Identification of the gut bacteria of the greater wax moth

Identificación de las bacterias intestinales de la polilla de la cera mayor

Identificação das bactérias intestinais da traça-cera

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Abstract

Throughout the world, the use of industrial polymers derived from fossil fuels is practically inevitable because they have such a wide range of applications; however, the environmental problems arising from this practice have led to a search for alternatives which will allow their use to be reduced, as well as strategies for their control by degradation using biorganic active agents. Insects have been a focus of special interest, as some species consume plastics and may serve to biodegrade them through the action of bacteria in their digestive tracts. In this context, the object of the present study was to characterise bacteria present in the intestine of wax moth larvae (*Galleria mellonella*). Thirty larvae were subjected to a diet based on polystyrene foam and thirty larvae in natural diet for 7 days. Gastrointestinal tracts were extracted and PCR was run. The results showed the presence of bacterial cells of *Carnobacterium maltaromaticum, Brevibacterium sandarakinum, Pseudomonas psychrophila, Pseudomonas* sp., *Providence* sp., *Corynebacterium* sp. However, the real action of these groups of bacteria in the effective degradation of polymers must be verified.

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Resumen

A nivel mundial la utilización de polímeros industriales de origen de combustibles fósiles es prácticamente inevitable debido a la diversidad de aplicaciones; sin embargo, los problemas medioambientales que esto genera han motivado la búsqueda de alternativas que permitan reducir el uso de estos, así como estrategias para el control mediante la degradación, con la participación de algunos agentes activos bio-organicos. Los insectos son de especial interés ya que algunas especies consumen plásticos y son posibles biodegradadores debido a la acción de bacterias de su tracto digestivo. Considerando estos antecedentes, este estudio tuvo como objetivo identificar bacterias presentes en el intestino de las larvas de la polilla de la cera (*Galleria mellonella*). Treinta larvas fueron sometidas a una dieta a base de espuma de poliestireno y treinta larvas a una dieta natural por un período de 7 días. Posteriormente, se tomaron las larvas para hacer el estudio del tracto gastrointestinal mediante PCR. Los resultados obtenidos, demostraron la presencia de células bacterianas de *Carnobacterium maltaromaticum, Brevibacterium sandarakinum, Pseudomonas psychrophila, Pseudomonas* sp.*, Providencia* sp., *Corynebacterium* sp. Sin embargo, es necesario verificar la real acción en forma aislada de estos grupos de bacterias sobre la degradación efectiva de polímeros.

Palabras clave: *Galleria mellonella*, plástico, secuencia ADN, microorganismos, *Pseudomonas.*

Resumo

Globalmente, o uso de polímeros industriais de fontes de combustíveis fósseis é praticamente inevitável devido à diversidade de aplicações; porém, os problemas ambientais e ambientais que estão levando à busca de alternativas que permitam reduzir o uso destes, bem como estratégias para controlá-los por meio da degradação, com a participação de alguns agentes ativos bioorgânicos. Os insetos são de interesse, como possíveis biodegradantes devido à ação de bactérias no trato digestivo. Levando em consideração esses antecedentes, este estudo teve como objetivo identificar as bactérias presentes no intestino das larvas da traça-cera (*Galleria mellonella*). Trinta larvas foram alimentadas com dieta de isopor y trinta larvas em dieta natural por um período de 7 dias. Posteriormente, as larvas foram retiradas para estudo do trato gastrointestinal por PCR. Resultados obtidos mostraram a presencia de células bacterianas de *Carnobacterium maltaromaticum, Brevibacterium sandarakinum, Pseudomonas psychrophila, Pseudomonas* sp*., Providencia* sp*., Corynebacterium* sp. Porém, é necessário verificar a real ação isolada desses grupos de bactérias na degradação efetiva dos polímeros.

Palavras-chave: *Galleria mellonella,* plástico, sequência de DNA, microrganismos, *Pseudomonas.*

Introduction

Plastics are synthetic polymers, derived from fossil petroleum, which are highly resistant to biodegradation (Bombelli *et al.*, 2017). They include polypropylene and polyethylene, which represent around 92 % of the plastic produced. The latter is used for plastic bags and sheets, plastic films, bottles and containers, and is considered the most durable plastic. It accumulates in the environment, creating an ecological threat (Santo *et al.*, 2013).

