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Research Paper

Attachment of potential cultivable primo-colonizing bacteria and its implications on the fate of low-density polyethylene (LDPE) plastics in the marine environment

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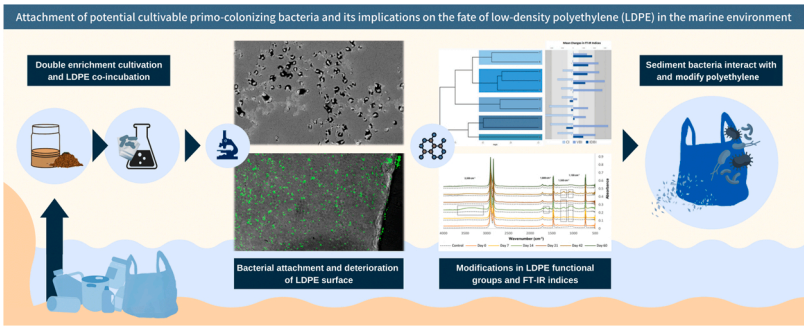
HIGHLIGHTS

- Sediment bacteria isolated from Manila Bay were able to utilize LDPE as the sole carbon source.
- Bacterial clusters modified different moieties in the polymer backbone.
- Attachment and proliferation of bacterial cells on the plastic surface were observed.
- Surface deterioration of the LDPE film was observed as shown by roughness, pits, and crevices.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastics released in the environment become suitable matrices for microbial attachment and colonization. Plastics-associated microbial communities interact with each other and are metabolically distinct from the surrounding environment. However, pioneer colonizing species and their interaction with the plastic during initial colonization are less described. Marine sediment bacteria from sites in Manila Bay were isolated *via* a double selective enrichment method using sterilized low-density polyethylene (LDPE) sheets as the sole carbon source. Ten isolates were identified to belong to the genera *Halomonas*, *Bacillus*, *Alteromonas*, *Photobacterium*, and *Aliishimia* based on 16S rRNA gene phylogeny, and majority of the taxa found exhibit a surface-associated lifestyle. Isolates were then tested for their ability to colonize polyethylene (PE) through co-incubation with LDPE sheets for 60 days. Growth of colonies in crevices, formation of cell-shaped pits, and increased roughness of the surface indicate physical deterioration. Fourier-transform infrared (FT-IR) spectroscopy revealed significant changes in the functional groups and bond indices on LDPE sheets separately co-incubated with the isolates, demonstrating that different species potentially target different substrates of the photo-oxidized polymer backbone. Understanding the activity of primo-colonizing bacteria on the plastic surface can provide insights on the

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possible mechanisms used to make plastic more bioavailable for other species, and their implications on the fate of plastics in the marine environment.

1. Introduction

Plastics are ubiquitous, recalcitrant pollutants that have invaded almost every type of environment. Existing research on the fate of marine plastic debris has monitored its possible transport pathways [80], deposition [89], trophic transfer [16], and degradation [31] to determine its potential risks to different trophic levels and ecosystems. Understanding the mechanistic processes that drive and control the dispersal, transport, and fate of plastics in the environment will help in developing interventions and mitigation strategies to curb the problem. Notably, ubiquitous microorganisms at the base of the food web are the first biotic components that interact with the material when released in the environment [53]. However, there is still a lot to be understood about plastics-microbe interactions and their potential roles on the fate of the mismanaged plastics.

Once exposed to the environment, plastics are rapidly colonized by a variety of microorganisms. The distinct physical characteristics of plastics such as its hydrophobicity, surface charge, surface roughness, topography, crystallinity, and buoyancy make it a unique microenvironment that selects for microbial communities with specific niches [78, 93]. Certain bacterial groups were found to be enriched on plastic more than the surrounding environment, suggesting a potential metabolic adaptation by specific taxonomic groups to the substrate [13,24]. Members of *Alphaproteobacteria* and *Gammaproteobacteria* have been frequently reported to represent the primary colonizers of the marine plastic's surface, while groups under *Bacteroidetes* were identified to be secondary colonizers [21]. Although bacterial colonization of plastics in the marine environment has been well-studied, plastics-associated marine microbes and their potential functions within the plastisphere are yet to be fully elucidated [84].

Pioneer settlers are distinctly classified as primo-colonizers that interact with the surface and initiate attachment by formation of the first biofilm layer [24,78], which is important as they facilitate the attachment of other organisms. Majority of the early plastic-specific colonizers found in other studies occur in low abundance relative to the rest of the plastisphere communities [79]. This could imply that rare members of the plastisphere can provide specific functions that are necessary for the biofilm communities to thrive on the plastic substrate. Therefore, plastic primo-colonizers are a key to understanding the behavior and fate of plastics in the environment. However, even though pioneer microbial groups colonizing plastics are much described [18,50], existing literature on the activities through which primo-colonizers are able to prime the plastic and set the substrate up for successive colonizers remains underrepresented.

Phylogeny-based studies revealed core microbiomes in the plastic surface, especially in environments where plastics also tend to accumulate, such as in coasts [70], marine sediments [21], and ocean gyres [22]. A similar area is in Manila Bay in the Philippines, which is a catch basin for wastes and pollutants from the surrounding land areas in the southwestern part of the Luzon Island [41]. It has been reported to accumulate an average of 47,900 metric tons of plastic wastes or 6.43 % of the estimated overall national input, originating only from one major river system, the Pasig River [51,58]. When enriched *in vitro*, most bacterial OTUs found attached to low density polyethylene (LDPE) belonged to known hydrocarbonoclastic groups [33]. However, insights on their direct involvement in the modification of the plastic were only based on correlations similar to most molecular-based studies. Current research on the environmental fate of plastics in relation to the functionality of pioneer microbial species involved in the initial stages of plastic colonization and the mechanisms that allow them to transform and assimilate plastics remains limited.

There is a need to investigate the activities of bacteria able to grow directly on plastic surfaces distinct from those that are only co-associated with the biofilm community to pinpoint biotically-mediated interactions directly affecting the plastic substrate. In this study, we isolated bacteria capable of growing on a commonly found plastic type (low density polyethylene; LDPE) from the sediments of Manila Bay *via* selective double enrichment cultivation method and characterized their ability to alter the surface morphology and molecular structures of LDPE through incubation assays. LDPE sheets, serving as the sole carbon source, were co-incubated with each of the isolates for 60 days. The bacteria-incubated LDPE sheets were investigated for possible changes in surface texture using high-resolution confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). LDPE sheets were subjected to FT-IR spectroscopy to identify structural modifications in terms of formation or disappearance of functional groups in the polymer backbone, which can be detected as indices attributed to potential degradation. Results of this study provide new insights on how each species contributes to the transformation of plastics in the marine environment.

2. Materials and methods

2.1. Sample source and collection

Sediment samples were collected from sites with low and high plastic accumulation in the southern coast of Manila Bay (Fig. 1). Briefly, Site 1 was in Las Piñas-Parañaque Critical Habitat and Ecotourism Area (LPPCHEA; 14.49744°E,120.98185°N), a coastal wetland with mangrove forests, while Site 2 was in Ternate, Cavite (14.28523°E, 120.70441°N), a beach shoreline located beside a resort. Thus, the sediment characteristics also differed. Sampling was done during the intermonsoon months of May to June 2019 to allow accumulation of plastics and enrichment of hydrocarbonoclasts . Initial surveys showed that plastic accumulation increased towards the inner parts of Manila Bay ([33]; Supplementary Method 1). Site 1, adjacent to a densely populated coastal settlement and covered by mangroves with root systems that allow marine litter deposition, had the highest plastic percent cover. Site 2, located on the outer part of the bay and farther from riverine systems, had low plastic accumulation.

About 10 g of collected sediments were initially co-incubated with pre-weighed LDPE sheets for 90-days at 30 °C in 500 mL Erlenmeyer flasks containing 350 mL Bushnell-Haas medium (0.2 g $MgSO_4{\cdot}7H_2O$, 0.02 $CaCl_2{\cdot}2H_2O$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, and 0.05 g $FeCl_3$) and were subjected to partial biodegradation assays to observe changes on the plastic substrate over time *in vitro* [33]. LDPE was chosen as the plastic substrate since it was one of the most common polymers used for packaging, and it comprised most of the plastic waste in Manila Bay [73]. The 90-day incubation showed modifications in the plastics based on confocal laser scanning microscopy (CLSM) and changes in FT-IR spectra [33]. Given these initial observations, the LDPE sheets from the same 90-day enriched cultures were further processed and used as a source of the potential plastic primo-colonizers in this study.

