, Martin & Cunningham, 2010)
Class	Hexapoda (Blainville, 1816)
Subclass	Insecta (Linnaeus, 1758)
Infra-class	Pterygota (Brauer, 1885)
Cladus	Neoptera (Martynov, 1923)
Order	Coleoptera (Linnaeus, 1758)
Sub-Order	Polyphaga
Infra-Order	Cucujiformia
Super-Family	Tenebrionoidea (Latreille, 1802)
Family	Tenebrionidae (Latreille, 1802)
Sub-Family	Diaperinae (Latreille, 1802)
Tribe	Diaperini (Latreille, 1802)
Genre	*Ulomoides* (Blackburn, 1888)
Species	*Ulomoides dermestoides* (Chevrolat, 1878)

Microorganisms were identified comparing their sequences with those deposited in the NCBI website, identification was positive if they had similarity greater than 97% (Table 1). With the data of number of different microorganisms identified from the *Ulomoides dermestoides* microbiome fed with three polymeric diets, a cluster dendrogram based on the Braun Blanquet distance (the highest cophenetic correlation) was performed (Figure 1).

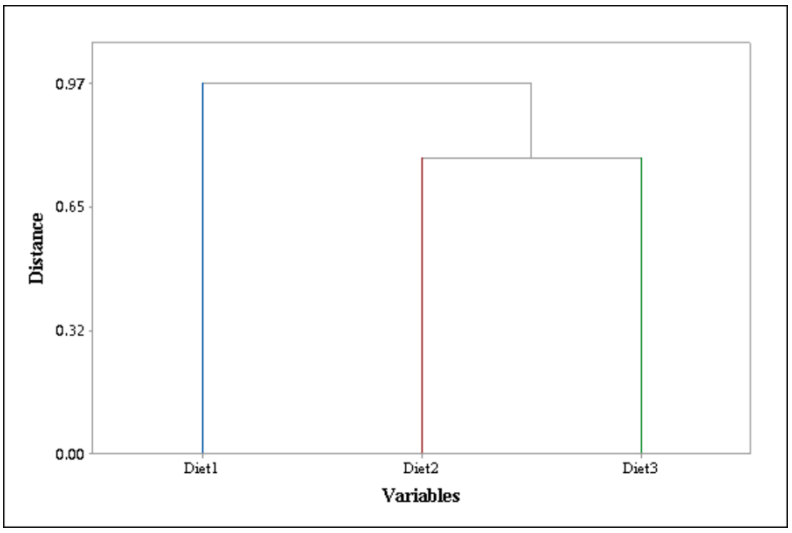

Figure 1. Dendrogram based on number of microorganisms identified from the intestinal microbiome of Ulomoides dermestoides Coleoptera undergoing three polymeric diets.

The horizontal axis of the dendrogram represents the dissimilarity between clusters. The vertical axis represents the samples. The dendrogram revealed that microbiome with the diets 1 and 2, and the diet 3 had significantly different (dissimilarity ≥1.0) communities. The effect of diets on the microbiome of the intestine of *Ulomoides* may be due to the physicochemical properties of each one, which may be due to two main reasons, the first being the inherent characteristics of the enzymatic activity of the insect. It has been reported that coleoptera of the Tenebrionidae family have proteases with natural ability to digest the gluten-rich plant products and possess stabile activity at acidic pH (Kannan et al. 2019), and the second is the composition of the diets to which it was submitted, oats, TPS and TPS/PLA. Which contain cellulose, amylose and amylopectin, and lactic acid, which is evidenced by the similarity between the microbiome of diet 1 and 2.

The diversity of bacterial communities was estimated using the Shannon-Weaver index, with diet 1 and the index was 1.95, for diet 2 it was 2.08 and for diet 3 it was 2.40, these results are similar to those reported by Jung et al., (2014). The exposure of these insects to different diets influenced the communities reducing biodiversity (Table 2).

Table 2. Shannon-Weaver diversity index of microorganisms identified from the intestinal microbiome of *Ulomoides dermestoides* Coleoptera undergoing three polymeric diets.

Diet	Estimator	Standard desviation of bootstrap
1	1,95*	±1,42
2	2,08	±1,57
3	2,40	±1,85

* Is the index estimation with its standard deviation of the bootstrap estimators. Each value was obtained with a 1050 bootstrap size.

