

## Research Article

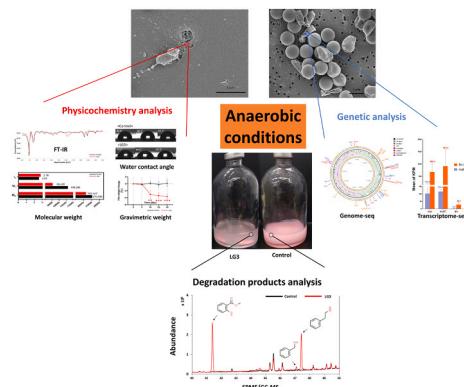
# Polystyrene microplastics biodegradation by gut bacterial *Enterobacter hormaechei* from mealworms under anaerobic conditions: Anaerobic oxidation and depolymerization

Min-Geun Kang <sup>a</sup>, Min-Jin Kwak <sup>a</sup>, Younghoon Kim <sup>a,\*</sup><sup>a</sup> Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Republic of Korea

## HIGHLIGHTS

- LG3 isolated from the mealworms biodegrades polystyrene under anaerobic and aerobic conditions.
- LG3 formed biofilm on the PS plastic surface under anaerobic conditions.
- Benzoic rings of PS were broken down, as confirmed by analyses using GPC, FTIR and metabolites.
- Nanopore sequencing identified degradative enzymes, including *tpx*, *ahpC*, and *bcp*, in LG3.
- Lipid A and biofilm-associated proteins were upregulated with PS as sole organic carbon source.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Synthetic plastic is used throughout daily life and industry, threatening organisms with microplastic pollution. Polystyrene is a major plastic polymer and also widely found sources of plastic wastes and microplastics. Here, we report that *Enterobacter hormaechei* LG3 (CP118279.1), a facultative anaerobic bacterial strain isolated from the gut of *Tenebrio molitor* larvae (mealworms) can oxidize and depolymerize polystyrene under anaerobic conditions. LG3 performed biodegradation while forming a biofilm on the plastic surface. PS biodegradation was characterized by analyses of surface oxidation, change in morphology and molecular weights, and production of biodegraded derivative. The biodegradation performance by LG3 was compared with PS biodegradation by *Bacillus amyloliquefaciens* SCGB1 under both anaerobic and aerobic conditions. In addition, through nanopore sequencing technology, we identified degradative enzymes, including thiol peroxidase (*tpx*), alkyl hydroperoxide reductase C (*ahpC*) and bacterioferritin comigratory protein (*bcp*). Along with the upregulation of degradative enzymes for biodegradation, changes in lipid A and biofilm-associated proteins were also observed after the cells were incubated with polystyrene microplastics. Our results provide evidence for anaerobic biodegradation by polystyrene-degrading bacteria and show alterations in gene expression patterns after polystyrene microplastics treatment in the opportunistic pathogen *Enterobacter hormaechei*.

\* Corresponding author.

E-mail address: [ykeys2584@snu.ac.kr](mailto:ykeys2584@snu.ac.kr) (Y. Kim).

## 1. Introduction

Polystyrene (PS) is a synthetic plastic that has a long hydrocarbon chain with a phenyl group attached to every other carbon atom, and it is widely used throughout daily life and industry. The COVID-19 impact induced plastic consumption and the production of global polystyrene and expandable polystyrene steadily increased, exceeding 73.7 million metric tons in 2021 [56]. In particular, expanded polystyrene (EPS) occupies the largest portion of the PS market due to its exceptional balance of lightness, shock absorption, insulation, and indefectibility [47]. EPS is used as food contact materials, product packaging containers, and aquaculture buoys, so they are often exposed to marine life as well as human life [38,39].

The use of EPS buoys in territorial waters has been banned by the Korean government due to their role in producing Polystyrene microplastics (PS-MPs) and the associated risks; however, many countries are still using EPS buoys for aquaculture [12]. Moreover, unretrieved EPS buoys eventually turn into environmental pollutants in the sea because EPS may be a microplastic supplier with vulnerability to abrasion [21, 38,4]. Microplastics (MPs) can be ingested by small organisms and can accumulate in the gut due to their high resistance to degradation and strong binding ability. Cole et al., [14,59,62]. Consequently, MPs are widely observed in not only fish and fish meals but also agricultural feedstocks and livestock, including pigs, cattle, and chickens, through transmission between organisms [101,46,81].

MPs, directly or indirectly transferred into the body, can contact the gastrointestinal tract and interact with the commensal bacteria, including *Enterobacteriaceae* and *Bacillaceae*, in the intestinal rumen, and are available resources to plastic-degrading gut bacteria [1,43,54,75]. However, recent studies have focused PS-degrading gut bacteria under aerobic conditions, rather than anaerobic conditions [55]. In previous studies, PS-degrading gut bacterium *Exiguobacterium YT2* was isolated [99] and 8 strains of gut microbes were identified from the guts of mealworms [6]. Kim et al. [35] isolated PS-degrading *Pseudomonas* strain DSM 50071 from the gut of Superworms (*Zophobas atratus* larvae) [35]. Both studies indicated that the isolated bacterial cultures degraded PS in the presence of oxygen i.e. under aerobic conditions. Therefore, further studies should focus on investigating novel bacteria capable of degrading plastics under anaerobic conditions.

Mealworms (larvae of *Tenebrio molitor*) are known to digest PS and polyethylene (PE) materials, and antibiotics studies have indicated that the gut microbiota of mealworms plays an essential role in the biodegradation of PS [5,97,99]. Researchers have also revealed that gut bacteria play an important role in mealworms' ability to adapt to plastic feeds [45,99]. However, previous studies with plastic-degrading microorganisms were performed in an oxygen-rich environment, which contrasts with the oxygen-limited gut environment of mealworms [30,6, 86,99]. There is an oxygen-limited condition across the intestinal tract of mealworms due to microbiota in the lumen and close to the gut wall, as the hindgut features diverse genera of anaerobes, including *Clostridium*, *Enterobacter*, and *Spiroplasma* [20,5,84,86]. Collectively, the possibility of PS biodegradation by gut microorganisms under oxygen-limited and even anaerobic conditions should be investigated to fill the scientific gap.

In this study, we isolated a bacterial strain of *Enterobacter* spp. from the gut of mealworms under an oxygen-limited environment, which functioned as facultative PS-degrading gut microbes. *Enterobacter* spp. are facultative gut bacteria and opportunistic pathogens, and their impact on the host can vary significantly depending on the environment [9,63]. In certain environments, *Enterobacter* spp. make probiotics effects by activating the host's immune system and enhancing immune response [40,42]. However, in different environments, they can also cause a decline in the host's health due to released endotoxins [15,37].

*Enterobacter hormaechei* LG3, which has PS degradation activity under both anaerobic and aerobic conditions, when utilizing microspheres PS (Mi-PS) as a carbon source under anaerobic conditions, exhibits physiological changes such as biofilm formation and endotoxin synthesis pathways based on whole genome and transcriptomes sequencing. This is accompanied by the upregulation of degradative enzyme genes such as *tpx*, *ahpC* and *bcp* associated with PS degradation.

Furthermore, we conducted a comparative analysis of biodegradation using different PS materials, including emulsified-PS, PS film, and Mi-PS, under both aerobic and anaerobic conditions. The *Bacillus amyloliquefaciens* SCGB1 was used to confirm the PS degradation of *Enterobacter hormaechei* LG3. Species of *B. amyloliquefaciens* is facultative gut bacterium that has been recognized for its influence on gut health in the host [18,31,49] and is known for their PS and PE degradation activity [26,92]. The comprehensive analysis including mass reduction of PS film, cell viability, GPC, WCA, FTIR, and SPME/GC—MS revealed metabolic differences in PS degradation between the two distinct strains, providing phylogenetic differentiation.

## 2. Materials and methods

### 2.1. Experimental model and subject details

#### 2.1.1. *T. molitor* larvae

Two groups of *T. molitor* larvae of different ages and sites were obtained. The early instar group (< 10 mm and 0.03 g) was purchased from Anifoodfarm (Insects Breeding Plant, Namyangju, South Korea). The late instar group (> 20 mm and 0.1 g) was received from Daehanfeed company (Icheon, South Korea). Both groups were maintained in an incubator at 25 °C and 60% humidity, and were fed only PS foams (provided by SWEPS company (Chungju, South Korea)) and water for 2 weeks.

#### 2.1.2. PS foams, PS films, and Mi-PS

PS foams were source from commercially available products. PS films were purchased from the Goodfellow company (Huntingdon, UK). Microspheres PS (Mi-PS) with a diameter of 1 μm were obtained as beads from the Polysciences company (Warrington, USA). Mi-PS are internally PC Red dyed using dye entrapment. The dyes remain trapped in the beads in aqueous environments. The PS materials (0.1 g) dissolved in tetrahydrofuran (THF). The weight average molecular weight ( $M_w$ ), number average molecular weight ( $M_n$ ), and z-average molecular weight ( $M_z$ ) of THF-dissolved PS materials were analyzed using gel permeation chromatography (GPC, Ultimate 3000, Thermo) [96]. The  $M_w$  values of PS foams, PS films, Mi-PS, PS emulsion were 247,800 Da, 314,700 Da, 276,700 Da, and 284,800 Da, respectively (Supplementary Table 1).

#### 2.1.3. PS emulsion

The following process was performed in a fume hood to prepare the PS emulsion [86]. First, 5 g of PS foams were dissolved in 50 mL of dichloromethane. Then, the 50 mL of 1% sarkosyl NL was added and mixed. The mixture was sonicated using an ultrasonic homogenizer (KUS-650, KBT) for 10 min. Next, 100 mL of distilled water was added to the PS emulsion and vigorously stirred for 30 min using a stirrer (MSH-20A, Daihan Scientific). To evaporate the dichloromethane in the mixed solution, the solution was stirred at 80 °C for 6 h. The PS emulsion volume was maintained between 100 and 200 mL by adding distilled water. After the filtration process using filter paper (HM.5012055, Hyundai Micro), the weight of the filtered PS was measured to calculate the PS content in the emulsion. The average particles size of PS emulsion was measured by using particle size measurement system (NanoSQAQ, Otsuka). Based on the intensity distribution the mean particle diameter of PS emulsion was 119.4 ± 36.9 nm (Supplementary Fig. S1).

## 2.2. Isolation and identification of PS-degrading bacteria

### 2.2.1. Enrichment culture

Separate experiments were conducted using two groups: an early instar larvae group and a late instar larvae group, with limited feeding of PS foams for 14 days, to establish enrichment cultures (Supplementary Table S2). We prepared four different variants as follows: 1) EG: using gut samples from early instar larvae, 2) EF: using frass samples acquired from early instar larvae, 3) LG: using gut samples acquired from late instar larvae, and 4) LF: using frass samples acquired from late instar larvae. Enrichment cultures were performed using emulsified PS (ePS) containing fungal minimum medium (FMM) with the following components: 2 g L<sup>-1</sup> sodium phosphate monobasic 0.5 g L<sup>-1</sup> magnesium sulfate heptahydrate 0.2 g L<sup>-1</sup> monopotassium phosphate, 1 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> L-cysteine hydrochloride, 0.05 g L<sup>-1</sup> Sarkosyl NL, and 5 g L<sup>-1</sup> PS foam [86]. After filling a 50 mL conical tube with 20 mL of FMM, each of the frass and gut samples was inoculated into the ePS-containing FMM medium. The inoculation process was conducted in a vinyl anaerobic chamber equipped with a vacuum airlock (032714, Coy Labs). The conical tube was then vortexed for 5 min, following a 10-minute waiting period to allow the tissue or feces to settle. The settled material was subsequently removed using a pipette. The conical tube containing the FMM suspension was incubated for 5 days under anaerobic conditions to provide a favorable environment for anaerobic PS-degradable bacteria [95,100].