2.2. Double selective enrichment and isolation of plastic-utilizing microorganisms

Double selective enrichment followed by isolation were successively conducted to isolate the potential primo-colonizing bacteria, with the assumption that only those that have the metabolic adaptation to the plastic substrate as an energy source and habitat can survive by

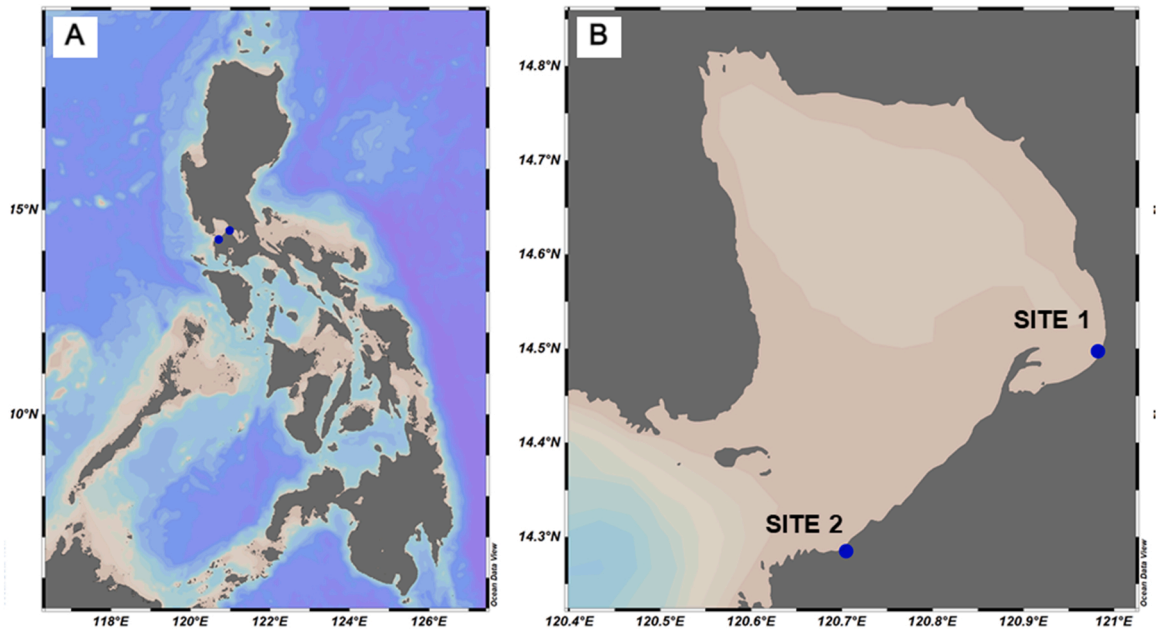

Fig. 1. Coastal sites of sediment samples from (A) Manila Bay, Philippines. (B) Site 1: Las Piñas-Parañaque Critical Habitat and Ecotourism Area (LPPCHEA; 14.49744°E,120.98185°N), a coastal wetland with mangrove forests; Site 2: Ternate, Cavite (14.28523°E, 120.70441°N), a beach shoreline located beside a resort. Sediment samples were collected from the submerged part of the coast at the low tide line using a seagrass corer.

attaching. To remove unattached cells, partially degraded LDPE sheets from the 90-day sediment incubation set-up (Section 2.1) were washed with sterile artificial seawater following the protocol of Nguyen [62], with some modifications. The washed sheets were placed in flasks containing 100 mL synthetic medium with 0.3 % LDPE powder as the sole carbon source to support the growth of and select for potentially plastic-utilizing microbes [20,23]. The synthetic medium (SM) broth was formulated according to the components used in the study of [38], with some modifications: K_2HPO_4, 1 g, KH_2PO_4, 0.2 g, $(NH_4)_2SO_4$, 1 g, $MgSO_47H_2O$, 0.5 g, NaCl, 1 g; $FeSO_47H_2O$, 0.01 g; $CaCl_22H_2O$, 0.002 g; $MnSO_4H_2O$, 0.001 g; $CuSO_45H_2O$, 0.001 g; $ZnSO_47H_2O$, 0.001 g; and 1 L artificial seawater. LDPE powder was made from LDPE plastic bags (SFig2; White Horse®, New Hi-Zex Plastic Manufacturing) purchased from a local supermarket, which were cut into 1.5 cm × 1.5 cm squares, disinfected by soaking in 70 % ethanol for 30 min, and air dried in a sterile laminar airflow chamber. To increase the available surface area for attachment and growth of bacterial cells, the LDPE squares were afterwards soaked in liquid nitrogen to increase polymer fragility and shredded into fine powder using an electric stainless-steel grinder (Yisino, Philippines). To make the particle sizes uniform, the ground LDPE particles were sieved through a 250 μm metal mesh, and the sieved powder was stored in a sterile lidded container until further use. These were all conducted under a sterile laminar airflow chamber to maintain an aseptic environment.

After adding the sheets containing the initial inocula, the flask was vigorously shaken to dislodge the biofilm. These were then incubated in an incubator shaker at 30 °C for 45 days. A second enrichment was performed, where the first enrichment set-up after 45 days was re-inoculated into a fresh LDPE-synthetic media and incubated for another 45 days. This was done to selectively cultivate the plastic-adapted bacteria and restrict the growth of other taxa. About 1 mL aliquot was collected from the set-up every 7 days during incubation and stored in a microfuge tube to monitor changes in the attached bacteria. A drop from the aliquot containing suspended LDPE particles was dispensed into a clean and sterile glass slide, stained with SYBRGreen DNA Gel Stain, and viewed using CLSM.

From the second selective enrichment culture after 45 days, 1 mL aliquot was subjected to 10-fold serial dilutions and the dilutions were spread plated on Zobell marine agar (in grams per liter of distilled water: agar, 15 g, peptone, 5 g, yeast extract, 1 g, ferric citrate, 0.1 g, NaCl, 19.45 g, $MgCl_2$, 8.8 g, Na_2SO_4, 3.24 g, $CaCl_2$, 1.8 g, KCl, 0.55 g, $NaHCO_3$, 0.16 g, KBr, 0.08 g, $SrCl_2$ 0.034 g, H_3BO_3, 0.22 g, Na_2SiO_3, 0.004 g, NaF, 0.0024 g, $N_2H_4O_3$, 0.0016 g, Na_2HPO_4, 0.008 g). All plates were incubated at 30 °C for 48–72 h. Growth was observed, and well-isolated colonies were picked and streaked for purification using the same medium. Pure isolates were stored in marine agar slants at 4 °C for succeeding analyses.

2.3. DNA extraction, sequencing, and phylogenetic analysis of the 16S rRNA gene

Genomic DNA was extracted from the pure isolates using the boiling lysis method of Englen and Kelley [25], with few modifications. A loopful of pure culture was obtained using a sterile applicator stick and resuspended in 100 μL sterile ultrapure water in a 1.5 mL microfuge tube. The suspension was heated at 100 °C for 10 mins, cooled down to 4 °C for 10 mins, and centrifuged at 12,300×*g* for 10 mins. The supernatant was collected and transferred to a separate sterile microfuge tube and stored at 4 °C. Concentration and purity of the extracted DNA were checked using a BioSpec-Nano spectrophotometer (Shimadzu, Japan), and stored at − 20 °C until PCR amplification. The 16S rRNA gene was amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [49] using 5 μL of the template DNA. Amplification was done with the following parameters: initial denaturation at 95 °C for 2 mins, 30 cycles of denaturation at 94 °C for 5 s, annealing at 50 °C for 40 s, extension at 72 °C for 60 s, and final extension at 72 °C for 5 mins [39]. The quality of the amplicons was evaluated and visualized using 1 % agarose gel stained with SYBRGreen. Single pass Sanger sequencing was performed in Macrogen Inc. (Korea).

The obtained sequences were deposited in GenBank (Accession Numbers OP854868-OP854877) and compared against similar gene sequences using BLASTn search in NCBI GenBank database. Most similar sequences were downloaded along with representative sequences from other clades and aligned with the query sequences using MAFFT v.7.450 (Multiple Alignment using Fast Fourier Transform; [45]). A model test

was performed in JModelTest2 [19], and a maximum likelihood phylogenetic tree was generated in FastTree ver 2.1.11 with bootstrap support repeated 1000 times [71].