It is evidenced for the diet 1 microorganisms such as *Enterobacter* sp., *Enterococcus durans*, *Enterococcus faecalis*, *Escherichia* sp., *Pantoea vagans*. For diet 2 the presence of non-cultivable bacteria similar to *Bacillus maritimus*, *Enterobacter* sp., *Enterococcus alcedines*, *Lactobacillus oeni*, *Enterococcus faecalis*, *Escherichia* sp. is observed. On the other side, in diet 3 there was a change in the microbiome evidencing the presence of Uncultured organism similar to *Bacillus simplex*, *Enterobacter* sp, *Enterococcus aquimarinus*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Escherichia* sp., and *Lactobacillus oligofermentans*, according to the microorganism species found with each diet, the most different microbiomas were those found with diet 2 and 3 (Figure 2).

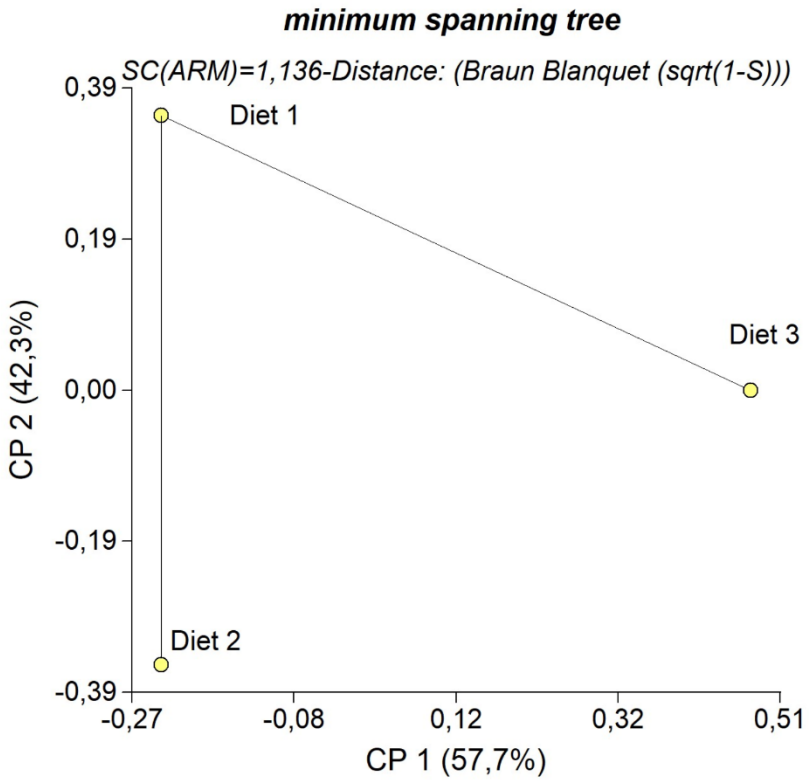

Figure 2. Distance of 3 groups of microorganisms identified from the intestinal microbiome of Ulomoides dermestoides Coleoptera undergoing three polymeric diets based on (1) oats, (2) thermoplastic cassava starch (TPS) and (3) an extruded matrix composed of TPS and polylactic acid (PLA), according to Braun Blanquet distance.

The Enterococci found are Gram-positive, facultative anaerobic bacteria (Archie, Theis, and Archie 2011), have the ability to colonize a wide range of different environments and are considered to be natural members of the intestinal microbiota of humans and other mammals. *Enterobacter* sp., *Enterococcus durans*, *E. faecalis* and *E. alcedinis* are organisms commonly found in soil, wastewater, plants, animal skins, in digestive tracts of farmyard animals such as birds (Devriese and Dutta 1984), cattle (Galvez et al. 2010), pigs (Aarestrup et al. 2002), rabbits (Coussement and Charlier 1984), sheep (Rougé et al. 2010), in urinary tract under infection, in dairy products (Wessels, Jooste, and Mostert 1989), so it is not uncommon to have found it in the digestive tract of the *Ulomoides dermestoides* due to the nature of the diets supplied.

Lactococcus species correlate directly with the percentage of body fat in animals that consume a diet high in fat and sugar (Parks et al. 2013; Zarrinpar et al. 2014), and TPS is a polymer formed by the union of glucose molecules (Moorthy and Padmaja 2002; Nafchi et al. 2013), this can explain the Lactobacilli found as *Lactobacillus oeni* in diet 2, *L. oligofermentans* and *Lactobacillus* sp. in diet 3, correspond to Gram-positive bacteria, which do not form spores, they are also catalase-negative belonging to lactic bacteria group. They are distributed in different habitats as well as Enteroccoci, they are reported in fermented food and drinks, mucous membranes and intestinal tracts of animals and humans, wastewater and plant material (Bernardeau et al. 2008; Mañez Lázaro et al. 2009). *Lactobacillus oeni* is also reported as a precursor of fermentation in the wine industry, this bacterium is a producer of acetic acid (Landete and Landete 2016), which can hydrolyse the starch present in the film of diet 2, and can transform L-malic acid into L-lactic acid (Mañez Lázaro et al. 2009), so it can be considered a viable synthesizer of polylactic acid (Felis et al. 2007).