### 2.2.2. Isolation of anaerobic polystyrene-degrading microorganisms

After 5 days of preincubation, the ePS-containing FMM medium inoculated with the specimen showed changes in turbidity. Cultured microorganisms were isolated through serial dilution. Aliquots (0.1 mL) of preincubated suspension in ePS-containing FMM medium were spread onto Luria-Bertani (LB) agar plates. After incubation for 48 h at 37 °C under anaerobic conditions, the grown colonies were isolated and streaked onto other plates with ePS-containing FMM agar. The colonies that formed clear zones were selected for further analysis. A total of ten strains were isolated from the gut and frass of the larvae. The strain names of the isolated bacteria were assigned based on the samples. For example, EG1 and EF1 were named to represent strains isolated from the gut and frass of early instar larvae, respectively.

### 2.2.3. L-cysteine hydrochloride

The FMM medium used in this experiment contains 0.5 g L<sup>-1</sup> of L-cysteine hydrochloride for microbial cultivation under anaerobic conditions. L-cysteine hydrochloride is utilized as a reducing agent to enhance efficiency of anaerobic conditions by scavenging minuscule amounts of oxygen in medium [91]. The presence of trace amounts of L-cysteine hydrochloride in the FMM medium did not significantly affect PS degradation under anaerobic conditions (Supplementary Fig. S2).

### 2.2.4. Atmosphere conditions of anaerobic chamber

Nitrogen gas and hydrogen gas were introduced into the chamber through two lines: the background gas line and the pre-mixed gas line. The background gas line supplies pure nitrogen, while the pre-mixed gas line supplies a mixture of 5% hydrogen with nitrogen as the balance. Two heated fan boxes with a palladium catalyst and a hydrogen sulfide removal column are placed inside the anaerobic chamber. The heated fan boxes with a palladium catalyst remove traces of O<sub>2</sub> from the atmosphere of chamber using H<sub>2</sub>, maintain temperature, and provide a homogeneous mix of gases in the chamber. The hydrogen sulfide removal column removes H<sub>2</sub>S by continuously recirculating the chamber's atmosphere through the column. Throughout the experiment, the temperature was consistently maintained at 37 °C, and the humidity level ranged from 30% to 40%. The levels of O<sub>2</sub> and H<sub>2</sub> equilibrated to 0–25 parts per million (ppm) and 2.5–3%, respectively.

### 2.2.5. Identification and phylogenetic analysis

The selected bacteria, which formed clear zones on ePS-containing FMM agar plates, were identified through 16 S rRNA gene sequencing. The HiGene™ Genomic DNA prep kit for Bacterium (GD262–060, BIOFACT) was used according to the manufacturer's manual. Amplification of the 16 S rRNA gene was performed with the universal primers 27-F (5'-TACGGYTACCTTGTACGACTT-3') and 1492-R (5'-CCAG-CAGCCGCGTAATACG-3'). The obtained approximately 1500 bp sequences were aligned with organisms present in the GenBank database using BLAST (<https://blast.ncbi.nlm.nih.gov>) created by the National Center for Biotechnology Information, USA. The 16 S rRNA gene-based evolutionary tree was constructed using the neighbor-joining method and with the Kimura two-parameter Model using MEGA X. The 16 S rRNA sequences of ten isolated strains from mealworms (EG1, EG2, EF1, EF2, LG1, LG2, LG3, LF1, LF2, and LF3) have been deposited in GenBank with the following accession numbers: OQ253539, OQ253542, OQ253543, OQ253544, OQ253545, OQ253550, OQ253551, OQ253553, OQ253554, and OQ253563, respectively.

## 2.3. *Bacillus amyloliquefaciens* SCGB1

*B. amyloliquefaciens* SCGB1, which was isolated from traditional Korean soybean-fermented foods, was provided from the Microbial Institute for Fermentation Industry (Sunchang, South Korea). This strain SCGB1 can affect intestinal health of mice [31] and is capable of growing under anaerobic conditions in a nutritionally rich medium, such as LB broth, or on LB agar plates (Supplementary Fig. S3).

### 2.4. Biodegradation assay

#### 2.4.1. Biodegradation assay in liquid FMM medium

To prevent PS films from overlapping and increase the available surface area for microorganism adhesion, three pieces of crumpled PS films weighing 93 ± 0.1 mg were used as a carbon source in 20 mL of liquid FMM medium. The PS film was disinfected with 70% ethanol and underwent an air-drying process in a laminar-flow clean bench before being placed into the FMM medium. The FMM medium containing 2 g L<sup>-1</sup> sodium phosphate monobasic 0.5 g L<sup>-1</sup> magnesium sulfate heptahydrate 0.2 g L<sup>-1</sup> monopotassium phosphate, 1 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> L-cysteine hydrochloride. The incubated bacterial cell suspensions (10<sup>9</sup> CFU mL<sup>-1</sup>) were resuspended three times in sterile saline water (SW). Aliquots (0.01 mL) of washed cell suspensions (10<sup>9</sup> CFU mL<sup>-1</sup>) were inoculated into the FMM medium. To establish aerobic and anaerobic conditions, different caps were used: an air-permeable silicone rubber cap for aerobic conditions and a sealed butyl cap for anaerobic conditions (Supplementary Fig. S4). The samples were cultured for 15 days at 37 °C in shaker incubators (120 rpm) or stationary incubators. After 15 days of inoculation, the bottles containing the PS films were shaken on a vortex mixer for 5 min to detach the bacteria from films. The bacterial cell numbers in the medium were determined using the series dilution method of plate counting. Next, a PS film was transferred into another centrifuge tube containing 40 mL of aqueous solution with 2% (w v<sup>-1</sup>) sodium dodecyl sulfate (SDS) for 4 h and then rinsed with 70% (v v<sup>-1</sup>) ethanol solution. Following treatment, it was confirmed that no cells were observed on the surface of the air-dried PS film. The change in the weight of the PS film was determined by measurement. (PX224KR, OHAUS) [8]. The specifications of this model are as follows: Draftshield Included, Readability of 0.1 mg, Repeatability of 0.0001 g, Stabilization Time of 2 s, and Linearity of ± 0.0002 g.

#### 2.4.2. Biodegradation assay on FMM agar plate

The biodegradation of the PS film was characterized by measuring its weight loss on the FMM agar plate containing 2 g L<sup>-1</sup> sodium phosphate monobasic 0.5 g L<sup>-1</sup> magnesium sulfate heptahydrate 0.2 g L<sup>-1</sup> monopotassium phosphate, 1 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> L-cysteine

hydrochloride, and 15 g L<sup>-1</sup> bacto agar. The incubated cell suspensions were resuspended three times in sterile saline water (SW). Aliquots (0.01 mL) of washed cell suspensions (10<sup>9</sup> CFU mL<sup>-1</sup>) were inoculated onto FMM agar. A pre-weighed 20 mm × 20 mm polystyrene film was then placed above the inoculated position. The sterile control was inoculated with sterile saline water (SW) instead of bacterial cell suspensions. The PS films were weighed, disinfected in 70% ethanol, and subsequently air-dried in a laminar-flow clean bench prior to use. Four samples were prepared for each incubation period of 0, 5, 10, 20, and 30 days at 37 °C, under either anaerobic or aerobic conditions. For the zero-day incubation, samples were collected after 3 h of inoculation. The number of cells attached to the PS film and the weight of the PS film were measured using the following procedure. The PS film sheet-attached bacterial cells were transferred into a 50 mL centrifuge tube containing 20 mL of sterile SW, and the tube was shaken on a vortex mixer for 5 min. The cell number of the suspension was counted using the series dilution method of plate counting. Next, a PS film was transferred into another centrifuge tube containing 40 mL of aqueous solution with 2% (w v<sup>-1</sup>) sodium dodecyl sulfate (SDS) for 4 h and then rinsed with 70% (v v<sup>-1</sup>) ethanol solution. After the treatment, the absence of cells on the surface of the air-dried PS film was confirmed. The change in the weight of the PS film was measured using an analytical balance (PX224KR, OHAUS).

## 2.5. Characterization of cell viability on PS film

Aliquots (0.01 mL) of washed cell suspensions (10<sup>9</sup> CFU mL<sup>-1</sup>) were inoculated on FMM agar, and subsequently covered with a PS film sheet (20 mm × 20 mm), as described above. Four samples were tested after incubation for 30 days at 37 °C in anaerobic conditions. The cell viability on the PS film surface was characterized according to the manufacturer's instructions (MP 07007). Microscopy and quantitative assays were conducted using a fluorescence microscope (IX53, Olympus) after staining with the LIVE/DEAD BacLight Bacterial Viability Kit (L7012).

## 2.6. Microscale chemical analysis of the PS film surface

After a 30-day incubation period at 37 °C in an anaerobic chamber, the biofilm on the PS film was completely removed using 2% (w v<sup>-1</sup>) aqueous SDS solution for 4 h and rinsed with 70% (v v<sup>-1</sup>) ethanol solution. The PS films in the sterile control were also treated using the same procedure. Consequently, the surface chemistry of PS films was characterized by Fourier transform infrared spectroscopy (FTIR, Vertex-80 V/Hyperion2000, Bruker Inc.) and pendant drop tensiometer (DSA100, Krüss Inc.). The FTIR analyses were performed using the following settings: wavenumber range of 4000–600 cm<sup>-1</sup>, resolution 8, scan 32. Pendant drop tensiometer analyses were performed with the following parameters: substance - water, solid/probe - B1, and gas phase - air. At the end of the test, the washed PS films (0.05 g) were dissolved in THF solvent. Subsequently, the THF solvent containing the dissolved PS films was analyzed using gel permeation chromatography (GPC, Ultimate 3000, Thermo) [96].

## 2.7. Microscale morphological analysis of PS films and Mi-PS

Strains LG3 and SCGB1 were cultivated in LB broth for two days at 37 °C in an anaerobic chamber. The cells were washed three times using SW. Aliquots (0.1 mL) of the washed cell suspensions (10<sup>9</sup> CFU mL<sup>-1</sup>) were inoculated into 10 mL of fresh liquid FMM medium in 20 mL serum vials, which were then sealed with butyl rubber stoppers. Each serum vial contained 0.075% (w v<sup>-1</sup>) PS film sheets or Mi-PS with a diameter of 1 μm. The sterile control was added sterile SW instead of cell suspensions. After 20 and 30 days of incubation, the morphologies of PS materials and bacterial cells were confirmed using a field-emission scanning electron microscope (FE-SEM, SUPRA 55VP, ZEISS).