2.4. Incubation assays using pure bacterial cultures

The identified isolates were further tested for their ability to attach and later change the physical and chemical properties of LDPE. LDPE sheets used for the incubation assays were first exposed to direct sunlight for two weeks to simulate initial abiotic degradation in the environment [31]. After exposure, the LDPE sheets were dried overnight at 60 °C, cut into 1.5 cm × 1.5 cm squares, weighed, disinfected by soaking in 70 % ethanol for 30 mins, and air-dried in a sterile laminar airflow chamber [20], and were then used in the treatments.

Flasks containing 90 mL Bushnell-Haas (BH) broth were added with the treated 0.3 % LDPE sheets, and each flask was inoculated with 10 mL of a 24-hour pure culture. The inoculum was prepared by growing it in 0.3 % LDPE-BH broth prior to use to acclimatize the culture and avoid a lengthened lag phase. The cell density of the 24-hour culture was determined pre-inoculation using a UV-Vis spectrophotometer set at 600 nm, and it was adjusted to 0.5 OD_{600} to standardize the inoculum size. The cultures were incubated at 30 °C for 60 days, with each set-up done in triplicates. A set-up with a flask containing sterile, non-inoculated SM broth and pre-weighed plastic sheet was used as negative control. Modifications in the LDPE structure were evaluated using the following indices: sheet surface morphology and polymer chemical structure.

2.5. Monitoring changes in plastic's surface topography

Visible physical changes in the surface of the plastics such as roughening and formation of holes, pits, or cracks were assessed after 60 days of incubation. Surface topography of the LDPE sheets was visualized through CLSM (CLSM 710, Carl Zeiss, Germany), with sample preparation following the methods of Harshvardhan and Jha [38] and Zettler et al. [93]. The sheets were washed with 2 % SDS followed by warm distilled water to eliminate surface-adhered bacterial cells. Prior to microscopy, the sheets were stored in 1 % glutaraldehyde to preserve the surface polymer ultrastructure. To visualize the plastic surface, the differential interference contrast (DIC) setting of the CLSM was used by turning on the T-PMT, then the obtained image was viewed using the 2.5D setting. To visualize surface erosion, select pre-processed LDPE sheets were sent to DOST-ITDI Advanced Device and Materials Testing Laboratory (Taguig, Philippines) for field emission scanning electron microscopy (SEM) imaging. The imaging was done using the following parameters: Dual Beam Helios Nanolab 600i for the instrument, 2.0 kV accelerating voltage, and 86 pA beam current. Samples were casted in aluminum foil and stored in a desiccator for drying overnight. The samples were analyzed in a clean room at relative humidity 50 ± 10 % and 20–25 °C.

2.6. Detection of structural changes in plastic surface

Changes in the polymer structure of the LDPE sheets were analyzed by FT-IR spectroscopy with ATR (Bruker, Ettingen, Germany), following Dela Torre et al. [23] and Jung et al. [43]. Prior to analysis, LDPE sheets were washed with 2 % SDS, followed by a sterile distilled water rinse, and subsequently dried at 60 °C overnight. Spectra from the LDPE sheets were collected by the instrument from 4000 cm^{-1} to 450 cm^{-1} with a data interval of 1 cm^{-1} and resolution of 4 cm^{-1}. The ATR diamond crystal was cleaned using 70 % 2-propanol, and a background scan was conducted between each sample.

To ensure proper contact between the ATR crystal and the LDPE sheet, each sheet was compressed against the diamond with the minimum force recommended by the instrument manufacturer. Characteristic absorption peaks were determined using a peak height algorithm from the equipment's software (OPUS 8.5, Bruker Corporation). Relative absorbance intensities of the carbonyl bond (1740 cm^{-1}), terminal double bond (vinyl) (1650 cm^{-1}), and internal double bond (908 cm^{-1}) to that of the methylene bond (1465 cm^{-1}) were evaluated using the following equations [2,3,48]:

Carbonyl Bond Index (CI) = I1740/I1460.

Vinyl Bond Index (VBI) = I1650/I1465.

Internal Double Bond Index (IDBI) = I908/I1465.

2.7. Statistical analyses

Polyethylene chemical structure indices were expressed as means ± standard deviation of three replicates, and significance was determined among all mean values by one-way analysis of variance (ANOVA) and between isolates and the control by Tukey's post-hoc test ($p < 0.05$). FT-IR spectra results were pre-processed by calculating the difference of each index to the control index per isolate and standardized using the scale function in R. Clustering of isolates based on changes in FT-IR indices was determined by calculating Euclidean distances among indices and using Ward's method for hierarchical clustering. All statistical analyses were done in RStudio (version 1.4.1106; RStudio Team, 2021) and Past 4.03 [36].

3. Results

3.1. Isolation and phylogenetic identification of LDPE primo-colonizers

Using a novel double selective enrichment cultivation method, 10 distinct colonies were isolated (hereafter referred to as MB1 to MB10). The isolated colonies were characterized as having circular and raised morphologies, with white to yellow and pink pigments (Supplementary Figure 1).

Analysis of the 16S rRNA gene sequences revealed that majority of the isolates belonged to phylum Proteobacteria (eight out of ten isolates), putatively identified as *Halomonas maura* (MB1), *H. denitrificans* (MB2), *H. pacifica* (MB3), *H. cerina* (MB8), *H. gudaonensis* (MB10), *Alteromonas oceani* (MB5), *Photobacterium ganghwense* (MB6), and *Aliishimia ponticola* (MB7). The two other isolates were classified under phylum Firmicutes, namely *Bacillus megaterium* (MB4) and *Bacillus vietnamensis* (MB9) (Table 1, Fig. 2). High percent similarity values between the query sequences and the most similar NCBI reference strains were observed (98–99 %) with strong bootstrap support.

3.2. Attachment and growth of isolates on LDPE sheets

High resolution imaging using both CLSM and SEM revealed aggregated growth of cells on the plastic surface (Figs. 3 and 4). Periodic monitoring showed attachment of bacteria to the plastic surface as early as the 7th day of incubation (Fig. 3B, F). As incubation progressed, cells started growing in patches with colony-like formations (Fig. 3D, H) but notably denser within the pits, forming cell-shaped indentations across the area of attachment. Physical interactions of the bacterial cells with the plastic surface were visualized as erosions, crevices, and pits. Differential interference contrast (DIC) images rendered using the 2.5D setting of the CLSM showed the roughened surface of the film characterized by profuse dents and pits after 60 days of incubation (SFig. 3A), in contrast to the relatively smoother and even surface of the sheets in the control (SFig. 3B).

SEM further revealed initial stages of surface deterioration. Fig. 4A–C showed the physical changes that occurred on the plastics after photo-oxidation by UV treatment and possible hydrolysis with no bacterial action (control; Fig. 4). After 60 days, imprints of the same deterioration were observed as rough patterns in the surface of the plastics (Fig. 4A). A closer look further revealed smaller, irregular crevices without bacterial cells (Fig. 4B, C), consistent with the surface topography rendered using the CLSM (Fig. 3A, E). In comparison, the same type of material co-

Table 1
Putative identities based on 16S rRNA gene of the cultivable biodegrading bacteria isolated from Manila Bay sediments.

No.	Sample Code	Phylum/Class	Putative Identity	NCBI Accession No.	Similarity (%)
1	MB1	Proteobacteria, γ-proteobacteria	*Halomonas maura* S-31	NR.116946.1	98.32
2	MB2	Proteobacteria, γ-proteobacteria	*Halomonas denitrificans* M29	MZ276310.1	99.04
3	MB3	Proteobacteria, γ-proteobacteria	*Halomonas pacifica* NBRC	NR_114047.1	98.56
4	MB4	Firmicutes, Bacilli	*Bacillus megaterium* ATCC 14581	MK508856.1	99.03
5	MB5	Proteobacteria, γ-proteobacteria	*Alteromonas oceani* S35	NR_159349.1	98.08
6	MB6	Proteobacteria, γ-proteobacteria	*Photobacterium ganghwense* FR1311	NR_043295.1	98.79
7	MB7	Proteobacteria, α-proteobacteria	*Aliishimia ponticola* MYP11	NR_170429.1	98.49
8	MB8	Proteobacteria, γ-proteobacteria	*Halomonas cerina* SP4	NR_044316.1	98.06
9	MB9	Firmicutes, Bacilli	*Bacillus vietnamensis* NBRC101237	NR_113995.1	99.50
10	MB10	Proteobacteria, γ-proteobacteria	*Halomonas gudaonensis* SL014B-69	NR_025773.1	98.08

incubated with the isolated bacteria had proliferation of cells within these crevices, with the holes appearing to be larger with rougher edges (Fig. 4F, I).

