

Tenebrio molitor and its gut bacteria growth in polystyrene (PS) presence as the sole source carbon

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Abstract

Although polystyrene (PS) is considered a non-biodegradable material, recent work has shown the degradation capacity of this material by microorganisms, especially those that are part of the natural microbiota of the digestive tract of some invertebrates. The present work sought to evaluate the growth of the larva of the mealworm (*Tenebrio molitor*) and its bacteria, using PS as the sole source of carbon. In this way it was possible to demonstrate the consumption of PS plates by the larva, found in holes and tunnels in the material, however, nutritionally it is not enough for the larva to gain biomass, notably reducing its size and time survival. Similarly, bacteria isolated from the digestive tract of *T. molitor* presented the ability to generate biofilms on PS sheets, generating changes (cracks, holes, etc.) in them, which were observed under scanning electron microscopy (SEM), indicating the possible use of this material as a carbon source for its growth.

Keywords: beetle; gut bacteria; polystyrene; Styrofoam; *Tenebrio*.

Introduction

Plastic is one of the most used materials in modern human societies. A derivative of oil, plastic has a light weight and is resistant, flexible, and relatively inert. These features make it ideal as a raw material for bags, packaging, construction material, thermal and electrical insulation products, etc. Annually, 250 million tons of plastic are produced in the world and 40 % of which is destined to the production of packaging (Cardoso *et al.*, 2017). However, only 9.5 % is recycled or reused. The remaining reaches landfills and dumps (Zalasiewicz *et al.*, 2016). Most of the massively consumed plastic serves its purpose in a short time and, depending on the complexity of the material, its degradation time can be short or long. This leads to an accumulation of plastic in aquatic systems and soils that negatively impact the biosphere and generate foci of contamination leading to public health issues (Barnes *et al.*, 2009). Polystyrene (PS) is a plastic that, given its versatility and low cost, has been used in a wide range of consumer products, such as insulation materials, food packaging, cutlery and kitchen utensils, among

others. In Colombia, PS is produced at a capacity of 110 000 tons per year and is the third most-produced petroleum-derived plastic, after polyvinyl chloride and propylene polymers (Acoplásticos, 2017). The expanded form of polystyrene is perhaps the most produced PS type thanks to its low density, hygroscopic properties, and innocuousness, making it a sought-after supply for the production of packaging items and construction and insulation materials (Ferrándiz-Mas & García-Alcocel, 2013). However, these characteristics also make it a highly resistant material to microbial degradation.

Taking into account the complexity of this material and its accumulation in ecosystems, methods of thermal decomposition have been proposed. However, these techniques, besides being expensive, generate large quantities of dioxins and aromatic compounds that magnify the damage to the environment (Desmet *et al.*, 2005; Moqadam *et al.*, 2015). Different studies have focused on the biological degradation of plastics, reporting on the capacity of soil invertebrates to use these materials as food, observing partial or complete transformation (Bombelli *et al.*, 2017; Yang *et al.*, 2015a). Similarly, the ability of various microorganisms to use plastic as the sole source of carbon has been evaluated. Such is the case of Orr *et al.*, (2004), who evaluated the degradation capacity of polyethylene by a strain of the fungus *Rhodococcus ruber*, observing an 8 % biodegradation of this material. Likewise, Velasco *et al.*, (1998) studied a molecular biology of *Pseudomonas* sp. and the enzymes involved in the transformation of styrene to phenylacetate. Similarly, Yang *et al.*, (2015b) established that the bacterium *Exiguobacterium* sp. from the digestive tract of the mealworm, or *Tenebrio molitor* beetle larvae, has the ability to degrade PS.

In the present study, we build on the body of work on PS degradation by the gut bacteria of *T. molitor* larvae. We sought to test the hypothesis that the gut bacteria of *T. molitor* larvae have the ability to use PS as sole source of carbon, as has been reported in other studies, however, this ability does not vary in relation to geographic location where mealworm is studied. Specifically, we evaluated the growth capacity of *T. molitor* and the bacteria isolated from its digestive tract in the presence of PS as the sole carbon source, to establish the role of these microbiota in the utilization of the PS and to evaluate whether this capacity is generalized for all the species, regardless of the geographic location of the organism.