## 2.8. Solid-phase microextraction gas chromatography (SPME/GC—MS) mass spectrometry analysis

Triplicate bottles were prepared for each sample, with each bottle containing 10 mL of FMM supplemented with 0.075% Mi-PS. The cell suspensions with a concentration of 10<sup>9</sup> CFU mL<sup>-1</sup> were resuspended three times in SW. Aliquots (0.1 mL) of washed cell suspension (10<sup>9</sup> CFU mL<sup>-1</sup>) were inoculated into the FMM solution containing 0.075% Mi-PS. The bottles were incubated for 10 days at 37 °C in shaker incubators (120 rpm) or stationary incubators, under either aerobic or anaerobic conditions as described previously. The incubated solution was detected using the SPME/GC—MS method [71]. Chromatographic analyses were performed using a Thermo TRACE 1310/TSQ 8000 gas chromatography & mass spectrometer equipped with a DB-Wax (122–7063, Agilent) capillary column. Pure helium gas (He, 99.999%) was used as the carrier gas. The GC conditions were as follows: the sample (1.6 mL) was injected to inlet at 250 °C in split mode (10:1) under a flow rate of 20 mL min<sup>-1</sup>, the GC oven temperature was programmed to hold at 40 °C for 2 min, then to increase from 40 °C to 150 °C at 4 °C min<sup>-1</sup>, then to hold at 150 °C for 10 min, then to increase from 150 °C to 200 °C at 4 °C min<sup>-1</sup>, then to hold at 200 °C for 5 min, then to increase from 200 °C to 230 °C at 4 °C min<sup>-1</sup>; then to hold at 230 for 5 min. The 1,2,3-Trichloropropane was used as the internal standard for quantitative analysis. The spectral clusters were identified by searching against the NIST/EPA/NIH Mass spectral library (v. 2.4 g) using the NIST mass spectral search program.

## 2.9. Bacterial-generated methane (CH<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>) concentration analysis

The CH<sub>4</sub> and CO<sub>2</sub> concentrations were detected using gas chromatography (GC, ChroZen GC System, Youngin Chromass) equipped with a PDD detector and a continuous column of molecular sieve 13X (13047-U) and Porapak-N (13052-U). Aliquots (0.1 mL) of cell suspension (10<sup>9</sup> CFU mL<sup>-1</sup>) were inoculated into serum vials containing FMM medium with 0.075% (w v<sup>-1</sup>) Mi-PS, following the procedure described above. The GC equipped with a PDD detector had a detection limit (DL = 3σ/S) of 743 ppm for CO<sub>2</sub> and 397 ppm for CH<sub>4</sub>. Triplicate samples were tested at incubation periods of 0, 2, 5, 10, and 20 days at 37 °C in anaerobic conditions. For the zero-day incubation, samples were collected after 3 h of inoculation. Aliquots (250 μL) of headspace were collected using a gastight syringe (1750 RN) and injected into the GC inlet. Pure He was employed as the carrier gas. Standard curves for CH<sub>4</sub> and CO<sub>2</sub> were constructed using a standard gas mixture consisting of known concentrations of the gases dissolved in pure He as the background gas. The inlet temperature was 150 °C; the detector was at 220 °C; the oven was programmed to hold at 40 °C for 5 min, then to increase from 40 °C to 220 °C at 15 °C min<sup>-1</sup>, then to hold at 220 °C for 5 min. The retention time for H<sub>2</sub> was 2.630 min; N<sub>2</sub>, 4.267 min, CH<sub>4</sub>, 9.243 min; CO<sub>2</sub>, 12.052 min

## 2.10. Whole genome sequence analysis

The strain LG3 was grown on 1.5% LB agar for 2 days at 37 °C. A single colony from the plate was transferred into a 1.5 mL microcentrifuge tube containing 1 mL SW. Genomic DNA (gDNA) extraction was performed using the HiGene™ Genomic DNA prep kit for Bacterium (GD262–060). The obtained gDNA was processed according to the Oxford Nanopore Technologies (ONT) protocol (SQK-LSK109) for ligation sequencing and purification [34,76]. The gDNA library of strain LG3 was sequenced in an R9.4.1 flow cell on a MinION Mk1B instrument provided by ONT. Bacterial whole genome analysis was based on a constructed pipeline ([Supplementary Fig. 11A](#)). The reads with a size of at least 1000 bp were assembled into a single genome using the Flye (v\_2.9.1) assembler. The single genome was annotated using the Prokka (v\_1.13.4) gene prediction method through automated computational analysis. The detailed command codes used for whole genome analysis

can be found in the supplementary file for nanopore sequencing analysis. A sequence alignment was generated to find the genes of biodegradable PE and PS in the LG3 genome with MEGA X (v.10.2.1) using ClustalW with default settings. Nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/>) was utilized for identity calculation between the aligned sequences. Circular maps were drawn with proksee of the CGView Server (<https://proksee.ca/>). The complete genome of LG3 has been deposited in NCBI. The complete genome of LG3 has been deposited in NCBI. The GenBank accession numbers are CP118279.1. The accession numbers for the BioProject and BioSample are PRJNA934675 and SAMN33277205, respectively. The raw sequence has been submitted to the Sequence Read Archive (SRA) under the accession number SRR23339334.

### 2.11. Transcriptomic analysis

Strain LG3 was incubated on LB agar for 2 days at 37 °C. A single colony from the plate was transferred into a 1.5 mL microcentrifuge tube that contained 1 mL SW and vortexed for 1 min. Aliquots (10 µL) of the cell suspension were inoculated either on a petri dish containing 20 mL of FMM medium with 1 µm microspheres polystyrene (0.075%, w v<sup>-1</sup>) or on FMM agar containing maltose monohydrate (0.075%, w v<sup>-1</sup>). After a 10-day incubation at 37 °C in an anaerobic jar, the colonies were harvested and immediately combined with TRIzol reagent (Invitrogen). RNA extraction was performed using a RNeasy Mini kit (74104, Qiagen). The quality of the RNA was assessed using a SpectraMax ABS Plus instrument (Molecular Devices). Total RNA was quantified using a Qubit 4 fluorometer (Invitrogen) with the Qubit RNA HS Assay kit (Invitrogen) following the manufacturer's instructions. The extracted total RNA was reverse transcribed and amplified using Eppendorf Mastercycler Pro Thermal Cyclers (EP950040025, Eppendorf) and a cDNA-PCR Sequencing Kit (SQK-PCB109, ONT). The cDNA library of strain LG3 was loaded into an R9.4.1 flow cell and sequenced on a MINION Mk1B provided by ONT. Bacterial total RNA analysis was performed using a constructed pipeline (Supplementary Fig. 11B). The reads with a size of at least 200 bp and above Q10 were retained during basecalling (Option: FLO-MIN106/FLO-FLG001 DNA – super – accurate). The Salmon (v.1.9.0) and Prokka tools were utilized to perform quasimapping of sequencing reads for gene expression quantification. Detailed command codes for this analysis can be found in the supplementary nanopore sequencing analysis file. The raw transcriptome sequencing data has been deposited in NCBI under the SRA, and the data accession number is SRR23339333.

### 2.12. Statistical analysis

If not specified otherwise, all values are presented as the mean ± Standard Deviation (SD) and were derived from three or more experiments. GraphPad Prism 9 software was used to statistically analyze the majority of experimental data by one-way ANOVA. Multiple comparisons were performed using Tukey's test, and the results were considered statistically significant at  $p < 0.05$ . Multiple t tests using the two-stage step-up method were applied to analyze the results from SPME/GC–MS. The significance levels are indicated by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ ).

## 3. Results

### 3.1. Enrichment culture and screening for anaerobic PS-degrading isolates

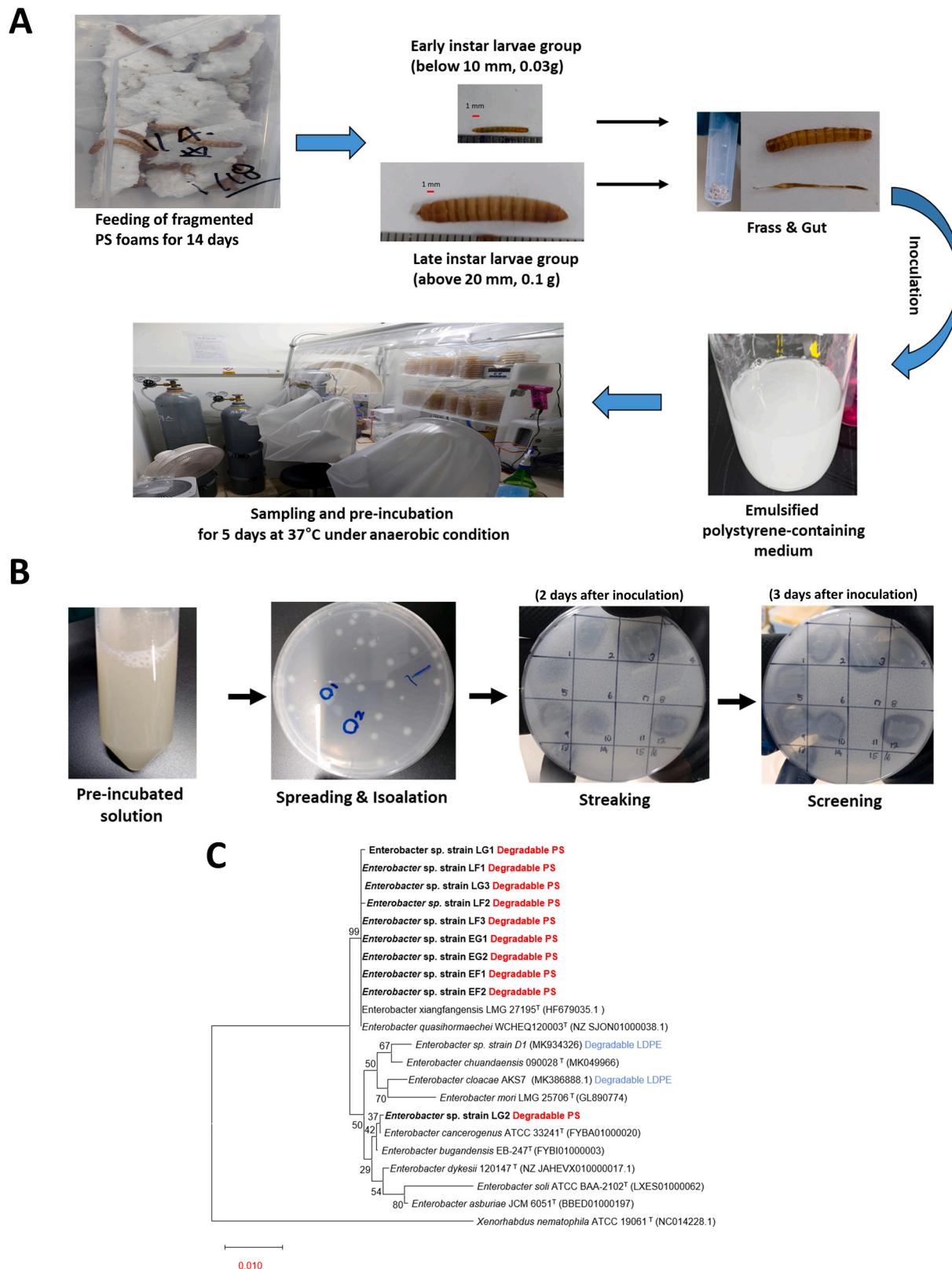
Enrichment culture procedures were employed, to isolate anaerobic PS-degrading bacteria from the gut and frass specimens of mealworms fed with PS foams (Fig. 1A). The isolates were incubated on emulsified PS (ePS)-containing FMM agar plates, where certain bacterial strains form distinct clear zones around colonies under anaerobic conditions (Fig. 1B). Strains unable to degrade ePS under anaerobic conditions

either do not form colonies on ePS-containing FMM agar plates or, if they do, fail to produce a clear zone (Supplementary Fig. S5A). However, certain strains capable of ePS degradation under anaerobic conditions expand the size of the clear zone around the colonies as they grow, resulting in the formation of a distinct clear zone by day 3 of incubation (Supplementary Fig. S5B). Additionally, these strains showed ePS degradation activity not only under anaerobic conditions but also under aerobic conditions (Supplementary Fig. S6A and S6B, and Supplementary Table 3).

