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Biodegradation of untreated plasticizers-free linear low-density polyethylene films by marine bacteria

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ABSTRACT

Polyethylene significantly contributes to marine plastic pollution. This study focuses on isolating bacteria from sea water and microplastic samples collected from the Tyrrhenian Sea and evaluating their ability to degrade virgin plasticizers-free linear low-density polyethylene (LLDPE) films. The isolates grew on the plastic film under aerobic conditions in shaken flasks leading to LLDPE mass losses of up to 2.597 ± 0.971 % after 60 days incubation. Biofilm formation on the film surface was confirmed by adhered protein quantification while film surface erosion and appearance of functional groups were revealed using SEM and FTIR analyses confirming biodegradation capabilities especially for isolates *Bacillus velezensis* MT9, *Vreelandella venusta* MT1 and *Vreelandella titanicae* MT11.

This is the first report on the biodegradation of plasticizers-free non pretreated LLDPE films by marine *Bacillus* sp. and *Vreelandella* sp.; most of the LLDPE biodegradation studies have been so far performed on plasticizer containing, pre-treated, or naturally weathered films.

1. Introduction

In 2021, the global production of plastics reached a total output of 390.7 million tons (Mt), with Europe contributing for 57.2 Mt. of this amount (Janssens, 2022). In the same year, the packaging and building & construction sectors used the 44 % and 18 %, respectively, of such an amount worldwide. The major end markets for plastics in Europe were similarly driven by packaging (39.1 %) and building & construction (21.3 %) along with automotive industry (8.6 % in Europe and 8 % globally) as third-largest market (Janssens, 2022). The widespread and multisectoral use of plastics is due to their multifaceted properties, including versatility, transparency, durability, stability, lightness, cost-effectiveness, etc. In 2010, between 4.8 and 12.7 million tons of plastic waste were released into the oceans and seas (Gao et al., 2022; Jambeck et al., 2015) where they have been found floating from the polar regions to the equator (Anand et al., 2023). The post-consumer plastic waste disposed in landfill are often subjected to photooxidation and degradation (Miri et al., 2022; Niu et al., 2023) with the production of microparticles (e.g., particles with a size from 1 μm to 5 mm) (MPs) that then enter marine ecosystems (Bitalac et al., 2023), rivers (Dong et al., 2023) and lakes (Fischer et al., 2016; Su et al., 2016). Two main sources of MPs are reported, primary and secondary MPs. The first group includes microbeads, fibre fragments from in-use wear or fabric washing and exfoliants used to remove dead cells from the external layer of the skin. Furthermore, MPs are also generated by plastics used in air blasting technology or in the petroleum industry. Secondary MPs originate from fragmentation or weathering of larger plastics that have entered the marine environment and here, they represent the prevalent source of MPs (Miri et al., 2022). In the marine environment, MPs can act as adsorbents and carriers of persistent organic and hydrophobic pollutants (POPs) such as polychlorinated biphenyls (Rodrigues et al., 2019), polycyclic aromatic hydrocarbons and organochlorine pesticides (Dong et al., 2023; Jiménez-Skrzypek et al., 2021). These toxic pollutants at concentrations 10–100 times higher than in the water, combined with plasticizers and plastic additives enter the food chain where they exert potential lethal effects (Ghatge et al., 2020; Miri et al., 2022; Naqash et al., 2020; Raddadi and Fava, 2019).

MPs along with those from polyethylene (PE) have recently been recognized as a significant threat to the marine ecosystem (Ammar et al., 2022; Hamed et al., 2022). Additional MPs sources are polypropylene,

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polyvinylchloride, polyethylene terephthalate, polylactic acid, polyvinyl alcohol, polycaprolactone, polyurethane (PUR) and polyhydroxybutyrate (PHB) (Miri et al., 2022). Notably, Suaria et al. (2016) reported that approximately 52 % of the MPs recovered from water of the Mediterranean Sea is represented by PE. PE finds extensive application in the production of plastic bags, bottles, plastic tubes, water pipes, and more. Indeed, it is the largest plastic produced globally. Specifically, in 2021, (LLD, LD) PE and (MD, HD) PE represented 14.4 and 12.5 % of the total fossil-based plastic production, respectively (Anand et al., 2023; Janssens, 2022). The chemical/physical features that render PE convenient in many everyday life applications render it also recalcitrant to degradation because of its insolubility in water, degree of crystallinity, hydrophobicity and high molecular weight (Ghatge et al., 2020; Harshvardhan and Jha, 2013; Wang et al., 2023).