Materials and Methods

Environmental adaptation of the *T. molitor* larvae

A total of 100 individual *T. molitor* larvae (14 days old) were used. Mealworms were collected in the locality of Nilo, department of Cundinamarca (Colombia) (altitude of 336 m.a.s.l. and annual average temperature of 26.5 °C) and transported alive in a cardboard box covered with insulating materials to

facilitate the mealworms acclimation to temperature and weather conditions in the city of Bogota (altitude of 2630 m.a.s.l. and annual average temperature of 14 °C), where the experiments were carried out. Once in the laboratory, the mealworms were left to acclimate to the new environmental conditions for one week. Then, they were placed in a glass container of 13×15×15cm (H × L × W) with a supply of 500 g of wheat flour as a source of food. During this period, room temperature was recorded daily (23 °C - 26 °C) and light was kept at a minimum due to the mealworms photophobic behavior.

Evaluation of PS intake

After the acclimation week, a total of 60 *T. molitor* larvae were separated in groups of 15 individuals and deposited in four glass containers of 10x10x15 cm (H x L x W). The acclimation conditions, previously described, were maintained for the first group hence becoming the control group; whereas the other three groups were fed with and an expanded PS plate of 5x9x1 cm of known weight (1.5 g). During 4 months and on a biweekly basis, the change in mass of control and treatment larvae were recorded with an analytical balance. The mass of the PS plates in the treatment groups was likewise assessed. In parallel, one PS plate of 4x7x1 cm (1.0 g) was placed in fifth glass container to track the effect of the environmental conditions on the mass of the PS plate.

The mealworms that were not used in the experiments were kept until reaching their imago or adult stage, and were then used to evaluate their capacity to ingest PS as a food source. To do this, a total of 15 *T. molitor* adults were placed in contact with a PS plate of 5x9x1 cm (1.5 g) which was visually inspected for two weeks to find changes in its surface that were signs of ingestion by the beetles.

Isolation and identification of the *T. molitor* gut bacteria

After the fourth month, the digestive tracts of the *T. molitor* larvae, fed on PS plates, were excised under a stereoscope and sterile conditions with the help of a scalpel. The obtained digestive tract contents were subjected to serial dilutions up to 10^{-7} , and the last three dilutions were sown on nutritive agar (agar-agar 15.0 g/l, beef extract 3.0 g/l, and gelatin peptone 5.0 g/l). These sowings were incubated at (30 °C), in the presence and absence of oxygen, to promote the growth of aerobic and anaerobic bacteria. To obtain pure cultures from each strain, multiple peels were made on a Petri dish until a single colonial morphology was observed.

Subsequently, each bacterial isolate was coded and biochemically characterized through TSI, MRVP, Citrate, Urease, Catalase, Oxidase, and motility tests as well as Gram staining. Additional isolate identification was performed with molecular barcoding via 16S rRNA sequencing and subsequent comparison of

the obtained sequences with databases such as RDP, Greengenes, and NCBI RefSeq / RNA. All bacterial isolates were tested for growth on PS (see the following section) and cryopreserved for future research. Adult *T. molitor* microbiota was isolated, grown, and tested following the same procedure above.

Evaluation of the growth of bacteria on PS as the sole carbon source

To evaluate the growth of mealworm gut bacteria isolates on PS, 0.1 ml of each resuspended isolate were cultured at 30 °C for 3 weeks in plates containing minimal salt medium (KH_2PO_4 0.7 g/l, K_2HPO_4 0.7 g/l, $MgSO_4 \cdot 7H_2O$ 0.7 g/l, NH_4NO_3 1.0 g/l, NaCl 0.005 g/l, $FeSO_4 \cdot 7H_2O$ 0.002 g/l, $ZnSO_4 \cdot 7H_2O$ 0.002 g/l, $MnSO_4 \cdot H_2O$ 0.001 g/l), agar (15 g/l), and PS sheets. Each PS sheet was prepared from 2.3 g of expanded PS dissolved in a benzene solution and recovered after solvent evaporation. During these 3 weeks, the formation of colonies on the agar and PS sheet surfaces was monitored. The plates showing colonial growth were observed under a scanning electron microscope (SEM) at magnifications of 17500X, 18000X, 22000X, 26500X, 29000X and 31000X to evaluate changes in the material imputable to microbial growth.