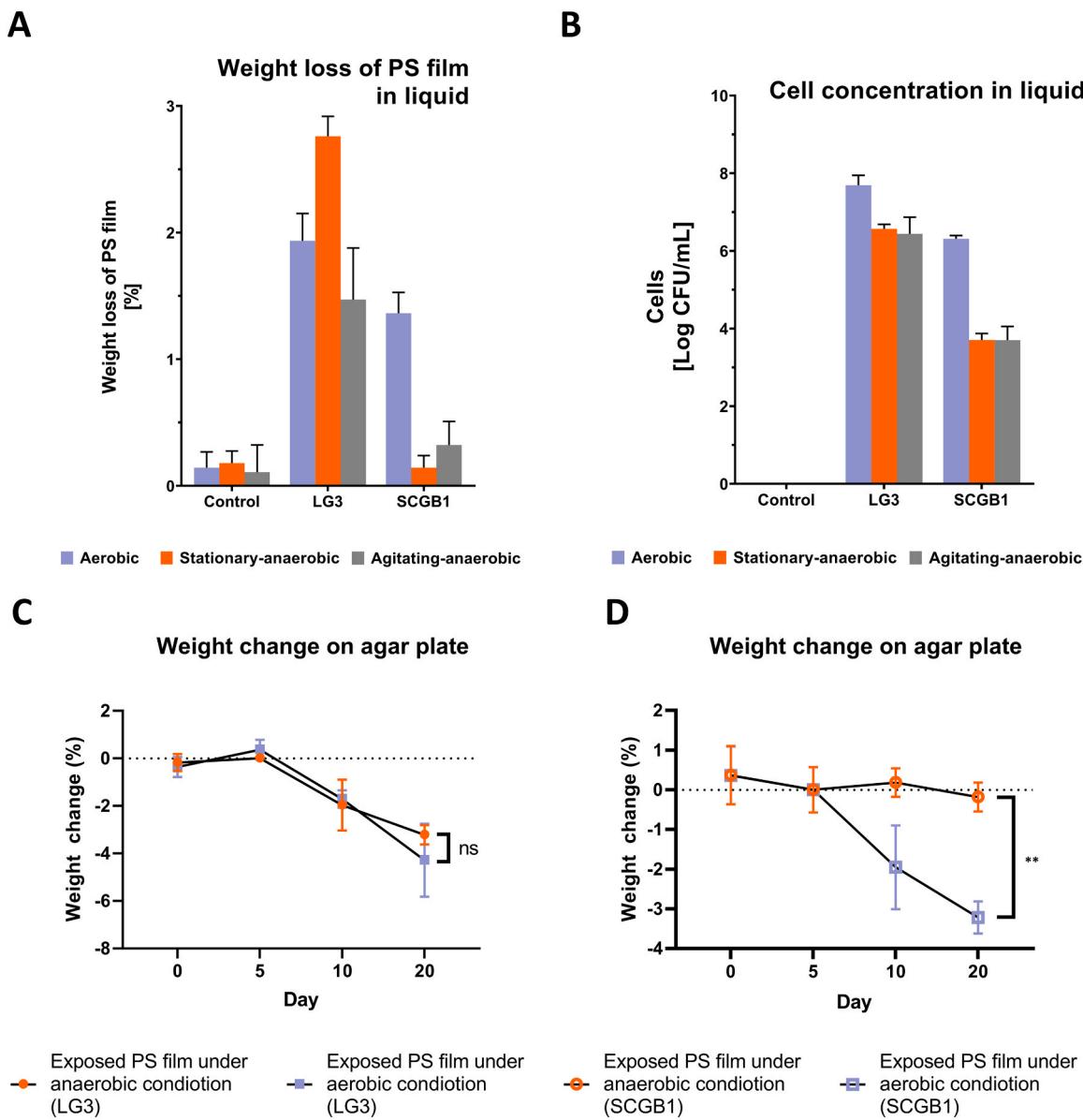
Subsequently, six strains (LF1, LF2, LF3, LG1, LG2, LG3) from the late-instar larvae group and four strains (EF1, EF2, EG1, EG2) from the early-instar larvae group were selected for identification. Based on the phylogenetic analysis using sequence of 16 S rRNA, these strains were classified within the *Enterobacter* genus (Fig. 1C, and Supplementary Table 2). The facultative PS-degrading isolates obtained in this experiment were identified as the same species as previously reported bacteria, with their 16 S rRNA sequences showing a sequence identity of  $\geq 98.7\%$  to *Enterobacter cancerogenus*, *Enterobacter quasihormaechei*, or *Enterobacter hormaechei* subsp. *Xiangfangensis* [16,48,80,89]. Among the isolated strains, *Enterobacter* sp. LG3, which showed a high sequence similarity to *Enterobacter hormaechei* based on the 16 S rRNA sequence, formed colonies with a diameter of  $12.30 \pm 0.30$  mm and clear zones with a diameter of  $23.73 \pm 0.84$  mm on ePS-containing FMM agar plate after 120 h of incubation under anaerobic conditions (Supplementary Table 3). The strain LG3 exhibited the highest ePS-degrading activity under anaerobic conditions compared to other isolated strains. Considering that strain LG3 has high anaerobic PS-degrading activity in the gut of late-instar PS-fed larvae, further research was conducted to investigate bioreactions for PS.

### 3.2. Weight loss of PS film by PS-degraders under aerobic and anaerobic conditions

The gravimetric determination of weight loss is widely used as an indicator to assess biodegradation. Wu, Criddle [94]. A comparative analysis was conducted to compare the PS degradation activities between strain LG3, which exhibited the highest activity among the PS-degraders isolated from the mealworm gut, and strain SCGB1, a bacterium belonging to the same species as *Bacillus amyloliquefaciens* and reported as both a PE- and PS-degrading organism [26,92]. To evaluate the PS degradation capabilities of the two strains, changes in the PS film weight were examined under aerobic and anaerobic conditions in FMM medium. Aerobic conditions were maintained using a shaking incubator (120 rpm), while anaerobic conditions were established by incubating the samples either in a shaking incubator (120 rpm) or a stationary incubator for a duration of 15 days (Supplementary Fig. S4). Strain LG3 exhibited significant weight reduction of PS films under all conditions compared to the sterile control (Fig. 2A). Specifically, under stationary-anaerobic conditions, LG3 displayed the greatest weight reduction of  $2.76 \pm 0.22\%$ , followed by a reduction of  $1.94 \pm 0.18\%$  under aerobic conditions, and  $1.47 \pm 0.33\%$  under agitating-anaerobic conditions. In the case of SCGB1, it showed a significant weight reduction of  $1.36 \pm 0.13\%$  of the PS film only under aerobic conditions, indicating a significant difference compared to the control. However, no significant differences were observed for SCGB1 under any of the anaerobic conditions (Fig. 2A). When measuring the CFU concentration of these PS-degraders utilizing the PS film as a carbon source, both LG3 and SCGB1 exhibited the highest concentrations under aerobic conditions (Fig. 2B). Moreover, similar trends were observed on FMM agar plates with PS film, where LG3 was able to degrade the PS film under both aerobic and anaerobic conditions (Fig. 2C). The PS films exposed to SCGB1 exhibited a significant weight difference between aerobic and anaerobic conditions (Fig. 2D). The results demonstrated that strain LG3 is capable of biodegrading PS under both anaerobic and aerobic conditions while strain SCGB1 can effectively degrade PS only under aerobic conditions as expected.



**Fig. 1.** Plastic-degrading bacteria screening and identification. (A) Scheme of enrichment culture for PS-degrading isolates. Breeding conditions are temperature: 25 °C, humidity: 60%. The vinyl anaerobic chamber conditions were as follows: temperature, 37 °C; humidity, 40%. (B) Scheme of isolation of anaerobic polystyrene-degrading microorganisms. Culture medium: LB medium, screening medium: ePS-containing FMM medium. (C) Neighbor-joining phylogenetic tree based on 16 S rRNA gene sequences showing the phylogenetic relationships of plastic-degrading isolates and related taxa. The numbers around the nodes are the confidence levels (%) generated from 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. *Enterobacter* sp. strain D1 (MK934326) and *Enterobacter* cloacae AKS7 (MK386888.1), which belong to the same genus as the isolates from the late-instar PS-fed larval gut, have been reported to have the ability to degrade PE as a carbon source for cell growth under aerobic condition [61,65].



**Fig. 2.** Analysis of mass reduction rate under aerobic and anaerobic conditions. (A-B) Approximately  $10^7$  CFU of incubated bacterial cell suspension was inoculated into FMM medium. The FMM medium (20 mL) with three pieces of crumpled PS film (93 mg) was capped with an air-permeable silicone rubber cap or a sealed butyl cap. Aerobic and agitating-anaerobic samples were incubated in shaker incubator (120 rpm) at 37 °C. Stationary-anaerobic sample was incubated in stationary incubator at 37 °C. The control group refers to a sterile condition where all processes were kept the same except for bacterial inoculation. (A) Mass reduction rate in FMM medium (mean value  $\pm$  SD, n = 3). (B) Cell density in FMM medium when utilizing PS film as a carbon source (mean value  $\pm$  SD, n = 3). (C-D) Approximately  $10^7$  CFU of incubated bacterial cell suspension was directly exposed to the PS film ( $2 \times 2$  cm) on FMM agar medium and incubated for 20 days. (C) Changes in the weight of the PS films after exposure to *E. hormaechei* LG3 or (D) *B. amyloliquefaciens* SCGB1 (mean value  $\pm$  SD, n = 4). The ns mark indicates that there is no significance.