Plastic products are in everyday use thanks to their properties of thermal and electrical insulation, as well as their good resistance to acids, alkalis and solvents (Frías *et al.,* 2003); they are therefore manufactured in huge quantities. However, their degradation period is long and complicated, making them a world-wide environmental problem. Since 1950, around 8.3 billion metric tons of plastics have been produced. About 6.3 billion tons have been produced since 2015, of which around 9 % have been recycled and 12% incinerated, leaving 79 % in landfill dumps or scattered around the environment (Geyer *et al.*, 2017).

The risk implied by the presence of these compounds in the environment is well known, especially in the marine medium where all organisms are threatened when they interact with the microplastics that result from the continuous erosion of plastic rubbish (Silva *et al.*, 2018). Around 10 million tons of plastic has ended up in the oceans, where the very small particles are ingested by marine organisms (Ng *et al.,* 2018). The most abundant source of contamination by microplastics in the world is low-density polyethylene.

This type of pollution has become a major problem; it could be solved by biodegradation through the action of different types of microorganisms (Rani and Rao, 2012). Studies have been carried out in species like the earthworm (*Lumbricus terrestris*) (Oligochaeta, Lumbricidae) to investigate how this product is absorbed and its particle size reduced by passage through the worm's intestine (Huerta *et al.,* 2016; 2018). The gastrointestinal tracts of insects are also associated with microorganisms, controlled by the intestinal immune system (Mukherjee *et al.*, 2013). Each species presents a characteristic microbiota composition, able to exist in this medium; for example the intestine of the mealworm larvae (*Tenebrio molitor* L.) (Coleoptera, Tenebrionidae) contains an environment in which rapid biodegradation of polystyrene can occur (Yang *et al.,* 2017).

Among the insect species studied for their efficiency in degrading this polymer is the greater wax moth (*Galleria mellonella* L.) (Lepidoptera, Pyralidae) (Bombelli *et al.*, 2017). It is found in beehives and is the major pest affecting stored wax, since it feeds in part on wax, pollen and exuviae of bee larvae (*Apis mellifera*) (Torres De La Cruz *et al.*, 2014).

In fungi, the capacity to degrade polyethylene by enzyme action is limited to a few species (Yoshida *et al.,* 2016); however, some researchers, such as Abrusci *et al*. (2011), have based their work on studying the behaviour of bacteria of the genera *Pseudomonas* and *Bacillus* as possible degraders of this material. Studies indicate that although it was regarded as inert (Kumar and Raut, 2015), polyethylene can be used as an energy source and bacteria strains capable of doing so can be isolated (Hadad *et al.* 2005).

The object of the present study was to identify bacteria present in the intestines of wax moth larvae (*Galleria mellonella*) capable of consuming plastic (polystyrene), using a microbiological method and through PCR and sequencing.

Materials and methods

The trial was carried out in the Plant Health and Bromatology Laboratories of the Department of Agriculture and Aquaculture Sciences of Universidad Católica de Temuco, Temuco, Chile, between March 2021 and November 2021.

Experimental material

The experiment used *G. mellonella* in the larval stage, provided by Biobichos Ltda., Chillán, Chile. The larvae were deposited in

sterilised glass flasks, closed with a wire mesh. Thirty *G. mellonella* larvae were fed with expanded polystyrene (PS) y thirty with natural diet, beewax and bran (1:1) for 7 days (ten larvae per replicate). They were observed daily to identify any behavioural changes that might affect the trial.

Isolation of bacteria from the gastrointestinal tract

To identify gut bacteria, three larvae were taken at random of each repetition (nine larvae per treatment). They were sterilised by immersion in ethanol at 75 % for 1 min and then rinsed twice with sterile water, following the methodology described by Yang *et al.* (2015). The larvae were then dissected with a scalpel on Petri dishes to extract their intestinal systems. Bacterial development was obtained only from the intestinal contents of larvae fed with a natural diet, seeded in agar PC and TSA and incubated for 3 to 5 days in aerobiotic conditions at 25 to 28 °C. The analyses included end-point PCR amplification using specific primers for bacteria, 16S sequencing and estimation of taxonomic diversity.