Results and Discussion

PS intake by *T. molitor* larvae

All glass containers with *T. molitor* larvae and PS plates had signs of PS consumption. The PS plates exhibited holes and tunnels, where the *T. molitor* larvae were found (Fig. 1A). Also, the mass of the plates decreased in the presence of the larvae (Fig. 2). In contrast, in the container without larvae, the PS plate did not reveal any changes in its morphology or weight (Fig. 1B).

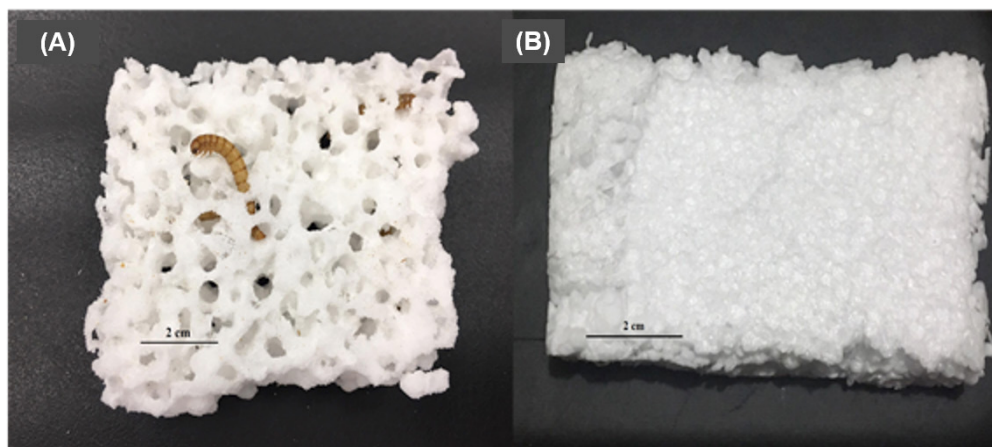


Figure 1. (A) Photograph of the mealworm (*Tenebrio molitor* larvae). The consumption of the polystyrene (PS) plate is evident by the holes and tunnels formed. (B) Photograph of the polystyrene plate used as a negative control.

