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Microbiome Analysis of the Superworm (*Zophobas morio*) gut in Styrofoam Feeding Experiments.

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ABSTRACT
Although plastics are commonly used and reasonably priced organic polymers, biodegradation is hampered by their extreme resilience. One of the most widely produced plastics in the world, polystyrene, including extruded polystyrene (often referred to as Styrofoam), is resistant to microbial deterioration. In this investigation, we evaluated the alterations in the intestinal microbiota of superworms (*Zophobas morio*) raised for three weeks on polystyrene. We found multiple encoded enzymes that have been shown to degrade styrene, which confirms earlier findings of bacteria in the gut of superworms that break down polystyrene. In summary, our findings offer the metagenomic understanding of the metabolic processes by which the superworm gut microbiota breaks down polystyrene.

INTRODUCTION:

The increasing use of plastics has transformed everyday life. According to World Economic Forum (2016), plastics manufacturing and processing could account for as much as 20% of the world's petroleum consumption and 15% of the annual budget for carbon emissions by the year 2050. Plastics are expected to have several positive social effects as well as, technological and medical advancements. Due to the diminishing oil reserves all over the world, the continuous use of plastics is generally not sustainable. About 4% of the world's crude oil is thought to be used to make plastics. The use of plastics raises a few concerns, one of which is the accumulation of plastic waste in landfills and other natural habitats, which can physically harm wildlife by ingestion or entanglement. Additionally, it is extremely risky for chemicals to be leached from plastic products and spread to humans and wildlife (Thompson *et al.,* 2009).

There are generally three types of plastic: high-performance plastics, commodity plastics, and engineering plastics. The six distinct polymer types of common plastics account for approximately 70% of global production: Polyethylene terephthalate (PET or PETE), High-thickness polyethylene (HDPE), Polyvinyl chloride (PVC), Low-thickness polyethylene (LDPE), Polypropylene (PP) and Polystyrene (PS). However, polyurethanes (PUR) and PP&A fibers are also components of common plastics. These common plastics are widely used and relatively inexpensive.

German apothecary Eduard Simon was the first person to discover polystyrene (PS). It is a common commodity thermoplastic made from petroleum, and its structure is very stable and has a high molecular weight. According to Plastics Europe, Polystyrene (PS) global production capacity is of 15.61 million metric tons in 2019 which represents approximately 0.04% of the 370 million metric tons of plastic produced worldwide in the same year. The German company BASF (IG Farben) began producing PS for commercial use at the beginning of the 1930s. In 1937, the product was made available in the United States. PS is accessible in two primary structures: a high-impact grade and a general-purpose grade in

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which polybutadiene is used to alter the PS. Expanded polystyrene (PS), which was developed in 1954 by Dow Chemical Company, is widely acknowledged for its capabilities as a moldable packaging material and as an excellent building insulating medium. Consumer goods typically use expanded PS cups and trays, whereas high-value goods like electronics, televisions, washing machines, and lighting are protected during transportation by industrial packaging (Andrady and Neil, 2009).

There are two types of PS: foam and rigid. Clear food containers are made with the rigid form, while single-use containers like disposable food boxes or cups are often made with the foam form (Styrofoam) (Ho *et al.,* 2017). PS compartments are abused since they are reasonable, modest, and sturdy materials, which brings about colossal measures of PS squander across the globe. According to Peng et al. (2018), the main ways used to dispose of plastic garbage now are landfilling, incineration, and mechanical and chemical recycling. Due to its practicality and affordability, landfilling is the primary method for disposing of plastic trash in most nations, especially developing ones. But the accumulating plastic debris has taken up a lot of space. The incineration of plastic garbage can lessen the requirement for landfill space and recover thermal energy, but it also has the potential to produce secondary pollutants that have an adverse impact on the environment, such as dioxins, carbon monoxide, nitrogen oxides, and others. Even though mechanical recycling has taken over as the main recycling technique and is used to reuse thermoplastic wastes, the qualities of most recycled materials are considerably impaired after several processing cycles, and the resultant commercial values are thus constrained. Chemical recycling is an alternative method for recovering monomers and other compounds from plastic trash, but its effectiveness depends on how affordable the procedures are and how effective the catalysts are (Rahimi and Garca, 2017). According to recent reports, only 9 to 12% of the world's plastic waste is currently recycled or burned, while up to 79% is dumped into landfills or the environment. This shows that there is a critical need to investigate cutting-edge recycling techniques for disposing of plastic waste (Garcia and Robertson, 2017).