Notably, the patterns on the deteriorated LDPE surface varied differently for each isolate. MB4, for example, which also exhibited the highest decrease in CI (19.2 %), produced smaller and more irregular patterns, with the cell aggregates only being observed along the edges of the formed pits (Fig. 4D–F). MB5 on the other hand formed larger pits with the cell colonies forming in dense aggregations in the center of the pits (Fig. 4G–I). Deterioration based on the average area of the pits formed ($n = 30$) after the same duration of incubation could also imply that MB5, which formed larger pits ($0.0130 \pm 0.004\ \mu m^2$), had higher activity than that of MB4 with smaller pits ($0.00677 \pm 0.003\ \mu m^2$). Moreover, higher magnification of the images revealed the presence of thread-like viscid components surrounding the bacterial cells (arrows) and along the roughened plastic surface.

3.3. Functional group modifications on the surface properties of LDPE sheets

Results of the incubation assay showed significant differences in the level of FT-IR indices for each isolate, namely CI, VBI, and IDBI. The isolates were grouped into five (5) different clusters based on the levels for which these indices were observed. Specifically, they were classified by having low (≤ 0.0059), moderate (0.006–0.0199), or high (≥ 0.02) mean FT-IR index change, with reference to the control samples. Clusters 1 and 2 were characterized by having very low to moderate carbonyl consumption (CI = 0.0003–0.015). Cluster 3 was characterized by low vinyl bond (VBI = 0.0037) and double bond production (IDBI = 0.002), and Clusters 4–5 by high carbonyl consumption (0.027) and low double bond production (0.0013; Fig. 5).

Specifically, LDPE sheets co-incubated with isolates from Clusters 1 and 2 exhibited the highest significant ($p = 0.047$) increase in the VBI and IDBI compared to the control after 60 days of incubation. The VBI of the sheets had at least 10.6 % and at most 24.2 % increase, while the IDBI showed a minimum of 7.72 % and a maximum of 12.1 % increase in all samples except in MB10 where a minimal decrease of 0.12 % in the index was observed (Fig. 5). Lowest decrease in CI was also noted for the two clusters (0.34–10.4 %), except in MB7 where an increase of 0.22 % was observed.

The FT-IR spectra were also used to note different chemical moieties within the polymer, each represented by distinct and specific infrared absorption bands observed as peaks (Fig. 6). For the control film, characteristic absorption bands were observed as medium doublet peaks at 720 cm^{-1} for CH_2 rocking deformation, slight peak at 1389 cm^{-1} for CH_3 bending, medium peaks at 1461 and 1465 cm^{-1} for CH_2 bending, low peak at 1692 cm^{-1} for C═O stretching, and strong doublet peaks at 2845 and 2913 cm^{-1} for C-H2 asymmetric stretching (Fig. 6A; [34]). LDPE sheets co-incubated with isolates from Clusters 1 and 2 showed the appearance of new and distinct peaks at 1100 to 1300 cm^{-1}, and a low peak at 3300 cm^{-1} (Fig. 6A-E).

The lowest increase in VBI was observed in Cluster 3, specifically for MB8 (3.05 %) (Fig. 5), with a decrease of 2.02 % and 1.19 % in IDBI for MB8 and MB2, respectively. The CI for this cluster also decreased, with the LDPE exhibiting 7.53 % less CI than the control for MB8 and 16.0 % less for MB2. From the mean changes of the FT-IR indices, Cluster 3 was characterized by moderate to high consumption of carbonyl groups indicated by the significant decrease in CI. Production of functional groups with vinyl bonds was classified as low, as the increase in VBI was not significant. Groups with internal double bonds were observed to be consumed, reflected by the slight decrease in IDBI, but the change was found to be also insignificant. The spectra of LDPE sheets co-incubated with Cluster 3 isolates was characterized by the disappearance of peaks at 1100 to 1200 cm^{-1} when compared to the control (Fig. 6F–G).

Cluster 4 showed the highest decrease in CI (19.2 %), which was observed in MB4, and the highest increase in VBI (26.8 %), exhibited by MB1 (Fig. 5). The IDBI between the two isolates of the cluster was noted to differ, wherein MB4 showed a very slight 0.088 % increase, while MB1 was found to decrease by 4.25 %. Cluster 5 was observed to have an increase in both VBI (17.1 %) and IDBI (6.39 %), while the CI was found to have decreased (10.3 %). Mean changes in the indices when compared to the control showed that Clusters 4 and 5 exhibited moderate to high carbonyl group consumption, moderate to high vinyl bond production, and moderate to low internal double bond production. After the 60-day co-incubation of LDPE sheets with Cluster 4 and 5 isolates, appearance of new peaks at 1100–1300 cm^{-1}, 1665–1710 cm^{-1}, and 3300 cm^{-1} was found (Fig. 6H-J).

4. Discussions

4.1. Plastics as a selective environment for surface-associated marine bacteria

Accumulating evidence suggests the significant roles played by primo-colonizers in the successful establishment and colonization of plastic surfaces by microorganisms. For example, Gomez and Onda [33] reported a high diversity of bacteria enriched on the surface of plastics from Manila Bay sediments *in vitro*, including some known hydrocarbonoclasts as potential primo-colonizers. However, since the community profiling of these types of studies only relied on the detection of 16S rRNA gene [68], their putative functions were only inferred from literature and have not been phenotypically characterized. While culture-independent molecular methods provide insights on potential roles of identified microbial taxa within a community, culture-based approaches are still necessary in investigating specific microbial characteristics and phenotypes.

Using the same setup of Gomez and Onda [33], we employed a double enrichment cultivation method with LDPE-SM media to isolate and characterize potential primo-colonizers. We were able to isolate and cultivate ten isolates belonging to five genera: *Halomonas, Bacillus, Alteromonas, Photobacterium,* and *Aliishimia*. Notably, Gomez and Onda [33] only detected *Halomonas, Alteromonas,* and *Photobacterium* using gene-based profiling with low number of reads. This is consistent with previous studies showing that most of the 'true' primo-colonizers - that is