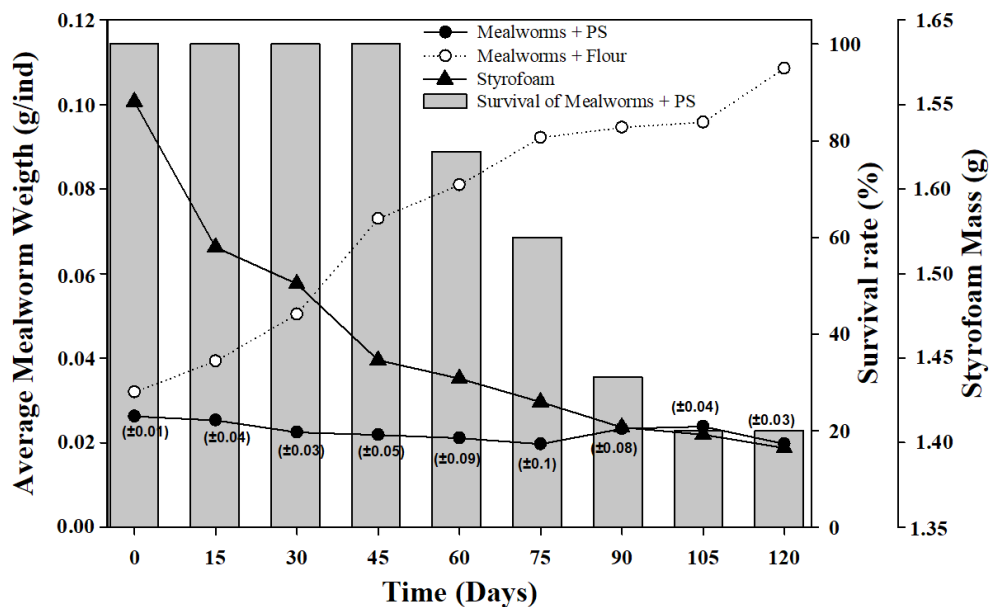


Figure 2. Relationship between average weight and survival of mealworms and polystyrene plates mass change throughout the experiment. Average biomass of a sample of 45 mealworms fed only on polystyrene (black circles) and 15 mealworms fed on wheat flour (white circles), weight of the PS plates (triangles), and survival rate of the mealworms under controlled conditions over a period of 4 months (grey bars).

Mealworms fed on wheat flour steadily gained biomass throughout the experiment. Whereas those fed only on PS tended to maintain or decrease their biomass despite evidence of plate consumption (Fig. 2). Our observations indicate that *T. molitor* larvae were capable of consuming PS, generating distinguishable changes in the plates. However, PS was of little nutritional value to these larvae as indicated by the presence of smaller-size surviving individuals at the end of the experiment. Since PS is not composed of organic molecules, it lacks the carbohydrates and proteins, otherwise found in wheat flour, necessary for larval growth (Catalán *et al.*, 2011).

Yang *et al.*, (2018) observed a biomass loss in *T. molitor* larvae fed on PS with a survival rate of 85 % after 32 days of incubation. These values are similar to those found in the present work, with a 100 % survival rate observed at day 60 (Fig. 2). Allegedly, mealworms are capable of consuming PS, managing to survive for a while. Yet, PS alone does not provide the necessary energy for biomass generation because the surviving larvae lost weight and shrunk.

Furthermore, after two months of feeding only on PS, larval survival dropped by (20 % or 40 %) despite having available PS plates. Survival could also have

been affected because of cannibalism. The complete or partial disappearance of mealworm bodies is a likely sign of cannibalism. This behavior can be triggered when the relative humidity or moisture content of foods is low (Ichikawa & Kurauchi, 2009). Since PS is a hygroscopic material, it may not help meet mealworm water needs.

T. molitor larvae have gained the attention of the scientific community due to their capacity to consume PS. However, it is not yet clear whether adults retain this capacity. In this work, *T. molitor* adults were allowed to feed on PS plates only, but after 15 days of incubation, none of the PS plate surfaces exhibited significant changes. The apparent inability of adults to feed on PS could be attributed to larval-to-adult jaw structure changes (Wilson, 1971), preventing adults from breaking and ingesting PS. Alternatively, changes in intestinal microbiota composition through the life stages of *T. molitor* (Wynants *et al.*, 2017) may lead to the adult's inability to process and transform PS.

Larval and adult *T. molitor* gut bacteria

Nine morphologically different bacterial strains were isolated from *T. molitor* digestive tracts. Five, of these isolates, were obtained from larvae and the remaining four from adults. Each isolate received a numerical code preceded by the letters G (for larval origin) or E (for adult origin). **Table 1.** shows the morphological and biochemical characteristics of the nine isolates. All of the isolates had bacillary form, and five of them were Gram-negative. In general, the isolates were facultative, fermenting bacteria, with negative mobility and urease test results. Furthermore, the sequencing of their respective 16S ribosomal RNA regions revealed that two of the strains from larval guts were members of the genus *Bacillus*, whereas the other three larval gut isolates belonged to the genus *Stenotrophomonas*. Similarly, two of the bacterial isolates from adult guts belonged to the genus *Bacillus*, and the remaining two, to the genera *Pantoea* and *Erwinia* (Table 1). The observed differences in the bacteria inhabiting the digestive tracts of larval and adult *T. molitor* could be related to the loss of the ability of the adult to use PS as a source of food.