In the present study, our objective was to verify that superworms raised only on PS survived and to look about the composition of the microbial communities in such a condition. We effectively identified the key microbial participants in the gut microbiome during this feeding regimen and the possible styrene breakdown genes

MATERIALS AND METHODS:

Superworms and polystyrene:

Superworms, the larvae of *Zophobas morio*, ranging in size from 25 to 40 mm, used in this study were purchased from Pisces Kolkata. Originally a registered trademark for a light-blue extruded polystyrene (XPS) foam used as insulation in buildings, Styrofoam has evolved into a colloquial term for white expanded polystyrene foam (EPA), which is widely used as packaging material, food containers, and coffee cups. A locally available Styrofoam was used, which contained a minimum of 92%–95% PS (CAS No. 9003-53-6), 4–7% pentane (CAS No. 109-66-0), an expanding agent added to PS beads, and 1% of the flame retardant hexabromocyclododecane (CAS No. 25637-99-4) were present in the styrofoam, according to the manufacturer. Since pentane diffuses to negligible quantities within a few weeks of manufacturing, the styrofoam was thought to contain only trace amounts of the agent because it was held in our laboratory for several months before to the feeding trials (Simpson *et al.,* 2020).

Feeding trials:

Our modified experimental design involved putting the starving control group animals in isolation while the animals in the other two groups were housed together during the feeding trial. This was done after preliminary testing with groups of starving superworms corroborated allegations of cannibalism (Maciel-Vergara *et al.,* 2018). We discovered that, despite the possibility that isolation may affect social behavior and the microbiota, this configuration is the only one that successfully inhibits cannibalism in starving scenarios.

One container held all 50 superworms for the first twenty-four hours after arrival. During the first week of acclimatization, the superworms were fed organic wheat bran supplemented with carrots. A customized water sprayer was used to regulate the moisture content in order to produce a fine mist layer on the underside of the superworm container lid. The experiment was conducted at room temperature, which varied during the experiment from 25 to 28 °C. A three-week feeding study was conducted with the superworms. Consequently, the superworms received a polystyrene feed, twice a week, the weight of all the foam blocks and superworms was measured. Following the three-week feeding trial, the superworms were preserved at -80°C for metagenomics by flash-freezing them in liquid nitrogen.

Dissection of the superworms:

To do dissections, the superworms were taken out of the freezer at -80°C, flipped over, and sliced from behind the legs to the final abdominal segment while

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still frozen. To retrieve DNA, a portion of the larvae's digestive tract, comprising the midgut and hindgut, was excised.

DNA extraction, sequencing and quality control:
The Power Biofilm DNA Isolation Kit (Qiagen) was used for all microbial DNA extractions from the frozen samples without any pretreatment. Genome sequencing was carried out on the Illumina NextSeq 500 platform using Nextera XT libraries (Biokart, Bangaluru, India), which were created following the standard Illumina protocol with 12 cycles of limited cycle PCR amplification, with the only modification of reducing the reagent volumes to 1/5th of the tagmentation and the PCR step. The libraries were sequenced with a 2×150 bp high- output v2 run chemistry, and a targeted sequence allocation of 3 Gb per sample. Quality control of raw Illumina reads (2×150 bp) from all 15 samples was performed with FastQC cersion 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Read QC:
Quality control of raw Illumina reads (2×150 bp) from all 15 samples was performed with FastQC Version 0.11.9

Ribosomal RNA fragments filtering
Any DNA in a collection of microorganisms is the target of short-gun Metagenomics sequencing. Ribosomal RNA sequences will make up the largest percentage of DNA sequences in any given organism. These rRNAs do not offer any functional information, such as which genes are expressed, but they are helpful for taxonomic assignment, or identifying which species are present. *SortMeRNA* has been used to sort the rRNA sequences in order to expedite the downstream functional annotation (Kopylova *et al.,* 2012). Large RNA datasets can be handled by it, and it can accurately and specifically sort out all fragments that fit the database.

Extraction of the community profile:
"A characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties" is what is meant by the word "microbiome." Consequently, the phrase incorporates both the microbes and their theatre of operation (Whipps *et al.* 1988). DNA from various organisms at a particular location where the sample was taken is present in metagenomic samples. It is possible to determine which organisms share that niche and which gene are shared by the various organisms by using metagenomic data. Classifying individual readings to reference taxa in order to determine the relative abundances of the various taxa is known as taxonomic profiling and it is a method for DNA metagenome analysis apart from taxonomic binning.

Using marker-based tools such as *MetaPhlAn* to look for marker genes (such as the 16S rRNA sequence) in reading metagenome data is rapid (Blanco-Míguez Aitor *et al.,* 2023). Here we have used *MetaPhlAn* through Galaxy website for taxonomic profiling of the gut metagenome data (Batut *et al.,* 2018).