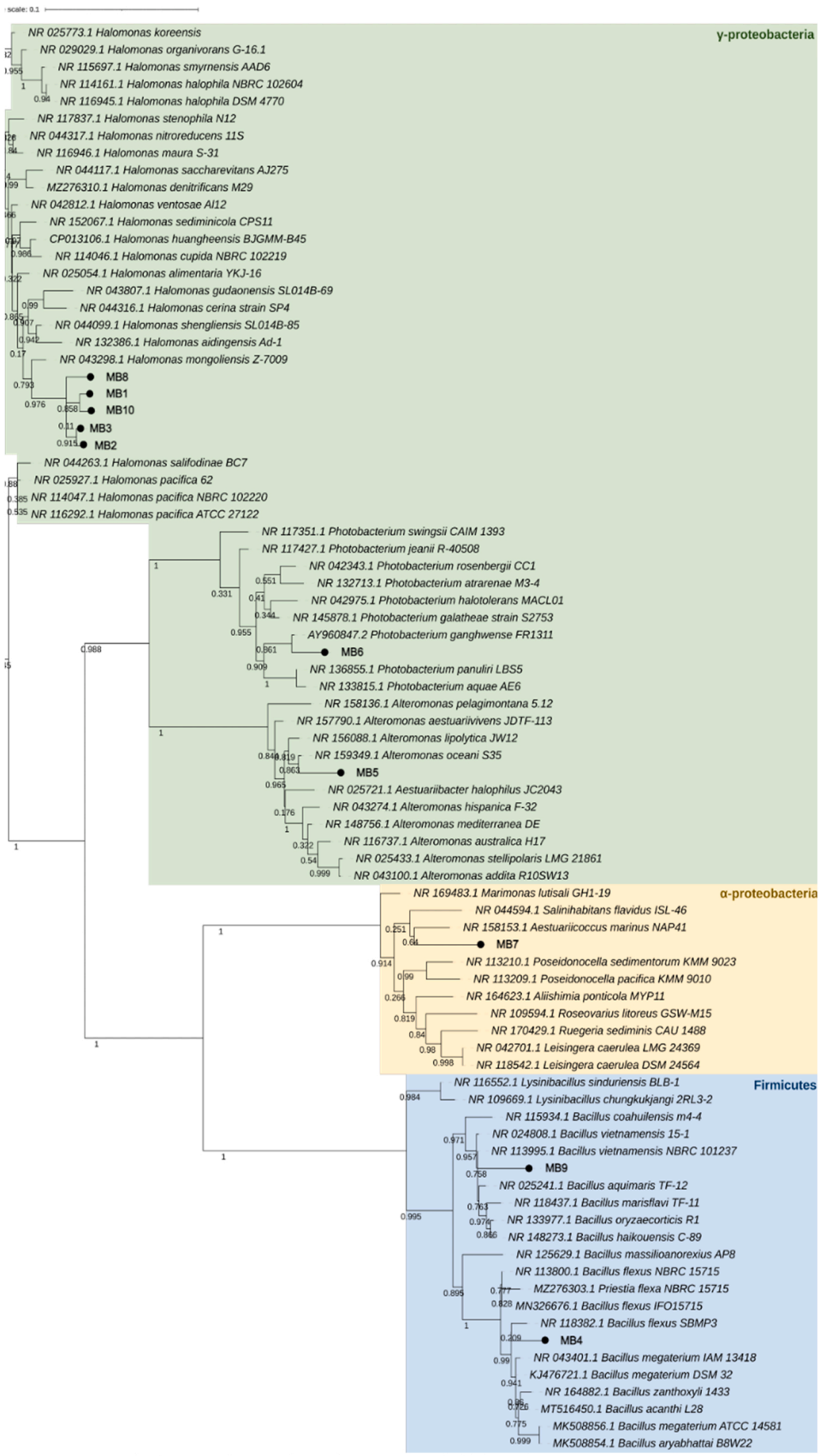

Fig. 2. A phylogenetic tree based on nearly full-length 16S rRNA gene sequences of bacteria isolated from sediment samples of Manila Bay. Isolates were highlighted (bold) and classified according to their nearest taxonomic affiliations. Major bacterial groups are represented in the tree, including γ-Proteobacteria (green), α-Proteobacteria (yellow), and Firmicutes (blue).

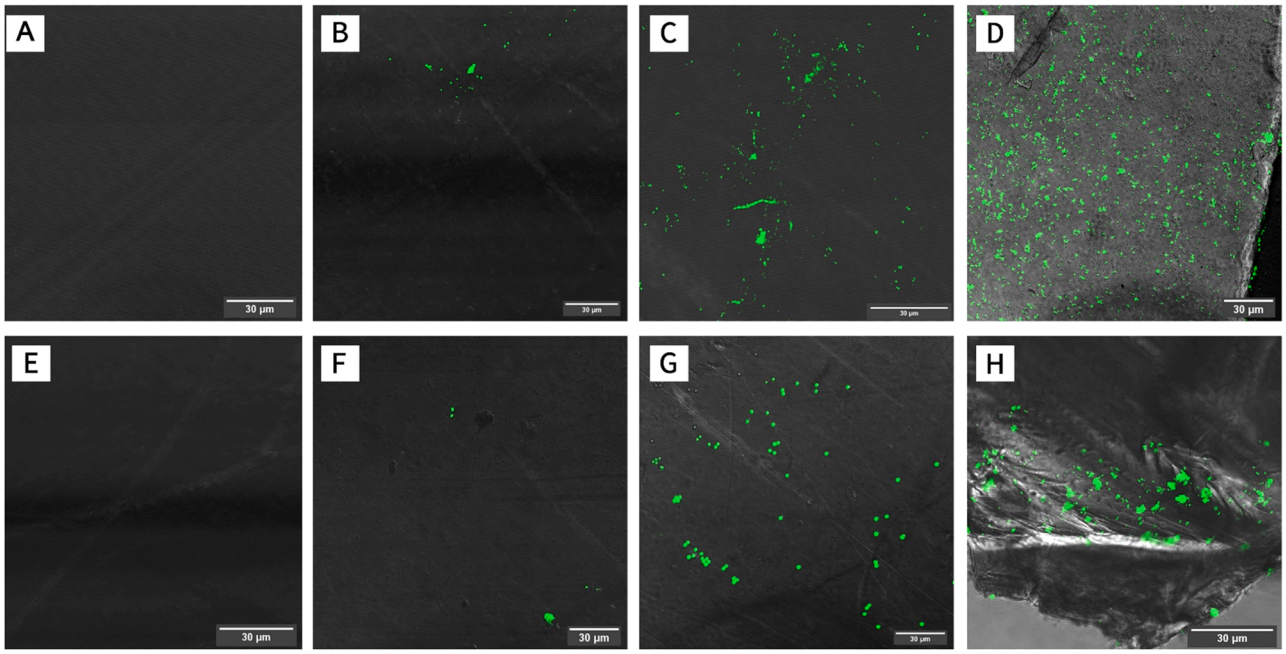

Fig. 3. Confocal laser scanning micrographs of pretreated (A, E) and biologically aged (B-D, F-H) LDPE sheets stained with SYBRSafe DNA (green). Bacterial cell attachments of Isolate MB4 (top) and Isolate MB5 (bottom) were observed on days 7 (B, F), 21 (C, G), and 42 (D, H). The images were viewed under 63X objective lens.

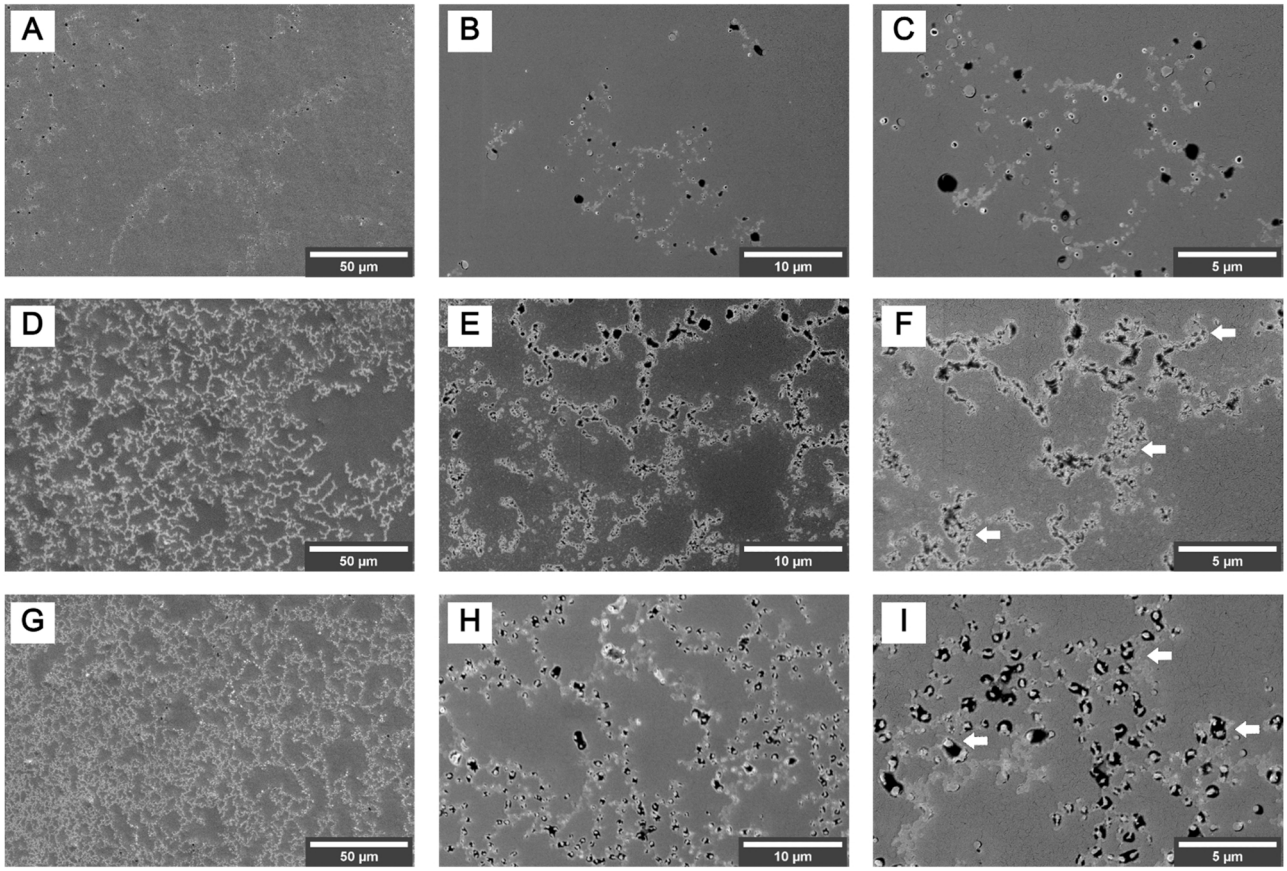

Fig. 4. Scanning electron micrographs showing LDPE surface morphology of control sheets (A-C) and sheets co-incubated with bacterial isolates MB4 (D-F) and MB5 (G-I) after 60 days of incubation. The images were viewed under 1,000x, 5,000x, and 10,000x magnification (from left to right, respectively). White arrows: viscid material observed around bacterial cells that can potentially be extracellular polymeric substances.