Yang *et al.*, (2015b) were the first to identify the bacteria in the digestive tract of *T. molitor* larvae involved in the transformation of PS. The authors obtained a total of 13 bacterial strains, chiefly *Enterobacteriaceae*, and determined that a member of the genus *Exiguobacterium* was the main responsible for the degradation of PS. In the present study, however, the most common isolates belonged to the genus *Bacillus*. This contrast highlights the effect of the diet on the composition of gut microbiota in insects of the same species. In the previously mentioned study, the acclimation diet of the mealworms consisted of oat flakes, whereas in the present work, it was wheat

Table 1. Morphological and biochemical characteristics of the bacterial strains isolated from the digestive tract of *Tenebrio molitor*. The molecular identification is based on the sequencing of 16s rRNA region.

Isolated Strains Code	Morphological Characteristics	Biochemical Characteristics	Molecular Identification
1G	Cells with bacillary morphology that generate white colonies, circular with wavy edge.	Gram-positive bacterium, lactose or sucrose fermenter with gas production. Positive indole or catalase, with motility.	<i>Bacillus anthracis</i>
3G	Cells with bacillary morphology that generate white colonies, punctate with full edge.	Gram-negative bacterium, fermenter of the 3 sugars with gas production. Positive citrate and catalase with motility.	<i>Stenotrophomonas</i> sp.
5G	Cells with bacillary morphology that generate yellow colonies, punctate with full edge.	Gram-negative bacterium, fermenter of the 3 sugars with gas production. Positive VP and oxidase with motility.	<i>Stenotrophomonas</i> sp.
8G	Cells with bacillary morphology that generate white colonies, circular with full edge	Gram-positive bacterium, glucose or sucrose fermenter with no gas production. Positive catalase and oxidase with motility.	<i>Bacillus</i> sp.
10G	Cells with bacillary morphology that generate white colonies, punctate with full edge.	Gram-negative bacterium, non-glucose fermenter, positive citrate and catalase with motility.	<i>Stenotrophomonas</i> sp.
11E	Cells with bacillary morphology that generate yellow colonies, punctate with full edge.	Gram-negative bacterium, lactose fermenter or sucrose with no gas production. Positive VP, citrate and catalase with motility.	<i>Pantoea agglomerans</i>
12E	Cells with bacillary morphology that generate white colonies, circular with wavy edge.	Gram-negative bacterium, lactose or sucrose fermenter with no gas production, with motility.	<i>Erwinia persicina</i>
13E	Cells with bacillary morphology that generate white colonies, circular with wavy edge.	Gram-positive bacterium, glucose or sucrose fermenter with no gas production. Positive catalase with motility.	<i>Bacillus</i> sp.
14E	Cells with bacillary morphology that generate white colonies, circular with wavy edge.	Gram-positive bacterium, lactose or sucrose fermenter with gas production. Positive catalase with motility.	<i>Bacillus anthracis</i>

flour. Multiple works with insects have shown that feeding substrates, as well as breeding and development conditions determine the individual's gut microbiota composition (Broderick & Lemaitre, 2012; Broderick *et al.*, 2004; Kane & Breznak, 1991; Santo Domingo *et al.*, 1998; Wynants *et al.*, 2017).

Bacterial growth on PS as the sole carbon source

Fig. 3 shows the growth of one of the isolates on PS, as the sole carbon source. The isolates formed colonies around, above, or below the PS sheets. Turbidity and darkening of the mineral medium were also signs of bacterial growth. The observed morphologies differed from those expressed by the strains isolated in nutritive agar. Isolates, revealing growth on PS sheets, were observed under a scanning electron microscope (SEM) (**Fig. 4**). Three of these isolates, namely



Figure 3. Photographs of colonial growth and formation of biofilm on PS sheets in minimal salt agar of some of the strains isolated from the digestive tract of *Tenebrio molitor*.