To isolate bacteria from the intestine samples, nine larvae were disinfected superficially in sodium hypochlorite at 5 % for 10 minutes and then rinsed five times with sterile distilled water and placed on sterile Petri dishes. The contents of the larvae's intestines were obtained by incision with a sterile scalpel, and inoculum was collected using an inoculation loop. It was seeded in grooves in Agar PC and Agar TSA and incubated at 25 °C, in aerobic and anaerobic conditions (anaerobic jars), for 3 to 5 days. Once the colonies developed, pure cultures were obtained which were characterised by Gram-staining prior to identification tests. As it was impossible to identify the pure cultures with the culture media and reagents available in the laboratory, a number were selected for molecular identification.

DNA extraction

Total DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturers' protocol. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) following the manufacturers' protocol (Thermo Fisher Scientific, USA). The integrity of the genomic DNA was confirmed by electrophoresis in agar gel. The DNA concentration was diluted at 20 $ng.\mu L^{-1}$ for further analysis. The samples were stored at -20°C until use.

PCR amplification

After DNA extraction, a PCR reaction was carried out in a SimpliAmp thermal cycler (Applied Biosystems, USA), under the following conditions: 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, followed by a final extension stage of 72°C for 2 min. The primers used were specific for amplification of the 16S RNA ribosomal gene in the bacteria: BACT1369F (5'-CGGTGAATACGTTCYCGG-3') and PROK1492R (5'-GGWTACCTTGTTACGACTT-3') (Suzuki *et al*., 2000). To discard polymerase errors, the reactions were performed in duplicate. Both *Taq* polymerase and Pfu polymerase were used for the amplifications.

Sequence analysis

The sequences obtained were checked manually and edited with BioEdit 7.0.9.0. The amplification sequences were aligned using Mega 4.0 to show polymorphisms. Genbank was then consulted to identify the bacteria.

Results and discussion

Gram positive and negative bacteria were observed among the strains isolated and Gram-stained; they were separated into bacillus and coco bacillus morphologies, and yeasts (table 1). The results of the mainly molecular identification indicate a diversity of bacteria, including non-fermenting bacilli and Actinobacteria.

Table 1. Bacteria obtained from the digestive tract of *G. mellonella* larvae with natural diet.

Bacteria	Presence
**Carnobacterium maltaromaticum*	+
**Brevibacterium sandarakinum*	+
**Pseudomonas psychrophila*	+
***Pseudomonas* sp.	+
***Providence* sp.	+
***Corynebacterium* sp.	+

* By PCR and sequencing.
** By microbiological method

Until recently, the importance of the greater wax moth (*G. mellonella*) in the degradation of microplastics was unknown. Bombelli *et al.* (2017) detected the diminution of polyethylene in the presence of this species, but we still had no information on the capacity of their intestines to degrade microplastic – or even potentially to produce it. Various studies stress the involvement of wax moths in the degradation of these recalcitrant wastes, but there is as yet no clarity as to the factors and mechanisms involved in this process. Investigations have reported different elements and actions, involving not only the digestive system of the insects studied, but also their development stages and the various microbial communities that develop in these systems in different development stages of the insect, in different habitats and under different feeding regimes or diets. This has opened up ample opportunities to find biotechnological uses through the design of biodegradation strategies for plastic in its many varieties.

Animals present associations with microorganisms in various places, and these relations are expressed in different degrees of association and sensitivity to environmental change. Diet is one of the factors that may alter interactions between host and microorganism; it has both short-term and long-term impacts on the sets of bacterial communities in the animal's gut, affecting their functional associations and the different species involved. Bacterial associations in the intestines of the larvae of Lepidoptera are particularly sensitive to changes caused by diet and environment, which also suggests that these bacteria are not simply temporary associates (Mason *et al*., 2020).