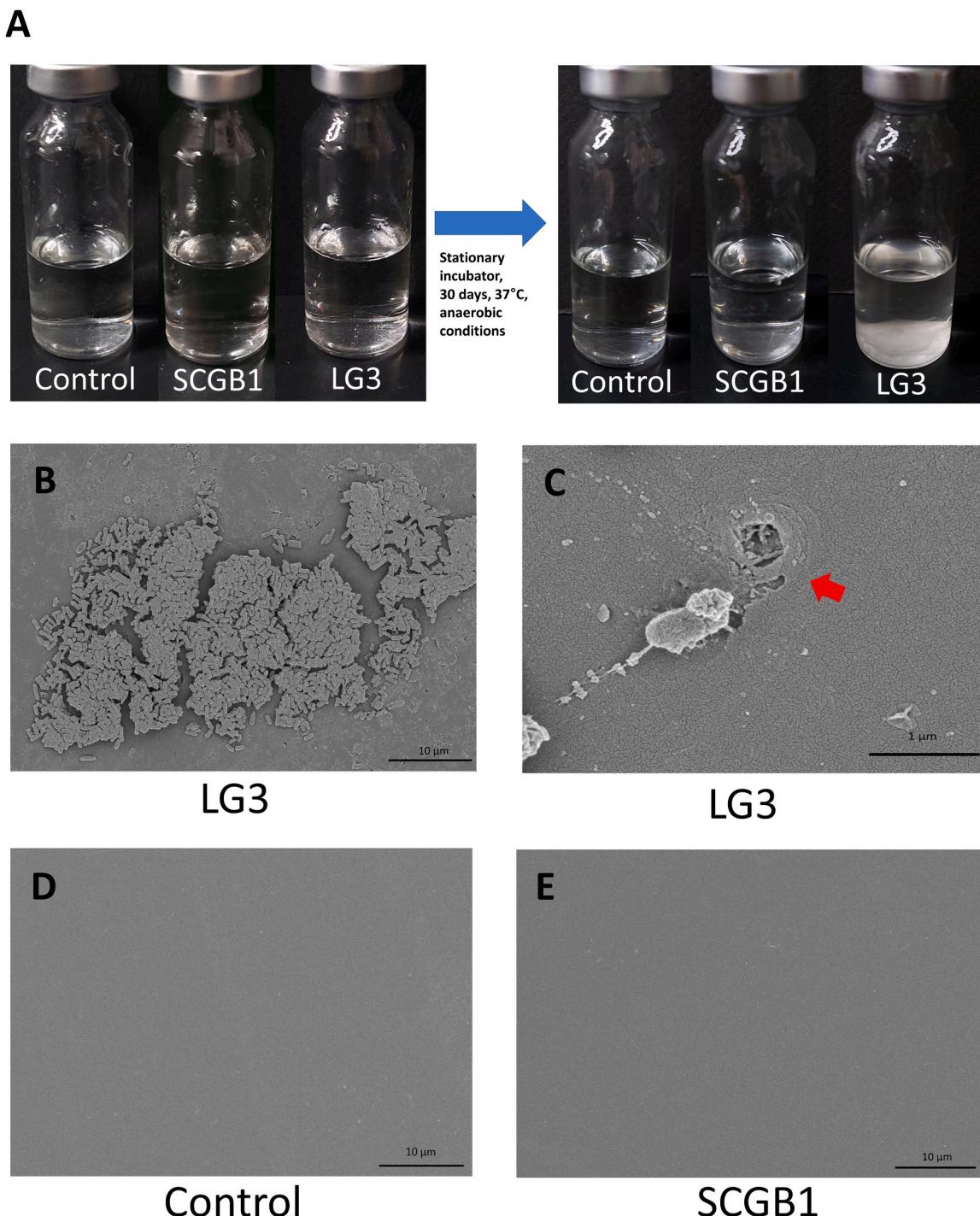
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### 3.3. *Enterobacter* sp. LG3 forms biofilms and exhibits activity on the PS film under anaerobic conditions

After 30 days of anaerobic microbial culture with the PS films, strain LG3 generated a noticeable microbial community at the periphery of the PS films (Fig. 3A). The cells of strain LG3 were observed on the surface of the PS films using field-emission scanning electron microscopy (FE-SEM), and they formed biofilms via intercellular attachment using appendages (Fig. 3B). In particular, the appendages of strain LG3 adhered to the PS film surface, and cavities due to biodegradation were also detected at the periphery of the attachment site (Fig. 3C). These types of cavities were not observed in the PS films exposed to *B. amyloliquefaciens* and the sterile control under anaerobic conditions (Figs. 3D and 3E).

### 3.4. Comparison of cell activities as PS-degraders under anaerobic conditions

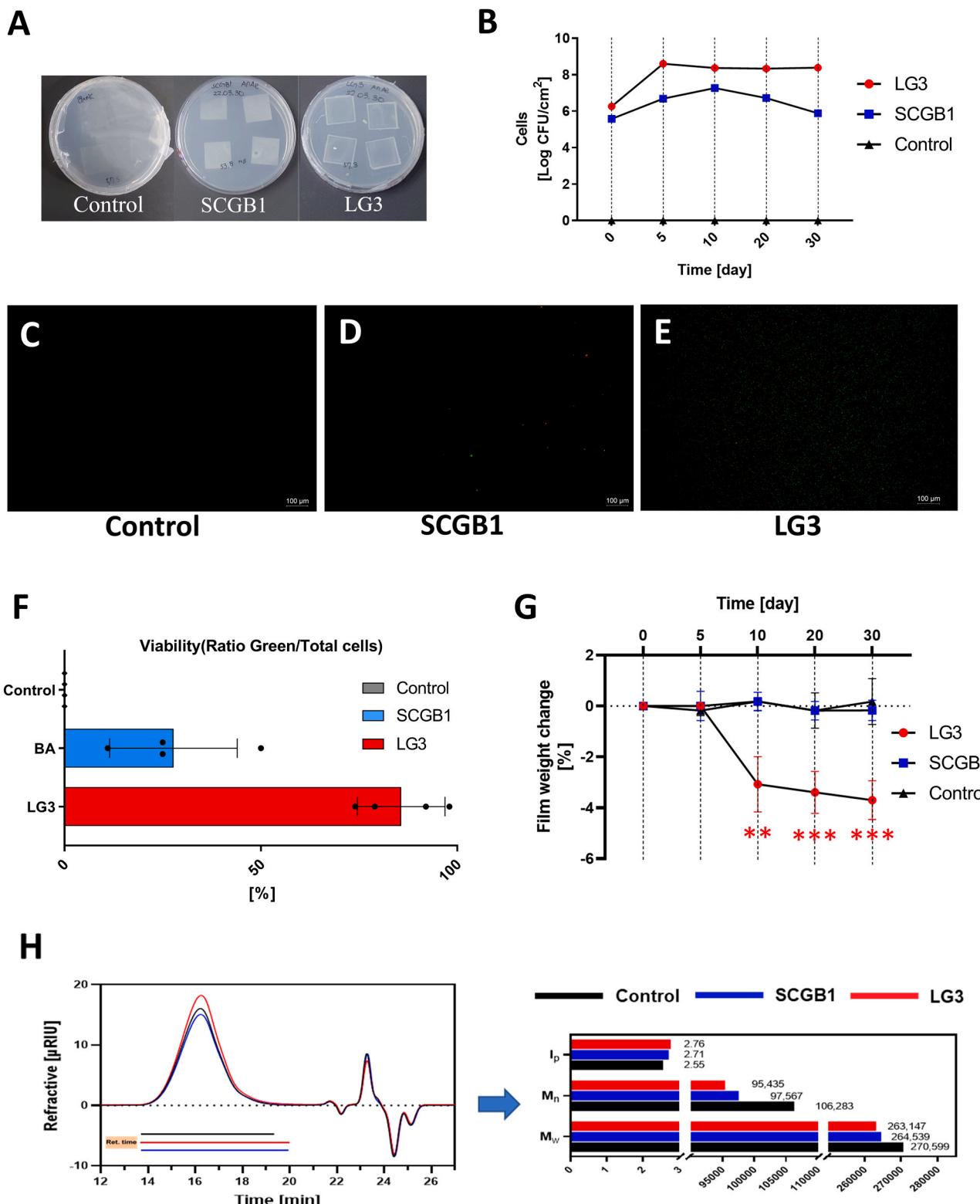
To assess the activity of an anaerobic PS-degrader, a homogenized bacterial cell suspension was directly exposed to PS films on FMM agar medium, and changes such as cell adhesion, viability, and PS film weight were observed. Strain LG3 formed white colonies in the space between the FMM agar medium and the PS film (Fig. 4A). When assessing the cell adhesion activity on the PS films, *Enterobacter* sp. LG3 peaked on the 5th day and then gradually decreased, demonstrating a 2.39% decrease from the peak by the 30th day (Fig. 4B). On the other hand, *B. amyloliquefaciens* SCGB1 reached the highest concentration on the 10th day and then displayed a strong inflection toward a decrease,



**Fig. 3.** Microbial growth on PS film and biodegradation of PS film by strain LG3. The FMM medium (10 mL) with PS films ( $20 \times 20 \times 0.3$  mm) was sealed with a butyl rubber stopper at  $37^\circ\text{C}$  under anaerobic conditions in stationary incubator. (A) Changes in the incubation bottle containing PS films. The formation of biofilm was shown in the FMM liquid medium inoculated with LG3. (B-E) Morphology of bacterial cells and PS film was observed using FE-SEM.

representing a 14.06% decrease from the peak by the 30th day (Fig. 4B). After the 30-day incubation, each biofilm was observed by staining live and dead bacterial cells (Fig. 4C-4E). The ratio of green-stained live cells was substantially higher in the LG3 samples (Fig. 4F). Next, the biofilm was removed from the PS film to analyze the unobscured PS film weight. For the PS films exposed to strain LG3 under anaerobic conditions, a significant weight change was observed, resulting in a net weight change of  $-3.82 \pm 0.77\%$  by Day 30 (Fig. 4G). These weight changes were not observed in the PS films exposed to *B. amyloliquefaciens* (Fig. 4G). The GPC analysis provided information on the molecular weight

characteristics of these PS films (Fig. 4H). The weight average molecular weight ( $M_w$ ) of the PS films exposed to strain LG3 and strain SCGB1 was 263,100 Da and 264,500 Da, respectively, which were 2.9% and 2.2% smaller than that of the sterile control. The number average molecular weight ( $M_n$ ) of the PS films exposed to strain LG3 and strain SCGB1 was measured at 95,400 Da and 97,600 Da, respectively, which represent a 10.3% and 8.2% reduction from the sterile control. The high polydispersity index ( $I_p$ ) of PS samples indicates an increased breadth of the residual PS polymers after biodegradation by LG3 cells. The cell adhesion, viability, and PS film weight change suggest that strain LG3



**Fig. 4.** Comparison of activities as a facultative PS-degrader. (A-H) Approximately  $10^7$  CFU of incubated bacterial cell suspension was directly exposed to the PS film ( $2 \times 2$  cm) on FMM agar medium and incubated for 30 days at  $37^\circ\text{C}$ . (A) Formation of colonies on PS film sheets in FMM plates under anaerobic conditions. No colony was present on the sterile control. (B) Changes in bacterial cell number on the PS film (mean value  $\pm$  SD, n = 3). (C-D) Fluorescence microscopic images of biofilms. Live cells are green, and dead cells are red. (F) Cell viability after the 30-day incubation. The ratio of green-stained cells among all observed cells was  $27.8 \pm 16.3\%$  for BA, while the rate for LG3 was  $85.8 \pm 11.1\%$  (mean value  $\pm$  SD, n = 4). (G) Changes in the dry weight of the PS films over the 30-day period (mean value  $\pm$  SD, n = 4). The significance levels compared with the sterile control are indicated by asterisks (\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001). (H) Molecular weight distribution and molecular weight of PS film (Ret. time: retention time, I<sub>p</sub>: polydispersity index, Mn: number-averaged molecular weight, Mw: weight-averaged molecular weight).