Estimates of Microbial Diversity
A diversity index is a numerical metric used to evaluate the degree of variation or diversity in each system, like a population, a biological community, or a workplace. It offers a means of recording and measuring how various types or categories are distributed inside a system. The term "α diversity" refers to the variety found in a community. It considers the variety of species present in an area, which is also known as species richness. Furthermore, species evenness—a measure of how equally individuals are dispersed throughout the sample—can be calculated by accounting for each species' abundance.

The alpha diversity of the sample was calculated using the *KrakenTools* suite (Lu et al., 2022).

Retrieval of the functional information:
We can access the genes that the community expresses through the short-gun metagenomics data. Using *HUMAnN*, we may use that to discover genes, evaluate their roles, construct pathways, and look at how they contribute to the community (Franzosa *et al.,* 2018). The program *HUMAnN* was created to effectively and precisely profile the abundance and presence/absence of microbial pathways in a community using metagenomic or meta-transcriptomic sequencing data.

Since rRNA sequences lack gene-coding sections and slow down the process, we do not require them to determine the functions performed by the community. We used both the *SortMeRNA* output and the *MetaPhlAn*-identified community profile. This will assist *HUMAnN* in concentrating on the recognized organisms' known sequences.

The metagenome data has been submitted to NCBI SRA website with the accession number: **(SRR27217060).**

RESULTS AND DISCUSSION:

Bacterial diversity:
With metagenomic shotgun sequencing data (i.e., not 16S), *MetaPhlAn* (Truong *et al.,* 2015) is a computational tool that profiles the species-level composition of microbial communities (Bacteria, Archaea, and Eukaryotes). *MetaPhlAn 4* is based on over 5.1 million distinct clade-specific marker genes

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that have been found in approximately 1 million microbial genomes over 26,970 species-level genome bins (~236,600 references (bacterial, archeal, viral, and eukaryotic) and 771,500 metagenomic assembled genomes. It enables: clear classifications of taxa; precise assessment of the relative abundance of organisms; species-level resolution for bacteria, viruses, eukaryotes, and archaea; and orders of magnitude faster strain identification and tracking than current techniques. *MetaPhlAn* is mostly used to identify the clades (from phyla to species and strains in specific circumstances) that are present in the microbiota that is extracted from a microbiome sample of the 7,76,02,847 quality sequencing reads produced by Illumina NextSeq 500 platform sequencing, 99% were assigned to bacteria, 0.07% to viruses, 0.001% to archaea, and 0.9% to eukaryote (Fig. 1a).

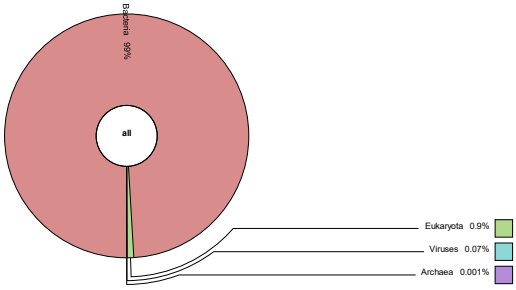

Fig. 1a. Krona graph of the total microbial community from the short-gun metagenomic analysis of the superworm gut.

The most prevalent bacterial taxa at in the gut of the superworm feed with styrofoam were the Proteobacteria (of which most of the proteobacterial sequences belong to the class Gammaproteobacteria), which was abundantly (88%) represented by the family *Hafiniaceae* (Fig. 1b, 1c).

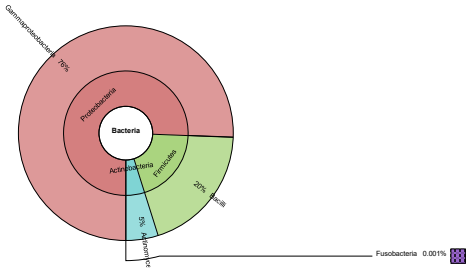

Fig. 1b. Krona graph of the bacterial community from the short-gun metagenomic analysis of the superworm gut.

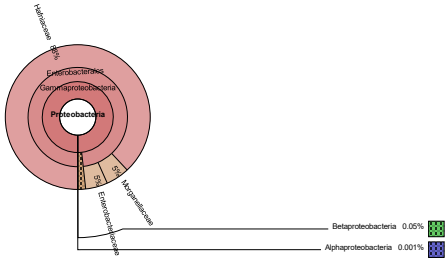

Fig. 1c. Krona graph of the Proteobacteria distribution from the short-gun metagenomic analysis of the superworm gut.

The phylum Actinibacteria accounted to 20% of the total bacterial community and was mostly (99%) represented in the form of genus *Corynebacterium* (Fig. 1d).