Various investigators are helping to expand knowledge of the microbial communities that colonise the middle and posterior intestines of insect larvae and adults; they are very varied and several phyla of bacteria are involved (Kong *et al.,* 2019; Lou *et al.,* 2020; Ruiz *et al*., 2022). According to the results of the present work, the development of Gram-positive and negative bacterial colonies included one Actinobacteria, a lactic acid bacillus and a psychotrophic bacterium known to form biofilms; these were identified by molecular techniques as *Brevibacterium sandarakinum*, *Carnobacterium maltaromaticum* and *Pseudomonas psychrophila* respectively.

Bacteria that degrade polystyrene have been isolated, identified and reported in numerous previous studies (Ren *et al*., 2019; Lou *et al*., 2020; Ruiz *et al.,* 2022); it has been reported that the majority

of the bacteria that degrade PS exist in the soil. However, recent studies have reported that the intestinal bacteria of mealworm larvae and wax moth larvae are directly involved in the degradation of plastic, in a similar way to soil bacteria (Kim *et al*., 2020).

Brevibacterium sandarakinum (Actinobacteria or coryneform bacteria) is one genus of bacteria of the order Actinomycetales. *Brevibacterium* is the only genus of the family *Brevibacteriaceae*; it consists of Gram-positive soil micro-organisms belonging to the Class Actinobacteridae, *B. sandarakinum* was isolated from a wall colonised by fungi by Kämpfer *et al*. (2010), who were the first to describe and name it. Its presence in an insect's digestive tract has not been confirmed previously, so further study is required. Other species of *Brevibacterium* have been found related with soil and with marine and freshwater ecosystems (Lewin *et al*., 2016). A variety of animals and plants depend on Actinobacteria to complement their diets or help to digest complex sources of plant foods present in soil, sludges, compost, and in eukaryotic associations with cellulolytic capacities. Investigations based on sequencing of the 16S rRNA gene indicate that Actinobacteria are most abundant in the intestines of insects that consume detritus or decomposing wood, especially termites. In more general terms, the cellulolytic capacities of this phylum cover a surprising diversity of enzyme families and microbial associations which hydrolyse plant biomass; these bacteria bring about a series of metabolic reactions which lead to the degradation of carbonate compounds, producing various bioproducts (Lewin *et al*., 2016). Their presence in the intestines of moth larvae may be related with a particular degrading activity of these larvae; the mechanism would be through specific bacterial conglomerates which depend on the dietary substrate, and pass from the substrate to colonise biofilms present in the insect's habitat.

Carnobacterium maltaromaticum belongs to a genus of Gram-positive bacteria, of the family *Carnobacteriaceae*, Order Lactobacillales. This genus is one of the lactic acid bacteria (LAB), which produce a variety of bacteriocins and other antimicrobial compounds such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and fatty acids (used in biotechnology as probiotics) (Brandon *et al*., 2018). In general, it is optionally anaerobic, with some species presenting aerobic or microaerophilic growth. *C. maltaromaticum* is found, together with *C. divergens*, in a variety of habitats: marine, soil, compost, faecal matter, and the intestines of moth larvae (Shannon *et al*., 2001). Strains of this species express chitinase activity, which could facilitate their survival adhering to zooplankton, as well as in the middle intestine of larvae of different moth species (Leisner *et al*., 2007). These characteristics, and its extensive colonisation abilities, allow *C. maltaromaticum* to degrade material under differing environmental conditions. It produces a variety of bacteriocins (carnobacteriocin, piscicolin, piscicocin, carnocin) and causes oxidation of tryptophan waste products, which are essential for its inhibitory activity (Leisner *et al*., 2007). Shannon *et al*. (2001) identified a species of the same genus, *C. piscicola*, in the middle intestine of larvae of the keratinophagous species *Hofmannophila pseudospretella* (Lepidoptera), which presents similar physiological and metabolic characteristics to *C. maltaromaticum*.

Strains of this species or its antimicrobial metabolites have been used as food protecting additives (Agudelo-Londoño *et al*., 2015), since they can form bacterial consortia that control or even inhibit the decomposition of foodstuffs – for example foods rich in proteins and lipids – at low temperatures. Although the capacity of *C. maltaromaticum* to decompose PS has not been proved, it could help to establish biofilms of microbial consortia which colonise polymers of this kind and use them as nutrients. This aspect will be explored in a later phase of this investigation.