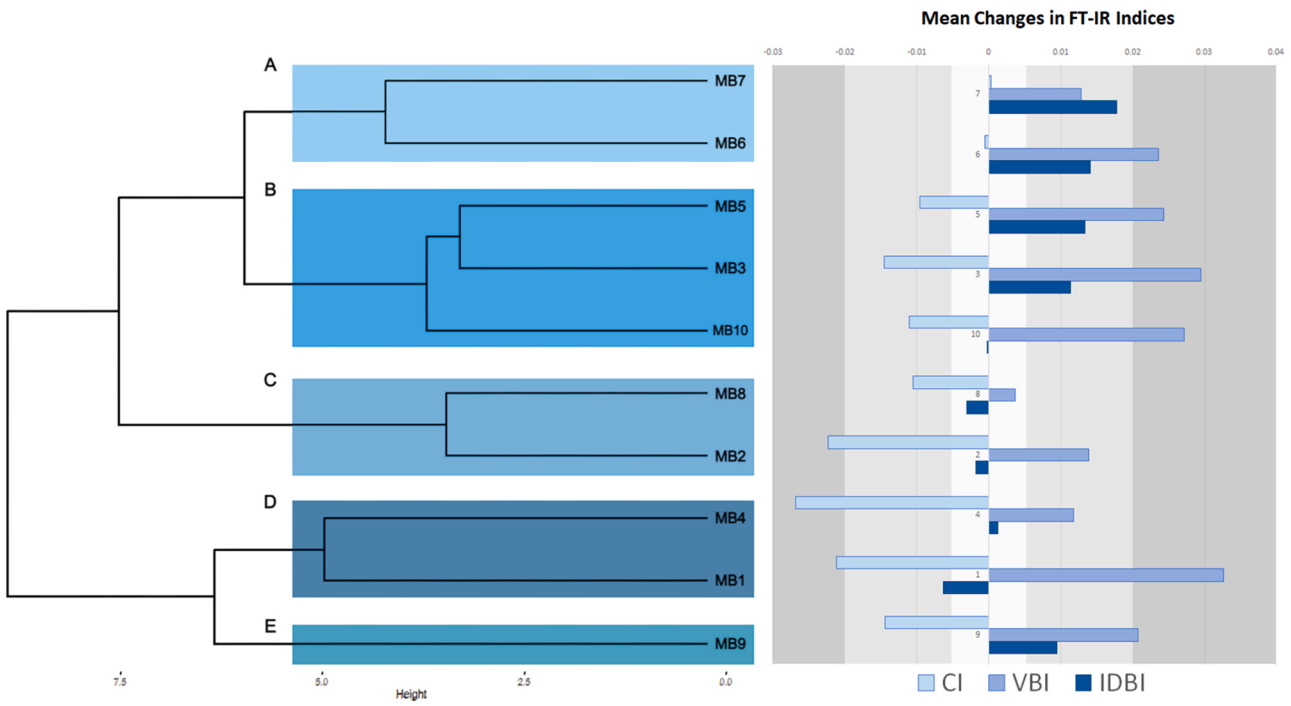

Fig. 5. Clustering of isolates based on mean changes in FT-IR indices (CI, VBI, IDBI). The isolates clustered into five groups: (A) Cluster I: MB7, MB6; (B) Cluster II: MB5, MB3, MB10; (C) Cluster III: MB8, MB2; (D) Cluster IV: MB4, MB1; and (E) Cluster V: MB9. Positive index changes were interpreted as an increase (appearance), while negative values were interpreted as a decrease (disappearance). Index changes were classified by having low ($\leq$ 0.0059; white area), moderate (0.006–0.0199; light gray area), or high ($\geq$0.02; dark gray area) mean FT-IR index change, with reference to the control samples.

opportunistic species with mechanisms for surface-sensing attachment, and biofilm development [24,52,54] - occur in low abundance relative to the rest of the plastisphere communities [79]. Here, double enrichment cultivation could have only allowed growth of taxa that can adapt to nutrient-limited media with LDPE as the sole carbon and energy source, a very specific niche that only some of the cultivated ones were able to adapt to. The successive two-step enrichment also eliminated other taxa that may be relying on other byproducts produced by some of the hydrocarbonoclastic bacteria, thus, reducing the number to cultivable strains that are directly interacting with the substrate. Previous studies also used PE as a substrate for cultivation and reported species that were not commonly detected in molecular data [20,26], emphasizing the need to conduct targeted isolation when investigating phenotypes of bacteria in specific niches.

Interestingly, most of the isolates enriched by the LDPE substrate were classified under Gammaproteobacteria, a phylum often identified as the dominant taxa found in plastic samples during the early stages of colonization [37,90]. In particular, *Halomonas* and *Alteromonas* were also previously described as pioneer colonizers among bacterial assemblages found in surfaces of inert materials in the marine environment [18,66]. Dominance of cultivable *Halomonadaceae* with significant colonization of different plastic types has been reported in varying environments [1,30]. Similarly, *Alteromonas* was also consistently found abundant in the early plastisphere community [90]. Meanwhile, this study is the first to report plastics-association of *A. ponticola*. These cultivated enriched taxa could be some of the 'true' pioneer bacterial groups or primo-colonizers that form the initial layer of biofilm on the plastic surface after its release into the marine environment. Primo-colonizers can overcome the poor accessibility of a hydrophobic substrate and commit cell proliferation on the surface entirely independent of other species [12], making them important key players in community succession. Studies exploring microbial succession in the plastisphere have identified different taxa involved in the early stages of plastic colonization through molecular detection of primo-colonizers at initial points of exposure [24,75]. However, functional capabilities of these primo-colonizers are not yet fully elucidated and the effect of these activities onto the plastic surface is less understood. This study presents a culture-based phenotypic characterization of primo-colonizers and *in vitro* observation of their interaction with the plastic (see succeeding sections).

4.2. *Attachment of isolated primo-colonizing bacteria*

Successful attachment and proliferation of colonies for all bacterial isolates in the LDPE surface over time were evident in the CLSM images, in contrast to the control which had no bacterial growth (Fig. 3). Specifically, colonies were observed to have progressively increased in abundance and have grown along and within the cracks formed from the initial abiotic weathering of the LDPE sheets. Bacterial growth and colony development on the weathered plastic surface establish irreversible attachment by mechanisms that cause surface modification [24].

In addition, SEM images revealed the presence of extracellular material (Fig. 4F, I) around the area where bacterial colonies proliferated, which may indicate production of surface-active exopolymers [15]. Higher magnifications of the images showed structures akin to extracellular polymeric substances (EPS) appear loosely bound and diffused, exhibiting a morphology similar to previous observations from studies exploring plastic-associated microbes [35,23]. Several studies have attributed this extracellular material to EPS, which is significant in surface adhesion and pioneer biofilm development [40,8]. Within the biofilm, the microcolonies of cells proliferate and the EPS facilitates adhesion by increasing the solubility and binding of hydrophobic substrates such as hydrocarbons in the plastic polymer [69].