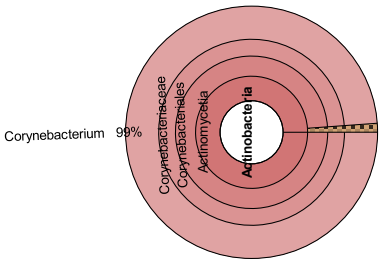

Fig. 1d. Krona graph of the Actinobacteria distribution from the short-gun metagenomic analysis of the superworm gut.

The Firmicutes group was predominantly represented by two genera *Lactococcus* (33%) and *Enterococcus* (67%) as represented in Fig. 1e.

The diversity that exists inside a community is referred to as α diversity. Because different indexes capture different features of diversity and have varied sensitivity to different conditions, α diversity is calculated using a variety of different indices. These indices have been created to highlight particular facets of variety, account for various ecological or population factors, or answer particular research problems.

The relationship between the number of species and the number of individuals within those species is described by the Fisher's alpha index. A parametric diversity measure based on the assumption that species abundance is distributed as a log series.

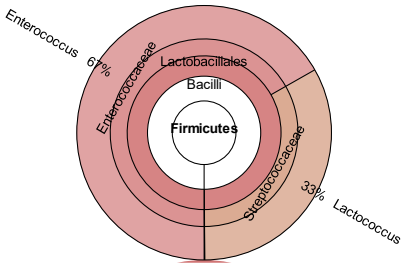

Fig. 1e. Krona graph of the Firmicutes distribution from the short-gun metagenomic analysis of the superworm gut.

The likelihood that two individuals chosen from a community will belong to the same species is determined by the Simpson's index. It yields significant values in low-diversity datasets and small values in high-diversity datasets. The Simpsons index is transformed into the Inverse Simpons index, which rises as diversity does. The most prevalent type's proportional importance is expressed by the Berger-Parker index. Extremely skewed by sample size and complexity. The Shannon's index determines the degree of uncertainty in identifying

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the species of an individual chosen from a community.

The microbial diversity analysis of the styrofoam feed superworm gut sample yielded the below results:

Fisher's index: 5242.283974884244
Simpson's Reciprocal Index: 2.506865182679442
Simpson's diversity: 0.6010954211222646
Berger-parker's diversity: 0.6195620202932504
Shannon's diversity: 1.7404551860049127

Functional Analysis:
Gene families with Pfam domains and pathway inference were examined using the *HUMAnN* v3.9 program (Beghini *et al.,* 2021; Abubacker *et al.,* 2012). The complexity of metabolic processes inside the Styrofoam fed superworm gut is demonstrated by 788 pathways, and 320972 gene families. Table 1 provides a summary of the top pathways found by average abundance. Furthermore, Supplementary Data Tables S1 and S2, respectively, present the relative abundance of the identified gene families and pathways. Overall, KEGG analysis of the gut metagenome showed presence of 75 genes involving the identified styrene degradation pathway, which were incorporated into 69 scaffolds.

To sum up, our metagenomic investigation of the gut microbiota of superworms yielded the understanding of how their microbial community gut reacts to a polystyrene diet. However, we still need to understand the genes involved in polystyrene breakdown pathway and bacteria possessing such genes in these microbial communities. Further, cultivating the bulk of superworm gut bacteria is still the ultimate objective in our quest to describe microbial polystyrene breakdown.

Table 1: Summary of major metabolic pathways by relative abundance (RPK)

Pathway	***Humann*_Abundance-RELAB**
PWY0-1586: peptidoglycan maturation (meso-diaminopimelate containing)	0.00142851
PWY0-1586: peptidoglycan maturation (meso-diaminopimelate containing) |unclassified	0.00142851
PWY-7185: UTP and CTP dephosphorylation I	0.00105586
PWY-7185: UTP and CTP dephosphorylation I|unclassified	0.00105586
NONOXIPENT-PWY: pentose phosphate pathway (non-oxidative branch) I	0.000718957
NONOXIPENT-PWY: pentose phosphate pathway (non-oxidative branch) I|unclassified	0.000718957
FAO-PWY: fatty acid & beta; -oxidation I (generic)	0.000678413
FAO-PWY: fatty acid & beta; -oxidation I (generic)|unclassified	0.000678413
PWY-5136: fatty acid & beta; -oxidation II (plant peroxisome)	0.000661711
PWY-5136: fatty acid & beta; -oxidation II (plant peroxisome) |unclassified	0.000661711
PWY-7013: (S)-propane-1,2-diol degradation	0.000657082

CONFLICT OF INTEREST:
The authors declare that there is no conflict of interest regarding the publication of this paper.

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AUTHORS ' CONTRIBUTION:
PV performed the experiments. SP conceived the study and was responsible for writing and critical revision of the manuscript for final publication.

FUNDING:
None.

ETHICS STATEMENT:
This article does not contain any studies on human participants or animals performed by any of the authors.

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