Source data are provided as a Source Data file.

degrades PS, acquiring energy to maintain physiological activity under anaerobic conditions [25,95].

### 3.5. Comparison of PS oxidation between LG3 and SCGB1 under anaerobic conditions

FTIR analysis confirmed the oxidation profile and revealed distinct physicochemical characteristics in PS films directly exposed to each strain under anaerobic conditions (Fig. 5A). All samples exhibited FTIR peaks at approximately 694.61 and 725.18 cm<sup>-1</sup>, which represented ring-bending vibrations of aromatic rings [22,27]. However, the LG3 sample had the weakest peak, which indicates that strain LG3 effectively destroyed the benzene ring of PS (Fig. 5A). Various peaks indicating PS oxidation, including alkyl/aryl structures, were observed in the PS film exposed to LG3 or SCGB1 under anaerobic conditions. The distinct peak at around 832.05 cm<sup>-1</sup> refers to the organic O-O peroxide bond [50,87]. Additionally, other distinct peaks were observed, such as the peak at around 1020.81 cm<sup>-1</sup> and 1076.48 cm<sup>-1</sup>, which were assigned to the C-O stretch of the ether bond [73,77], and the peak at 1217.85 cm<sup>-1</sup> was assigned to the C-O stretch of the ester bond [74,85]. Distinguishably, an O-H stretching peak at around 3464 cm<sup>-1</sup> was observed in the film exposed to SCGB1 under anaerobic conditions [35].

To evaluate changes in the hydrophobic properties of PS films exposed to PS-degraders under anaerobic conditions, water contact angle (WCA) measurements were conducted (Supplementary Fig. S7). Initially, the pristine PS film exhibited a WCA of 87.21 ± 2.47°. After a 30-day treatment, both samples exposed to LG3 or SCGB1 showed a significant decrease in hydrophobicity compared to the sterile control (Fig. 5B). The WCA of the PS films exposed to LG3 and SCGB1 measured as 71.48 ± 4.16° and 69.1 ± 4.55°, respectively, indicating a significant increase in hydrophilicity compared to the sterile control with a WCA of 85.72 ± 1.44°. The increased hydrophilicity suggests a higher chemical reactivity of the PS structure [78]. However, there was no significant difference in the WCA of the PS films between LG3 and SCGB1. Collectively, the formation of functional groups and the decrease in hydrophobicity indicate that both strains, *Enterobacter* sp. LG3 and *Bacillus amyloliquefaciens* SCGB1, induce PS oxidation under anaerobic conditions [35,64].

### 3.6. Comparison of PS metabolites between LG3 and SCGB1 under both aerobic and anaerobic conditions

To confirm the difference in the metabolites produced during PS degradation, microspheres polystyrene (Mi-PS) were exposed to the bacterial strains in a stationary incubator under anaerobic conditions, and the metabolic byproducts generated were analyzed to assess the ability to break down oxidized PS molecules into smaller molecules (Supplementary Fig. S8). The bacterial incubated solution with Mi-PS was analyzed by SPME/GC—MS and identified by the NIST/EPA/NIH library for untargeted compounds. Chromatograms represented specific elution peaks indicating aromatic alcohols and aliphatic alcohols (Fig. 6A). The specimens incubated with strain LG3 exhibited significant increases in the amounts of aromatic alcohols and aliphatic alcohols, including isoamyl alcohol, methyl 2-hydroxybenzoate, and 2-phenylethanol, compared to the sterile control as well as SCGB1 (Fig. 6B and 6C). Additionally, comparative experiments were conducted under both aerobic and anaerobic conditions in a shaking incubator (120 rpm) to examine the metabolic changes depending on the presence of oxygen gas (Supplementary Fig. S9). Differences in substance quantities were observed for LG3 between aerobic and anaerobic conditions; however, all substances detected under aerobic conditions were also detectable under anaerobic conditions. SCGB1 showed a different PS degradation pathway, with specific intermediates (isobutyric acid and isovaleric acid) detected only under aerobic conditions. Additionally, 2-phenylethanol, which was not found under anaerobic conditions, was detected under aerobic conditions.

Next, to measure the amount of CO<sub>2</sub>, the final metabolite of PS, GC analysis was conducted over a 20-day period under anaerobic conditions [33,98]. The concentration of CO<sub>2</sub> in the headspace of LG3 showed a rapid increase, resulting in a significant difference by day 20 (Fig. 6D and Supplementary Fig. S10). The headspace of the bottle containing LG3 exhibited a CO<sub>2</sub> concentration of 2.27 ± 0.13% on day 0, which increased to 4.08 ± 0.34% by day 20. Finally, the interaction between Mi-PS and bacteria under anaerobic conditions was visualized using FE-SEM (Fig. 6E–6 G). Identical 1 μm spherical beads were observed in the sterile control (Fig. 6E), whereas microsphere beads incubated with strain LG3 showed rough and dented surfaces due to PS biodegradation (Fig. 6F). Microsphere beads incubated with SCGB1 were not degraded, but disrupted cells and fragments were observed (Fig. 6G). These comparative experiments demonstrated that LG3 exhibited more effective depolymerization of oxidized PS than SCGB1 under anaerobic conditions.

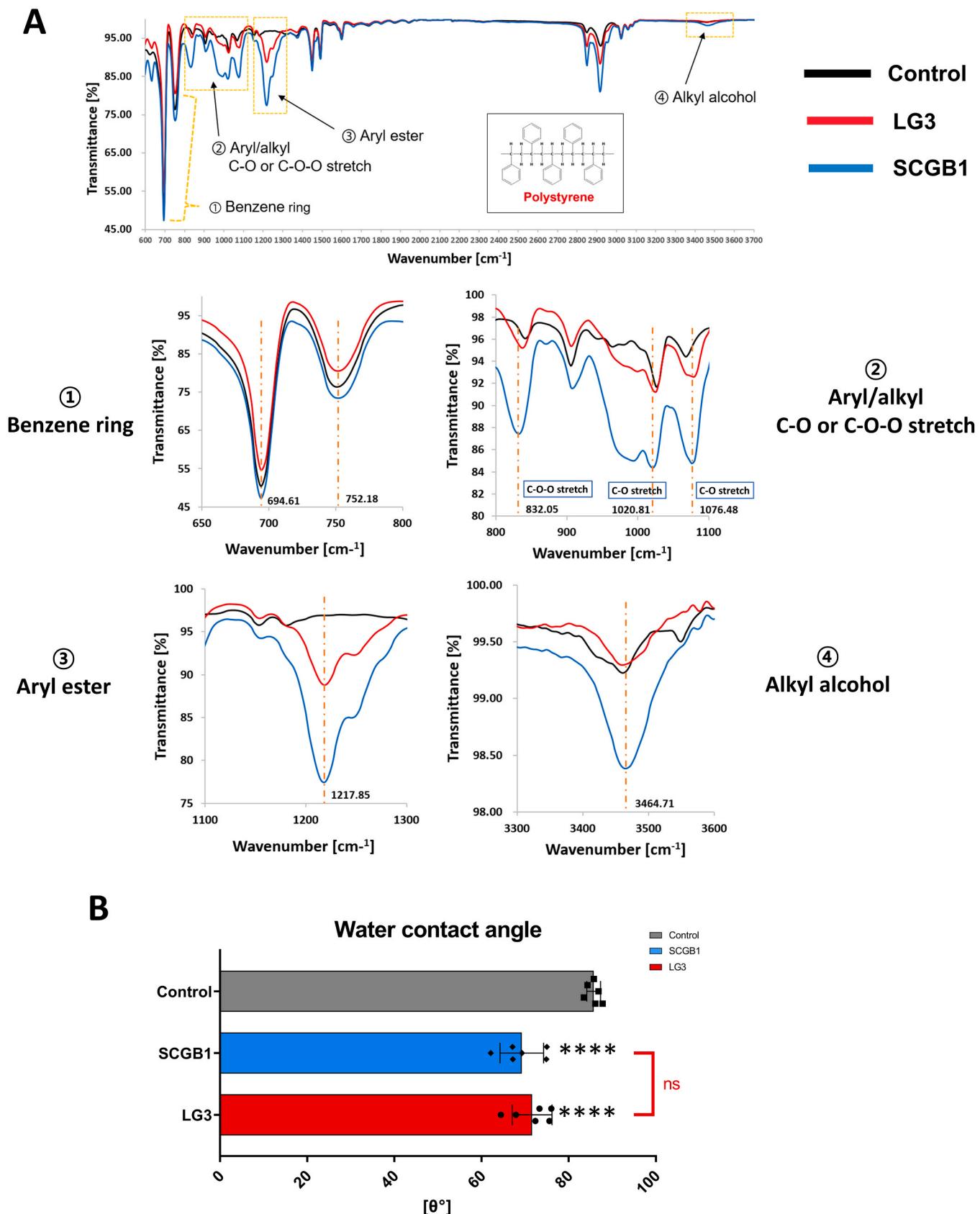
### 3.7. Genomic analysis of strain LG3 when metabolizing Mi-PS under anaerobic conditions

To identify the key enzymes involved in the cleavage of PS under anaerobic conditions in the LG3 genome, we utilized nanopore sequencing [90]. The bioinformatics methods were employed to exploit reads according to constructed pipelines (Supplementary Fig. S11A and S11B). A genome sequence-based analysis confirmed that strain LG3 is the same species as *Enterobacter hormaechei* (Supplementary Fig. S12A–C). The single circular genome, yielding 4514,131 bp with 55.54% GC content, was used to construct a genome map with potential PS-degrading enzyme genes (Fig. 7A). In addition, transcriptomes of LG3 using Mi-PS or maltose as carbon sources were sequenced to identify the enzymes related to PS biodegradation under anaerobic conditions (Supplementary Fig. S13 and S14). We selected specific enzymes reactive to aryl alcohol compounds among the potential PS-degrading enzymes and analyzed their expression levels (Fig. 7B). Peroxidase (*efeB*) and hydrolase (*ubi* genes) for aryl alcohol compounds were upregulated; however, oxidase (*yfiH*) and monooxygenase (*paa* genes) involved in aryl alcohol compounds were not upregulated when using Mi-PS. Next, differentially expressed genes were ranked regarding their fold change between Mi-PS and maltose (Supplementary Fig. S15A, B), and they were further categorized according to their functions (Fig. 7C, D and Supplementary Table S4 and S5). We observed differential expression of specific degradative enzymes as well as metabolic pathway enzymes (Fig. 7C, 7D).