The presence of *Lactobacillus oligofermentans* and *Lactobacillus* sp. in the digestion of diet 3 based on TPS/PLA film, indicates possibly a deterioration evidence and possible assimilation by this Coleoptera, since these bacteria are part of the obligatory heterofermentative lactobacilli, which degrade carbohydrates only through the phosphoketolase pathway (Andreevskaya et al. 2016), some authors report that Lactobacillus, have some enzymatic properties to convert starch in acid lactic (Giraud et al. 1991), thanks to the α-amylaze, which is then considered as amylolytic lactic acid bacteria (Armenta et al. 2018; Narita et al. 2006; Okafor, Ijioma, and Oyolu 1984; Scheirlinck et al. 1989), and can degraded the thermoplastic starch present in the film by enzymatic glucosylation (Wang et al. 2019), also have ß-galactosidase, enzyme hydrolyzes lactose (Gheytanchi et al. 2010), and can degraded the acid lactic chains of the polymer, by Transglycosylation and transgalactosylation properties (Sheik and Gunasekaran 2016). *Lactobacillus oligofermentans* is associated with the deterioration of poultry products packaged in modified atmosphere (Koort et al. 2005), it is also reported as a Gram-positive bacterium related to the deterioration of meat products due to rigor mortis processes and

production of acetic acid, such as hydrolysis production of polylactic acid to lactic acid and then smaller monomer.

For all diets. *Pantoea vagans* a Gram-negative cell, anaerobic facultative and oxidase negative (Brady et al. 2009; Maayer et al. 2014) and *Escherichia* sp were found. this condition means that Tenebrionidae coleoptera have been considered as potential disseminators of pathogenic bacteria (Krinsky 2019).

Some reports describe three types of symbiotic associations of bacteria in the insect gut: bacterial mutualists in the insect gut play an important role in regulating host metabolism, and also promote efficient digestion to extract maximum energy from ingested foods, those that protect the host from other potentially harmful microbes, and those that in addition to beneficial functions, the insect's intestinal microbiota can also have timely harmful interactions with its host (Bourtzis and Miller 2003; Yucheng and Sai 2015; Yun et al. 2014), in the case of *Ulomoides dermestoides* these associations are the promoters of the degradation of the TPS/PLA polymer.

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CHAPTER VI

CHAPTER VI

6.1 CONCLUSIONS

According with of the degradation evaluation of the film obtained from TPS/PLA, the structural changes at molecular and surface level of the TPS/PLA composite film were framed in the three phases of biodegradation: disintegration, fragmentation and mineralization. The fragmentation could be due to the effects of abiotic biodegradation (temperature increase), which makes available the monomers to be used in the biotic degradation that would provoke hydrolysis processes, therefore, changes on the polylactic acid in lactic acid or glycolic acid in the composting process, identified by the vibrations of C=O and as disintegration of the material the superficial changes were observed by the effect of the use of the microorganisms, with presence of holes in the films, that are evidenced during the mineralization of the film reaching a percentage of biodegradation of 65% in 32 days.

For the evaluation of the degree of disintegration and the determination of the degree of mineralization of a TPS/PLA, the *Ulomoides dermestoides* coleopter has the capacity of degrading polymers highly used in the plastics industry. The PLA presented a reduction in molecular weight and physicochemical changes by the effect of coleoptera intake in mixture with TPS, which gives a boost to waste management worldwide due to the ubiquity of the insect.

And for the evaluation of the effect of the assimilation of a TPS/PLA plastic film on the coleoptera, the microbiome in *Ulomoides dermestoides* individuals begins to have specificity to the change of diet by means of which it is evidenced that the TPS/PLA film is a polymer that possibly apart from being transformed by the enzymatic activity of the bacteria, as α-amylaze and ß-galactosidase, present in the coleopteran digestive tract and is assimilated in the form of sugars by hydrolysis and processed from oligomer to monomers.

6.1 FUTURE RESEARCH

Based on the results of the present investigation, it is recommended to investigate the enzymes present in each bacterium that was identified in the coleopteran microbiome, in this case it will have to develop the bacterial isolation and specialized metabolomics and proteomics investigations with mass spectrometry techniques (Maldi-Tof), which will serve to determine the potecial in which the microbial communities integrated to the inverted ones can be used.