Interestingly, most of the genus enriched and isolated in this study belonged to taxa which are known for surface attachment adaptations. Five *Halomonas* species were identified: *H. maura* (MB1), *H. denitrificans* (MB2), *H. pacifica* (MB3), *H. cerina* (MB8), and *H. gudaonensis* (MB10). *Halomonas* are recognized for producing EPS with biosurfactant-like properties that form an interface between cells and a hydrophobic

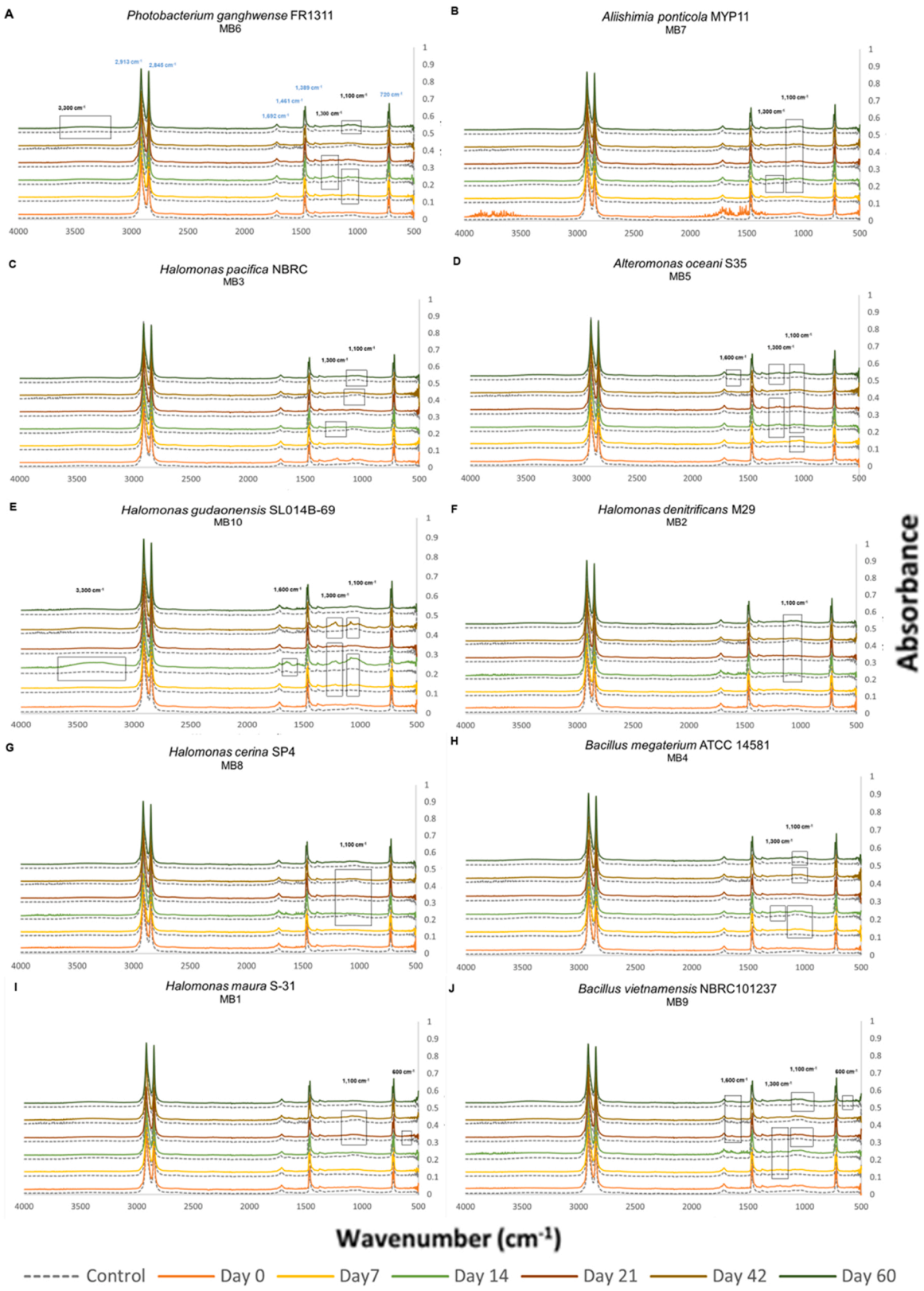

Fig. 6. FT-IR spectra of LDPE sheets after a 60-day co-incubation with Cluster 1 (A, B), Cluster 2 (C -E), Cluster 3 (F, G), Cluster 4 (H, I), and Cluster 5 (J) isolates. Characteristic peaks of the control LDPE are marked in blue values (A), while changes in peaks for the treated LDPE are marked in black values (A - J).

surface (Bouchotrouch et al., 2000). For instance, *H. maura* produces an acidic EPS known as mauran, a viscosifying compound that emulsifies hydrocarbons such as petroleum [6]. It is interesting to note that *Halomonas* bioemulsifiers are also able to stimulate the growth of other hydrocarbonoclastic bacteria such as *Bacillus* and *Pseudomonas* [14,57]. This suggests that aside from facilitating attachment, initial settlers on the plastic may also possess mechanisms that increase the bioavailability of the substrate for other taxa, which highlights the significance of

primo-colonizers in the recruitment of other plastisphere members during succession.

Isolate MB5, putatively identified as *A. oceani*, belonged under genus *Alteromonas* which is characterized by members commonly associated with biofilm-forming communities and hydrocarbonoclastic bacteria [17,42]. *A. oceani* was previously observed to produce robust biofilms in liquid media without supplements, suggesting its ability to settle on surfaces with minimal bioavailable nutrients such as plastic [56]. Two isolates, *B. megaterium* (MB4) and *B. vietnamensis* (MB9), were reported to cause surface damage on PE pellets and titanium alloy, respectively, through biofilm production [7,91]. Meanwhile, this is the first study to report on the other two isolates, *P. ganghwense* (MB6) and *A. ponticola* (MB7), as pioneer plastic colonizers that can grow solely on the substrate independent of a microbial consortium. Cultivation and co-incubation assays therefore allow *in vitro* observation of primo-colonizers' actual attachment and interaction with the substrate to understand how they are capable of modifying the plastic substrate and their implications on the fate of the plastic in the marine environment.

4.3. Surface morphological modification on LDPE

Attachment and establishment of colonies were coupled with changes in the morphology and topography of the plastic surface over time. This study used minimal medium in artificial seawater without initial stimulation of biofilm, which further emphasizes the efficiency of attachment of the isolated bacteria onto the plastic. Other studies that investigated bacterial colonization on plastics performed surface conditioning by adsorption of dissolved organic matter from natural seawater to encourage biofilm formation [9,77]. It has been shown that microbial attachment, proliferation, and biofilm formation facilitate the contact of enzymes possibly produced and secreted by microbes, which subsequently makes the hydrocarbon polymer structure more susceptible to microbe-mediated attack [82,83].

SEM analysis showing irregularly shaped crevices and holes on the plastic surface suggest that bacterial colonization could have caused damage to the polymer's physical integrity. The observed pits and cavities were non-uniformly scattered and were only present in areas where bacterial cells appeared to be embedded (Fig. 4F, I). This denotes the occurrence of surface erosion due to possible bacterial catalytic mechanisms resulting in the penetration of bacterial cells into the LDPE matrix during growth and colonization. Consistent with previous studies, surface morphological modifications caused by bacterial growth and activity have been reported [20,38,23].

It is important to note that bacterial proliferation was found to concentrate specifically within the areas where the plastic surface eroded. Substantial growth within the pits suggests that these crevices may have become a favorable microenvironment for subsequent cell recruitment. In the marine environment, crevices on the plastic surface could introduce new niches for other microorganisms to thrive on the substrate, specifically for bacterial groups that are unable to form biofilms [65]. These cracks contribute to an increase in roughness, which has been reported as a significant surface property affecting cell attachment and colonization since eroded areas provide physical spaces for non-pioneer microbes to proliferate [55,67]. Surface deterioration of the plastic is also a physical indication of macromolecule breakdown, subsequently exposing shorter polymer branches to which secondary colonizers can attach to [94,59]. With these observations, interactions between the surface of the plastic and the pioneer colonizing bacteria are then significant in driving the composition and successional recruitment of microorganisms, as previously described on other types of surfaces [18].

Changes in the plastic surface morphology would subsequently alter the buoyancy, roughness, hydrophobicity, and facilitate the sinking of the plastic debris [11,44]. Biofouling leads to deterioration and fragmentation of larger plastic items which can eventually result in the degradation of the plastic polymer [28]. Although analytical assessments of the plastic's physical properties are beyond the scope of this study, surface deterioration due to colonization of the bacterial isolates provide qualitative visual evidence of the pivotal role of primo-colonizers as main drivers of plastic biofouling in the real environment.