After Mi-PS treatment, the expression levels of thiol peroxidase (*tpx*), alkyl hydroperoxide reductase C (*ahpC*), and bacterioferritin comigratory protein (*bcp*) genes, which belong to the same peroxidase family [10], were substantially increased (Fig. 7E), and enzymes involved in lipid A synthesis pathway were also upregulated (Fig. 7F and Supplementary Table S6). In contrast, when using Mi-PS instead of maltose, flagellar protein-coding genes such as Flagellar brake protein (*ycgR*), Flagellar L-ring protein (*flgH*), and Flagellar biosynthetic protein (*flip*) were downregulated (Fig. 7G), and enzymes in the glycolytic pathway showed downregulation (Fig. 7H and Supplementary Table S7).

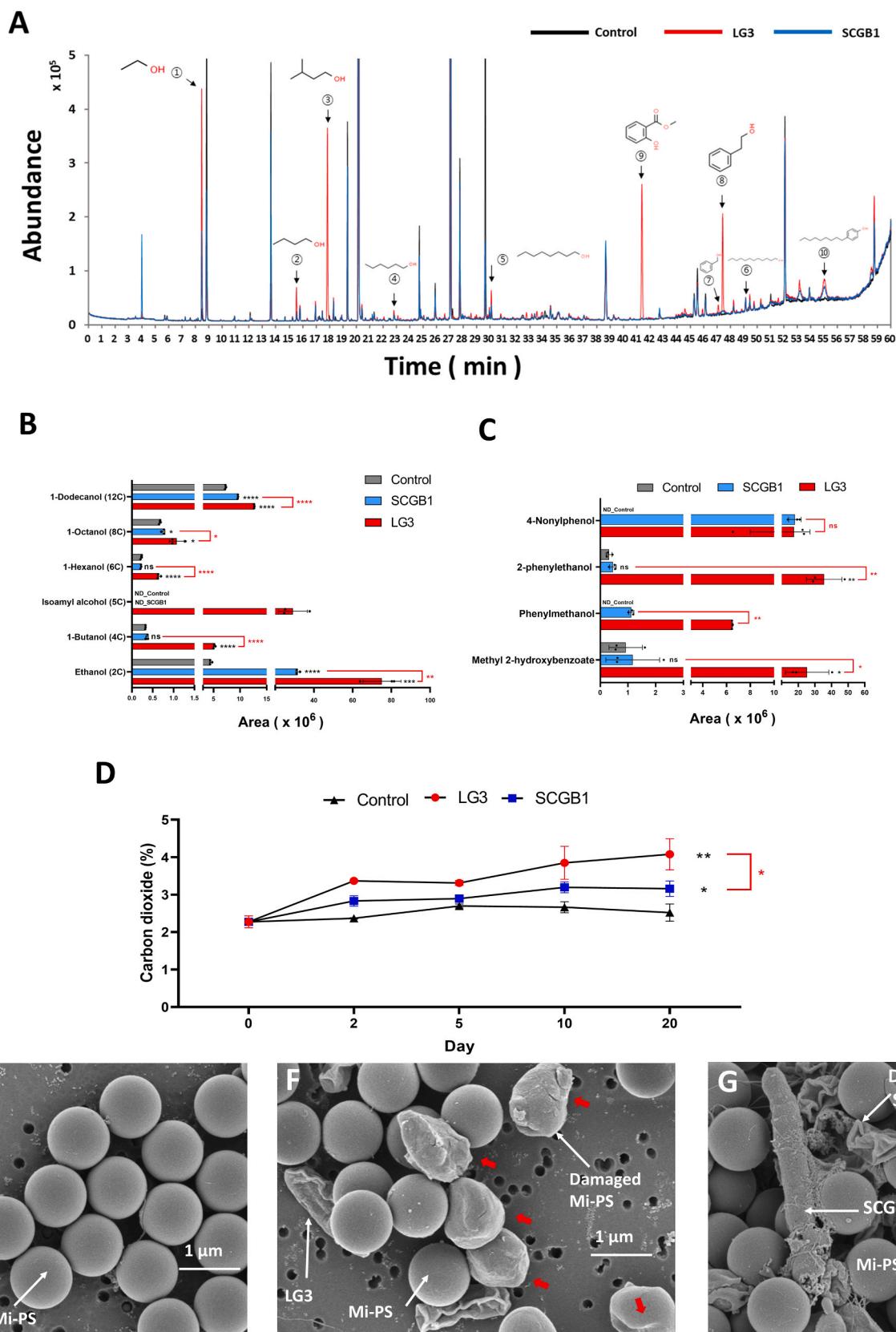
## 4. Discussion

In this study, we report that *E. hormaechei* LG3, isolated from the PS-fed mealworm gut, can effectively oxidize, and depolymerize PS under both anaerobic and aerobic conditions in comparison to *B. amyloliquefaciens* SCGB1. The facultative PS-degrader, strain LG3, exhibited remarkable biodegradation ability, forming a biofilm on the surface of various PS materials, including ePS, PS film, and Mi-PS, even in the absence of oxygen gas. Additionally, strain LG3 demonstrated efficient PS degradation under aerobic conditions as well. The PS materials resulted in noticeable morphological and physicochemical changes attributed to anaerobic biodegradation [25,86,99]. In addition,



**Fig. 5.** Oxidation of PS film exposed to strains. (A-B) Oxidation analysis of PS films after the 30-day incubation. (A) FTIR analysis of exposed PS film by strain. (B) When the PS film surface was exposed to strains, the WCA values were reduced compared to those of the sterile control. (Mean value  $\pm$  SD,  $n = 6$ ). The significance levels compared with the sterile control are indicated by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ ). The ns mark indicates that there is no significance.

Source data are provided as a Source Data file.



**Fig. 6.** Mi-PS degradation products and Mi-PS morphology. (A-G) The FMM medium, containing Mi-PS at a concentration of 0.075% ( $w\text{ v}^{-1}$ ), was sealed with a butyl rubber stopper and incubated at 37 °C under anaerobic conditions. (A-C) Identification of hydroxyl compounds via SPME/GC-MS analysis. (A) Chromatograms of PS treated with strains, indicating different compounds. (B, C) Increased abundances of aliphatic and aromatic alcohols were observed as degradation products (mean value  $\pm$  SD, n = 3). The ns mark indicates that there is no significance. (D)  $\text{CO}_2$  emission of strains and the sterile control (mean value  $\pm$  SD, n = 3). The significance levels compared to the sterile control at Day 20 are indicated by asterisks (\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001). (E-G) FE-SEM image of Mi-PS

and bacterial cells.

Source data are provided as a Source Data file.

through a comparison of the metabolic products under aerobic and anaerobic conditions, we confirmed that LG3 and SCGB1 exhibit differences in the process of PS metabolism depending on the presence of oxygen gas. In transcriptome and metabolite analyses, we observed the upregulation of degradative enzymes such as peroxidase and hydroxylase, along with the detection of corresponding depolymerized PS derivatives after anaerobic PS biodegradation [19]. Collectively, these results showed that *Enterobacter* LG3 effectively degrades PS under both anaerobic and aerobic conditions.

To date, there has been much controversy over the core bacteria involved in PS biodegradation in the mealworm gut microbiota because the isolated PS-degrading species are completely different in each experiment within the same host [20,30,84,86,99]. In this experiment, mealworm specimens were spatially and temporally divided (Supplementary Table S2), but all of the isolated strains with activity on PS under anaerobic conditions belonged to the *Enterobacter* genus [67,86,95]. *Enterobacter* spp. are mainly distributed in the oxygen-limited posterior gut section of mealworms [20,5,84,86], and when PS or PE is fed to mealworms, they increase their composition in the host gut microbiome [5,86]. The gradual decrease in oxygen levels that occurs when air-containing PS foam enter the oxygen-limited gut environment provides an advantage to *Enterobacter* spp., as they are capable of efficiently carrying out bioreactions under both aerobic and anaerobic conditions [20,84,86]. Collectively, *Enterobacter* spp. play an important role in anaerobic PS biodegradation in mealworms [67,86,95]. In addition, *Enterobacter* species, including *E. hormaechei*, which can widely exist not only in the gut of insects but also in the gut of marine fish, livestock, and even humans [53,57,72,88]. These results indicate that anaerobic degradation of PS can take place within the gut of various organisms. On the other hand, *B. amyloliquefaciens* SCGB1, another gut commensal bacterium, had significantly reduced PS degradation activity under anaerobic conditions. In the presence of oxygen gas, it produced specific metabolites, isobutyric acid and isovaleric acid, which are distinct from strain LG3. This indicates a difference in the mechanism for degrading PS between the two PS-degraders. The significant differences in PS activity and metabolism between LG3 and SCGB1 hold significant implications, indicating that the impact of biodegradation of PS on the host animal may also be different [7]. Further studies are needed to clarify the effects of bioreaction on Mi-PS by PS-degrading gut microbes in the actual intestinal environment of living organisms [68,69].