P. psychrophila has been isolated from various habitats including soil, the marine environment, decomposing muscle and the intestines of different species of insect, such as *Zophobas atratus*. Kim *et al*. (2020) state that bacteria of the genus *Pseudomonas* have been reported to degrade polystyrene. Various publications indicate that species of *Pseudomonas* produce bacteriocins (Agudelo-Londoño *et al*., 2015). They also have the unique capability of degrading and metabolising polymers with extracellular oxidative and/or hydrolytic enzyme activities; these enzymes facilitate the absorption and degradation of polymer fragments and control the water mediated interaction between biofilms and polymer surfaces (Wilkes and Aristilde, 2017; Ghatge *et al*., 2020) by facilitating a chemical change from hydrophobicity to hydrophilicity (Kim *et al*., 2020). Most Gram-negative bacteria, including *Pseudomonas*, use molecules of acyl homoserine lactone (AHL) as their QS signalling molecule (communication mechanism between bacteria or quorum of detection that regulates the expression of bacterial genes when the bacteria live in a large community), released into the environment by the cells. Although the role of these molecules, for example in the decomposition of foodstuffs, is not clear, it is interesting to note that lipolytic, proteolytic, chitinolytic and other enzyme activities have been related with AHL regulation in various bacteria (Wickramasinghe *et al*., 2019); this may go some way to explaining the presence of this *Pseudomonas* in the digestive tract of wax moth larvae (*G. mellonella*). When these molecules accumulate in the medium and reach threshold level, all the unicellular bacteria that identify the signalling molecule regulate their gene expression in unison and respond to environmental stimuli as if they were a single multicellular organism (Wickramasinghe *et al*., 2019). These are aspects that should be investigated in the strain isolated, to better characterise its biotechnological utility in PS degradation by these larvae.

The species isolated from the digestive tract of wax moth larvae (*G. mellonella*) in this work, and identified by molecular means, would be beneficial for the nutritional and integral health of the insect, providing it with a source of nitrogen (Rizzi *et al*., 2013). All this requires more complete studies of the functions that these bacteria perform in the digestive tract of the larvae, or later development stages. One of the most notable characteristics of the plastic degradation processes mediated by bacteria is the formation of a biofilm on the surface of the plastic, which accelerates degradation by increasing the contact area between the bacteria and the (hydrophobic) plastic. This increases the oxidative processes and the general degradation mediated by enzymes secreted by the bacteria, which in turn contribute to the formation of the biofilm (Kim *et al*., 2020).

According to Lou *et al*., (2020) PS is difficult to degrade; only few bacteria and fungi can colonize PS films or degrade it at a very low rate. Specially species of the genus *Pseudomonas* are among the most cited degraders for a wide range of plastics and diverse hydrophobic polymers recalcitrant (Lou *et al.,* 2020; Ruiz *et al*., 2022). Consequently, this isolate could be the most interesting among others. However, the isolation of intestinal microbiota has failed to provide significant insight into the role of intestinal microbiota metabolic processes (Kong *et al.,* 2019),

therefore we need more evidence to demonstrate direct degradation of PS by *Pseudomonas psychrophila, Brevibacterium sandarakinum, Carnobacterium maltaromaticum, Pseudomonas* sp.*, Providence* sp. *and Corynebacterium* sp.

The complex biodegradation mechanisms of both polystyrene (PS) and polyethylene (PE) have yet to be well established. The biodegradation process has been studied using pure bacterial cultures and complex associations, with results that indicate that various abiotic and biotic factors play a vital role in the biodegradation of these plastic polymers in the environment, and particularly in the digestive system of moths (Ghatge *et al*., 2020).

Conclusions

We were able to characterise the intestinal microflora of the wax moth (*G. mellonella*), detecting the presence of bacteria from genera linked with benefits to the health, viability and nutrition of the host organisms, namely *Carnobacterium maltaromaticum, Brevibacterium sandarakinum, Pseudomonas psychrophila, Pseudomonas* sp.*, Providence* sp.*, Corynebacterium* sp. These bacteria may play an important role in the formation of microbial biofilms that can foment the degradation of low density polymers. However further studies are needed to verify their action, in isolation or in consortium, in the degradation of these polymers under different conditions of larval development.

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