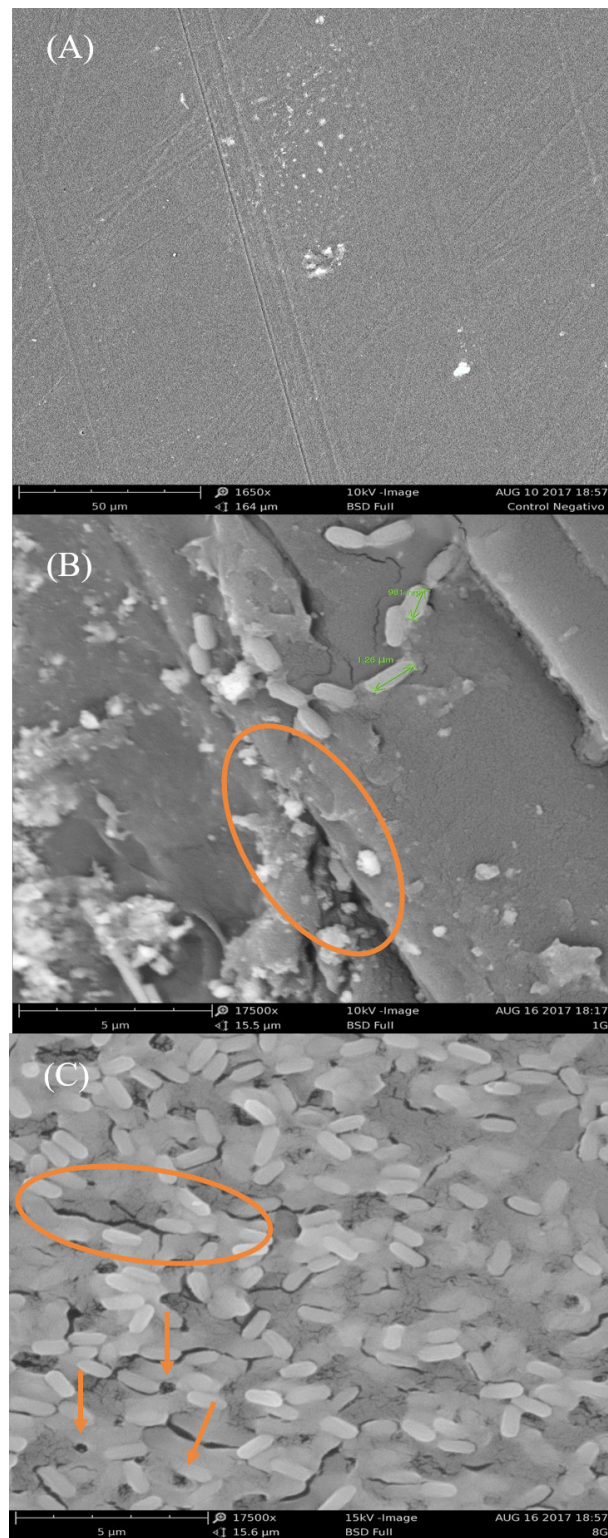


Figure 4. Microphotographs of the surface of the PS sheets after a period of 3 weeks of incubation. (A) Control sheet, without sowing of bacteria, (B) Modification of the surface of the sheet by the action of isolate 1G bacteria due to the proliferation of microorganisms and the appearance of furrows and holes in the material, not present in the negative control. (C) Modification of the PS sheet surface by the action of the isolate 8G bacteria. Grooves and holes in the observed PS sheet, absent in the negative control, evidence its consumption.

Bacillus anthracis, *Bacillus* sp, and *Stenotrophomonas* sp. formed tunnels, cracks, and cellular aggregates on the PS sheets. None of these changes were observed in the control sample (PS sheet without microorganism). Multiple SEM-assisted works have documented bacterial-driven transformations of PS and other plastics (Ho *et al.*, 2018; Sekhar *et al.*, 2016; Skariyachan *et al.*, 2016; Yang *et al.*, 2015a; Yang *et al.*, 2015b; Yoshida *et al.*, 2016). These transformations start with biofilm generation, on the material's surface, followed by material deterioration because of the enzymatic activity of the microorganisms.

The metabolic pathways involved in the transformation of PS are still unclear. Nevertheless, the oxidation of styrene to phenylacetate, via the citric acid cycle, is common among different microorganisms (Ho *et al.*, 2018). Bacteria of genus *Bacillus* stand out for their capacity to transform styrene to phenylacetate. *Bacillus* monocultures or consortia have been studied in different PS degradation experiments (Asmita *et al.*, 2015; Atiq *et al.*, 2010; Kiatkamjornwong *et al.*, 1999). Our results highlight the importance of *Bacillus* in the degradation of PS. Taking into account that four out of nine isolates obtained from the intestinal tract of *T. molitor* belonged to the genus *Bacillus* and two of them elicited signs of PS degradation, namely forming biofilms on the material and triggering its wearing.