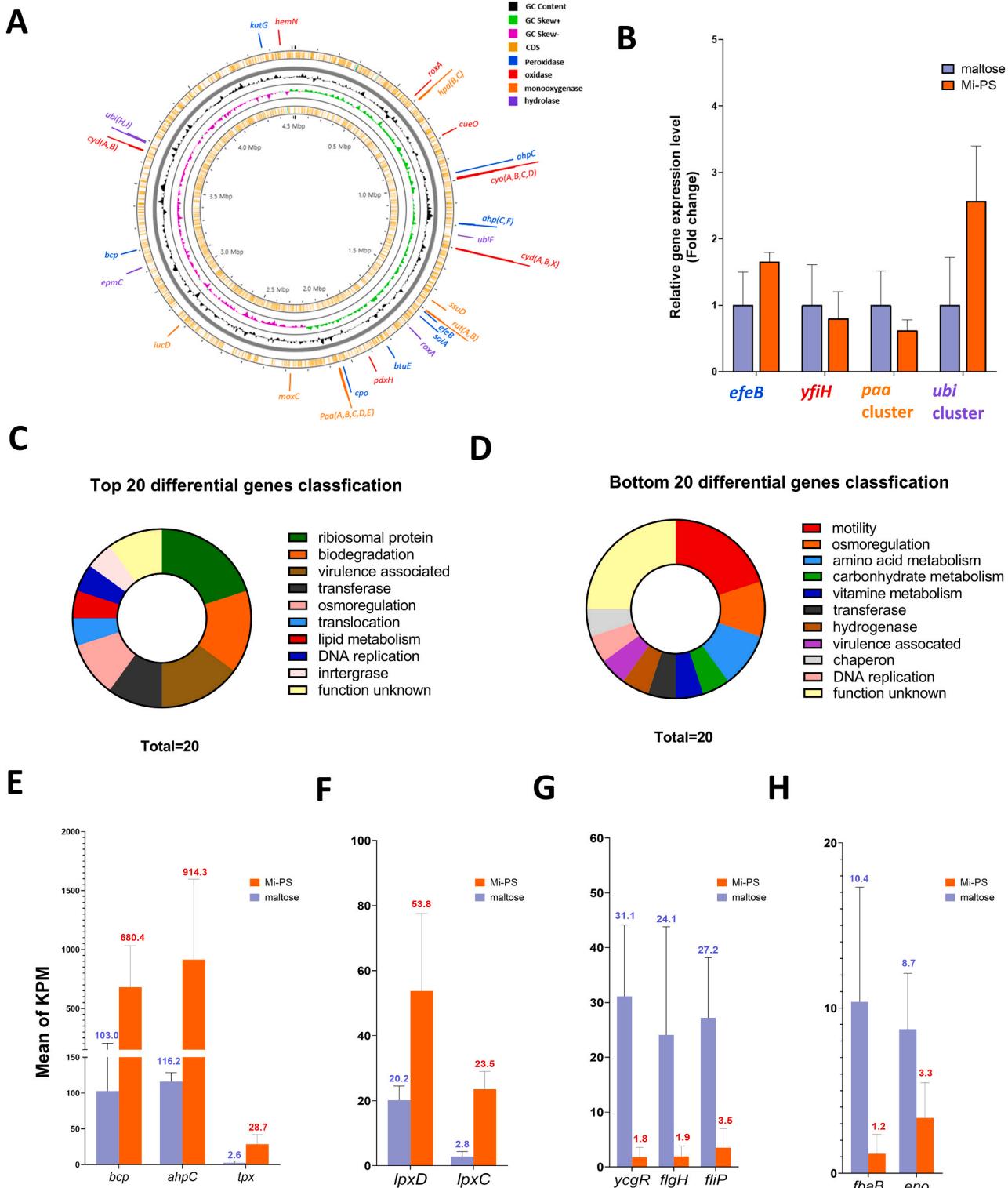
The two strains, LG3 and SCGB1, capable of degrading PS, showed a significant difference in the not only anaerobic but also aerobic biodegradation of PS. Regarding anaerobic oxidation, both strains oxidize the PS molecule, resulting in decreases in the WCA values. The WCA values were not significantly different between LG3 and SCGB1. Under anaerobic conditions, oxidizing chemically stable compounds such as benzene and methane requires sulfate ( $\text{SO}_4^{2-}$ ), nitrate ( $\text{NO}_3^-$ ), or nitrite ( $\text{NO}_2^-$ ) instead of oxygen ( $\text{O}_2$ ) [23,66,83]. Both PS-degraders are facultative anaerobic bacteria that can grow by means of anaerobic respiration using nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) as electron acceptors to create ATP through the respiratory electron transport process [17,29,41]. Furthermore, the expression of denitrification enzymes was observed in the transcriptome of strain LG3 exposed to Mi-PS (Supplementary Fig. S16), suggesting that anaerobic PS oxidation is coupled to microbial nitrate reduction [32,58]. However, LG3 produced more abundant secondary products of PS under anaerobic conditions, including 2-phenylethanol and methyl 2-hydroxybenzoate, which indicates that LG3 has superior degradative enzymes that effectively cleave oxidized PS to small molecules [25,61,95]. Peroxidase is known to be a degradative enzyme on oxidized PS [19]. The expression of *tpx*, *ahpC*, and *bcp* in the transcriptome of LG3 was substantially upregulated after treatment with Mi-PS, which are peroxiredoxins, and they have

activity against oxidized lipid molecules under anaerobic conditions [10]. The superior oxidized-PS depolymerization ability of strain LG3 contributed to acquiring sufficient resources and energy to maintain cell growth ability, adhesion ability and viability at high levels [25,61,95]. In conclusion, our results suggest that anaerobic PS biodegradation proceeds in two stages, oxidation and depolymerization, and that distinct enzymes, PS oxidative enzyme and oxidized-PS degradative enzyme, exist in the LG3 genome [17,19,23,29,41,44,60].

SPME/GC-MS was used to detect PS derivative molecules, such as aromatic and alkyl compounds containing hydroxyl groups, which could be produced by peroxidase and hydrolase reactions [19,102]. Strain LG3 showed that all aromatic alcohols and aliphatic alcohols were significantly increased compared to the sterile control under anaerobic conditions. Most *Enterobacter* species contain two anaerobic styrene biodegradation pathways: the 2-phenylethanol route and the 2-ethylphenol route [22,82]. The chromatogram of LG3 represented peaks of 2-phenylethanol and methyl 2-hydroxybenzoate, which are intermediates of the 2-ethylphenol route and route, 2-phenylethanol respectively, which means that the anaerobic biodegradation mechanisms of PS and styrene are partially shared [102]. Finally, LG3 produced  $\text{CO}_2$  as an end product arising from anaerobic biodegradation of PS [11,22,98].

RNA-seq analysis showed that PS-degradable bacterium, LG3 changed their physiological characteristics after treatment with Mi-PS. Downregulation of cell motility-associated proteins such as *ycgR*, *flgH* and *FlhA* provides an advantage in maintaining immobile microbial communities and biofilms [24], which is considered the crucial early step of microbe-mediated biodegradation of plastics [25,95]. Based on the observed decrease in PS film reduction rate under the agitating-anaerobic conditions, suggesting that stable binding is an important factor in anaerobic PS degradation. This trend was also observed when utilizing Mi-PS as a carbon source, and LG3 showed not only efficient Mi-PS degrader, but also effective Mi-PS flocculants by binding Mi-PS under stationary anaerobic conditions (Supplementary Fig. S17). Another noteworthy result from RNA-seq analysis is the observed changes in the lipid metabolism pathway (Supplementary Table S6) and accompanied upregulation of lipid A biosynthesis pathway enzymes, including *lpxC* and *lpxD* [13]. Lipid A is an essential toxic component in endotoxin of opportunistic pathogens such as *Enterobacter hormaechei* [3,70,79]. Collectively, the Mi-PS niche induces changes in metabolic pathways for PS metabolism and alters physiological activities, including biofilm and lipid A formation to LG3, gut commensal bacterium [25,52,95]. These findings indicate that PS-MPs themselves not only have negative effects on the host but also enhance toxicity of opportunistic pathogen [28,51,68].

This study provides evidence that anaerobic PS biodegradation can be achieved by microorganisms, offering potential applications in bioremediation fields such as wastewater treatment to remove small MPs, particularly in secondary wastewater treatment systems like anaerobic lagoon systems and aerobic lagoon systems [2,36,93]. Additionally, the presence of abandoned MPs in the ocean and soil poses threats to the health and life of living organisms [14,46,62]. Though a comprehensive understanding of the step-by-step mechanism of PS biodegradation is necessary for further enzymatic studies, our findings present a novel approach to addressing plastic pollution. By demonstrating the degradation of PS-MPs under anaerobic conditions - conditions which are prevalent in the majority of animal guts - we offer a new perspective on how to mitigate the impact of such pollutants. This study contributes to the exploration of PS-degrading gut commensal bacteria to mitigate the harmful effects of PS-MPs in host intestine. We further propose that the field of bioremediation be expanded to include the intestinal environment of living organisms. To date, most isolated



**Fig. 7.** Genetic characterization of anaerobic PS-degrading LG3. (A-H) Analysis of whole genome sequence data and genome map of strain LG3. (A) The single circular genome yields 4514,131 bp with a 55.54% GC content. A total of 4254 CDSs were identified using Prokka (v.1.v.9.0), and the genome encoded 39 potential PS-degrading enzymes, including peroxidase, oxidase, monooxygenase, and hydrolase genes. (B) Relative expression (fold change) values between maltose and Mi-PS as carbon sources. The enzymes have activity on aryl alcohol compounds (mean value  $\pm$  SEM, n = 3), which are guaiacol peroxidase (*efeB*), polyphenol oxidase (*yfiH*), 1,2-phenylacetyl-CoA epoxidase (*paa* cluster), and 2-octaprenylphenol hydroxylase (*ubi* cluster). (C, D) The top and bottom 20 differentially expressed genes after treatment with Mi-PS in the transcriptome were classified based on their functions (Supplementary Tables S4 and S5). (E-H) Differentially expressed genes upon Mi-PS treatment. (E) The expression levels of peroxiredoxin family enzymes (mean value  $\pm$  SEM, n = 3). (F) The expression levels of lipid A synthesis pathway proteins (mean value  $\pm$  SEM, n = 3). (G) The expression levels of flagellar-associated proteins (mean value  $\pm$  SEM, n = 3). (H) The expression levels of glycolysis pathway enzymes (mean value  $\pm$  SEM, n = 3).

Source data are provided as a Source Data file.

PS-degrading bacteria are facultative microbes [35,6,99]. It remains unknown whether bacterial strains, e.g., *Exiguobacterium* strain YT2, and *Pseudomonas* strain DSM 50071 have similar or different abilities for PS biodegradation like strain LG3. Further research is needed to evaluate previously isolated aerobic plastic-degrading cultures.

## 5. Conclusions

In this study, we successfully isolated PS-degrading bacteria from the gut and frass of PS-fed mealworms under anaerobic conditions. The isolated bacteria were identified as belonging to the *Enterobacter* genus. Notably, *E. hormaechei* LG3 showed the highest anaerobic PS-degrading activity among the other isolated strains and also effectively degraded PS under aerobic conditions. Furthermore, we compared the LG3 and SCGB1, another PS-degrading bacterium that exhibited distinct metabolic characteristics. The results showed that LG3 has higher anaerobic PS-degrading activity, including cell adhesion, viability, and PS film weight change than *B. amylolyticus* SCGB1 under anaerobic conditions. Further analysis using FTIR and SPME/GC—MS confirmed that the difference in biodegradation ability appeared in the depolymerization of oxidized-PS under anaerobic conditions. Nanopore sequencing confirmed that in the case of LG3, the expression of the *tpx*, *ahpC*, and *bcp* genes, which belong to the identical peroxidase family, was upregulated to depolymerize Mi-PS. Overall, this study identified and characterized *E. hormaechei* LG3 compared with *B. amylolyticus* SCGB1, another gut commensal bacterium having PS degradation activity. The discovery of anaerobic PS degradation by gut microbes of *T. molitor* has provided new insights to the mechanisms of plastic biodegradation in insect larvae as well as in natural ecosystems, which could help in development of bioremediation approaches for the solutions of plastic debris and MPs.

## Ethics declarations

Competing interests.

## Environmental Implication

Microplastics threaten organisms, accumulating in their guts, potentially entering the food chain. To date, research on plastic biodegradation has primarily focused on aerobic conditions. Anaerobic plastic-degrading has limited report although the discovery of anaerobic plastic-degrading bacteria would be of significance for potential bioremediation of Microplastics in environments, including wastewater treatment as well as understanding plastic degradation mechanism within the gut of living organisms, especially plastic-degrading insect larvae. Therefore, it is crucial to investigate the characterization of anaerobic plastic-degrading bacteria to gain a comprehensive understanding of the environmental implications of microplastic pollution.

## CRediT authorship contribution statement

M.G.K., and Y.K. designed the study. M.G.K., and Y.K. performed the experiments. M.G.K., and Y.K. undertook bioinformatics analysis. Y.K. supervised this study. M.G.K., M.J.K., and Y.K. validated the data of this study. M.G.K., M.J.K., and Y.K. wrote the manuscript, and all authors contributed to feedback and proofreading.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132045.

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