4.4. Functional group modifications in PE surface chemistry

Chemical modifications reflected as formation and disappearance of compounds suggest depolymerization of the polymer as a potential consequence of both biotic and abiotic factors. For example, photo-oxidation causes initial changes in the polymer backbone, such as chain scission and formation of carbonyls and vinyl groups [60,86]. Presence of these oxidation products render the plastic surface vulnerable to biotic factors, which can generate additional modifications in the PE structure. Notably, peaks observed after incubation varied between the isolates, indicating that different taxa or strain might be targeting different moieties of the LDPE backbone. New peaks at the 1100–1300 cm^{-1} region that correspond to C-O stretching in ether, ester, carboxylic acid, and alcohol groups were detected after LDPE sheets were co-incubated with most of the isolates. These functional groups have previously been ascribed to oxidative degradation of PE [60]. Microbial oxidation of carbonyl groups in the PE structure results in the formation of carboxylic acids as indicated by the decrease in the number of carbonyl groups observed in most isolates (Fig. 5). Carbonyl groups generated by exposure to UV radiation could have undergone hydrolysis by primo-colonizers, which suggest possible metabolic activity through the β-oxidation pathway [3,5,92]. The region also represents the presence of primary alcohols that are products of terminal hydroxylation of alkanes, a significant step in enhancing the hydrophilicity of alkane substrates [63,64]. Additional peaks in this region of the spectra coincide with previous studies [30,46], which confirmed capability for hydrocarbon degradation by most of the taxa found in this study.

Oxidized groups were identified as additional peaks in the 1600 cm^{-1} region, which may be attributed to the C═O stretch of ketone and aldehydes [87]. Four isolates (MB1, MB4, MB9, MB10) were found to induce these changes, which suggest further oxidation of the abiotically-generated carbonyl groups. Further oxidation of carbonyl groups facilitates their conversion into double bonds [61], as observed in the decreased CI and increased terminal double bonds (VBI; Fig. 5B, D, E). Additionally, production of alkene groups was also represented by the appearance of a new peak at around 600 cm^{-1} region (Fig. 6I and J; [60]). Formation of terminal C═C bonds is also distinguished by a significant increase in VBI, alongside a considerable decrease in CI (Fig. 5). These index changes are consistent with the evidence for biodeterioration reported by [2,3], who suggested the formation of terminal double bonds because of microbial enzymatic activity. Norrish type II reaction occurs when carbon double bonds are formed due to consumption of the carbonyl groups, releasing unsaturated chains that are detected as alkene groups [2].

The four isolates that were specifically found to form alkene groups upon interacting with the PE backbone were less explored for potential plastic degrading activity. *H. gudaonensis* has not yet been implicated in biodeterioration, however, the species was found to thrive in crude oil contaminated environments [85,88]. Existing reports on the plastic surface activity of *B. megaterium* were on the two strains isolated separately from a soil dumpsite [10] and a landfill [81]. *B. vietnamensis* was able to grow on minimal medium with LDPE powder and showed clearance on the media around the colony, indicating its potential to utilize PE as a substrate [72]. This study on the other hand is the first to report *H. maura* for its ability to induce modifications on the plastic surface, potentially *via* the production of mauran, implying it has mechanisms that could contribute to polymer degradation [6].

Interestingly, disappearance of peaks along 1100 to 1200 cm^{-1} of the spectra was observed for Cluster III isolates (Fig. 6F, G). Their absence denotes breakdown of functional groups represented by this region, such

as carboxylates, ester, ether, and alcohol moieties, and may be a consequence of preferential assimilation of oxidized polymer chains by bacteria [4]. Eyheraguibel et al. [27] showed evidence that ethanol, acetate, and formate, identified as oxidation products from abiotic treatment, are readily consumed by bacteria. Similar to the activity exhibited by Cluster III isolates, their study is in agreement with the proposed idea that oligomers containing at least one carboxylic group can be assimilated by the cells as fatty acid analogues and then metabolized further through the β-oxidation pathway [3,5,47]. In addition, a decrease in IDBI was observed (Fig. 5), possibly indicating decreased internal C═C bonds because of oxidation in the polymer backbone [92]. While this does not confirm that cleavage and assimilation of the polyethylene backbone did occur in the set-up, it can provide insights on the possible mechanisms of *H. cerina* and *H. denitrificans* within the plastisphere. To date, these species have not been reported to degrade plastics. However, in a simulated marine system tank experiment, *H. denitrificans* strain M29 was revealed to specifically associate with LDPE microplastics and was not isolated from other substrates other than MPs, possibly due to a plastic-specific metabolism [29].

The FT-IR analysis confirmed the ability of these isolates to modify the chemical structure of polyethylene after co-incubation *in vitro*. Spectroscopic studies on LDPE structure concurred that these types of changes are detected when biological activity on the substrate surface is present [74]. New functional groups are possible products of microbial enzymes that catalyze a succession of chemical modifications upon the polymer structure, such as hydrolysis, oxidation, reduction, and esterification [60]. Although the general pathway for PE utilization has already been established [32,76], it is not yet clear what taxonomic groups produce the new functional groups in the pathway. Identifying which part of the plastic backbone each primo-colonizing bacteria directly interacts with has implications on understanding their potential metabolic contributions within the plastisphere.

Incidentally, the observed shifts in the indices coincide with the previously noted changes reported as part of biodegradation [60,87]. To demonstrate occurrence of partial PE degradation, further analyses using more sensitive analytical techniques to investigate the chemical changes in the polymer should be carried out. Gel permeation chromatography (GPC) can be applied to analyze the possible changes in LDPE molecular weight. Growth rate quantifications of isolated strains on LDPE sheets can confirm assimilation of the plastic substrate. Measuring hydrophobicity changes of the LDPE sheets may indicate further surface deterioration caused by bacterial growth. Depolymerization of the polyethylene backbone may also reveal changes in the LDPE molecular weight, which can be evidence for partial biodegradation. While the results of this study do not necessarily present direct evidence for biodegradation, changes in the polymer backbone can indicate how these isolated primo-colonizers alter the structure of marine plastic debris. Omics approaches may also reveal the genes that are involved in the production of necessary metabolites for attachment and the biodegradation pathway, allowing them to be key primo-colonizers.

5. Conclusions

Primo-colonization has been recognized to be a key step that results in biofouling and modifications of the plastics, affecting its fate in the environment. Here, surface morphology of the plastic films showed bacterial attachment, increased roughness, pits, crevices, and EPS-like substance after co-incubation, confirming surface deterioration as a consequence of bacterial activity. The isolated bacteria were also shown to modify the chemical structure of PE by targeting different parts of the plastic backbone and its photo-oxidized by-products. These suggest that primo-colonizing bacteria may possess specific mechanisms for biodeterioration and bio-fragmentation, acting upon the oxidized and abiotically degraded surface of the polymer. Studying what role each of the pioneer bacteria play will provide an insight on the effect of initial microbial colonization to the plastic structure. Understanding the specific activity of a single species capable of modifying the plastic substrate may be used as a basis for selecting strains that possess genes functionally adapted to biodegradation of the target polymer and in the development of a microbial consortia that can degrade plastics more efficiently. Moreover, whole genome analysis of the isolates may reveal genes that confer metabolic capabilities adapted for plastics association. Ultimately, the functions exhibited by primo-colonizers within the plastisphere can be explored for further analyses on the impacts and fate of marine plastic debris to higher trophic levels, public health, and biogeochemical cycles.

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CRediT authorship contribution statement

Justine Marey S. Bitalac conceptualized and conducted project implementation, sample processing, data analysis and interpretation, prepared the draft, figures, and tables, and finalized the manuscript. Nacita B. Lantican participated in the conceptualization, technical advising, and finalization of the manuscript. Norchel Corcia F. Gomez reviewed some sections and helped finalize the manuscript. Deo Florence L. Onda conceptualized and designed the data analysis and interpretation, provided technical advising, reviewed, edited, and finalized the manuscript, and provided the funding through his grants.

Declaration of Competing Interest

The authors declare no competing interest.

Data Availability

Data will be made available on request.

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Environmental implication

Mismanaged plastic wastes resist decomposition and accumulate in the environment. Studies, however, also showed that naturally-occurring marine microorganisms associate with marine plastic debris. Here, cultivable primo-colonizing sediment bacteria from areas of Manila Bay with high plastics accumulation were isolated to detect their activity on the low-density polyethylene (LDPE) surface. Our isolates showed varying mechanisms for biodeterioration and bio-fragmentation of LDPE. Investigations on how pollutants are biotically modified provide insights on their portential fate and risks of possible products of bio-fragmentation in the marine environment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131124.

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