Finally, although PS sheet wearing was not evaluated with bacteria isolated from adult *T. molitor* digestive tracts, there is evidence that bacteria of the genus *Pantoea*, present in the adult gut isolates in our study, have the capacity to degrade plastics such as low-density polyethylene (Skariyachan *et al.*, 2016). Additionally, taking into account that our *T. molitor* adults also have *Bacillus* in their intestinal flora, it is likely that *T. molitor* can digest PS. But, due to the structural changes in their jaws, it may not be possible for them to ingest it.

Conclusions

In light of our results, we can conclude that *T. molitor* larvae can use PS as food. Though, PS alone cannot meet larval nutritional needs. Larvae feeding only on PS for extended periods reduced their body size and commenced exhibiting cannibalistic behaviors. Furthermore, this use of PS by *T. molitor* larvae is conceivable due to the presence of bacteria in their digestive tract with the ability to grow with this polymer as their sole source of carbon. Likewise, the larval capacity seems not to remain in adults, which could be attributed to larval-to-adult jaw changes, preventing from breaking and ingesting PS. Alternatively, changes in intestinal microbiota composition through the life stages of *T. molitor* may lead to the adult inability to process and transform PS.

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***Tenebrio molitor* y el crecimiento de sus bacterias intestinales en presencia de poliestireno (PS) como única fuente de carbono**

Resumen: Aunque el poliestireno (PS) se considera un material no biodegradable, trabajos recientes han mostrado la capacidad de degradación de este material por medio de microorganismos, especialmente de aquellos que hacen parte de la microbiota natural del tracto digestivo de algunos invertebrados. El presente trabajo buscó evaluar el crecimiento de la larva del gusano de la harina (*Tenebrio molitor*) y sus bacterias, usando PS como única fuente de carbono. De esta forma fue posible demostrar el consumo de placas de PS por la larva, evidenciado en agujeros y túneles en el material. Sin embargo, el PS no es suficiente nutricionalmente para que la larva gane biomasa, por lo que se redujeron notablemente su tamaño y tiempo de supervivencia. De forma similar, las bacterias aisladas del tracto digestivo de *T. molitor* presentaron habilidad para generar biopelículas en láminas de PS, generando cambios (grietas, huecos, etc.) en ellas, como fue observado bajo microscopía electrónica de barrido (SEM). Esto indica el posible uso de este material como fuente de carbono para su crecimiento.

Palabras clave: escarabajo; bacterias del tracto intestinal; poliestireno, espuma de poliestireno; *Tenebrio*.

***Tenebrio molitor* e o crescimento de suas bactérias intestinais em presença de poliestireno (PS) como única fonte de carbono**

Resumo: Ainda que o poliestireno (PS) seja considerado um material não biodegradável, trabalhos recentes mostram a capacidade de degradação de este material por meio de microorganismos, especialmente daqueles que fazem parte da microbiota natural do trato digestivo de alguns invertebrados. O presente trabalho buscou avaliar o crescimento da larva do verme da farinha (*Tenebrio molitor*) e suas bactérias, usando PS como única fonte de carbono. De esta forma foi possível demonstrar o consumo de placas de PS pela larva, evidenciado em buracos e túneis no material. Entretanto, o PS não é suficiente nutricionalmente para que a larva ganhe biomassa, visto que se reduziram de modo notável seu tamanho e tempo de sobrevivência. De maneira similar, as bactérias isoladas do trato digestivo de *T. molitor* apresentaram habilidade para gerar biopelículas em laminas de PS, gerando mudanças nas mesmas (ranhuras, buracos, etc.), como foi observado por meio de microscopia eletrônica de varredura (SEM). Isso indica o possível uso de este material como fonte de carbono para seu crescimento.

Palavras-chave: besouro; bactérias do trato gastrointestinal; poliestireno; *Tenebrio*.

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