PE-MPs pose severe concerns for the marine ecosystems and solutions to prevent, remove or degrade PE-MPs in such ecosystems have been investigated and assessed (López-Vázquez et al., 2024; Mustapha et al., 2024). Up to now, physico-chemical, microbial, or a combination of both methods have been tested to degrade PE (Ghatge et al., 2020; Niu et al., 2023; Wang et al., 2023). Thermal and UV pre-treatment or a combination of both are used to reduce the polymer chains and/or to oxidize them with the introduction of carboxyl, carbonyl, and hydroxyl groups thus enhancing their biodegradability (Khandare et al., 2021; Raddadi and Fava, 2019). Microorganisms can affect the physico-chemical structure of PE-MPs by degrading and/or modifying the polymer chemical structure and surface.

Various microorganisms, including algae (*Anabaena* sp., *Chlorella* sp.), fungi (*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Alternaria* sp., *Zalerion* sp.), and bacteria (*Alcanivorax* sp., *Ideonella* sp., *Mycobacterium* sp., *Bacillus* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Rhodococcus* sp. and *Azotobacter* sp.), exhibited degradative activities towards plastic compounds (Danso et al., 2019; Gao et al., 2022; Giacomucci et al., 2019, 2020; Khandare et al., 2021, 2022; Miri et al., 2022; Paço et al., 2017; Restrepo-Flórez et al., 2014; Wayman and Niemann, 2021). However, the biodegradation of PE by bacteria and fungi is not extensively documented. Most of the available reports deal with PE biodegradation in terrestrial habitat (such as soil from landfill sites and composting) and only a few are reporting the same processes in marine environments (Gao and Sun, 2021; Goudriaan et al., 2023; Mohanan et al., 2020; Raddadi and Fava, 2019; Wayman and Niemann, 2021). Therefore, a more comprehensive study on the biodegradation of PE in MPs polluted marine habitats is necessary.

Among the marine microorganisms described so far for the PE biodegradation, *Bacillus* sp. and *Halomonas* sp. strains were reported as able to degrade LDPE (Khandare et al., 2021; Kumari et al., 2019; Syranidou et al., 2019). It is worth noting that recently the taxonomy of the family Halomonadaceae has been rearranged and several bacterial species within the genus *Halomonas* have been reclassified to new genera as proposed by de la Haba et al. (2023) and then validated in list n°. 216 by Oren and Göker (2024). For example, *H. venusta* and *H. titanicae* have been moved to the genus *Vreelandella*. Harshvardhan and Jha (2013) isolated two LDPE degrading strains from pelagic waters of the Arabian Sea and identified them as *Bacillus pumilus* (isolate M27) and *Bacillus subtilis* (isolate H1584) based on 16S rRNA analysis. The isolates exhibited a mass loss of autoclaved LDPE commercial bags up to 1.5 % (M27) and 1.75 % (H1584) after 30-day incubation period. Khandare et al. (2021) reported the biodegradation potential of new marine *Halomonas* sp. which demonstrated a maximum weight loss of unpretreated commercial LDPE film of 0.78 and 1.72 % after 30- and 90-days aerobic incubation, respectively. No clarification on LDPE film additives was provided. Novotný et al. (2018) reported that a *Bacillus amyloliquefaciens* strain isolated from composted plastics was able to reduce the weight of ɣ-irradiation/high temperature-pretreated LLDPE within 40–60 days, while no biotic attack was reported for the virgin polymer.

In this context, the aim of our study was to isolate and identify novel marine bacteria from MPs-contaminated actual site. Then, we assessed their ability to degrade untreated plasticizers-free LLDPE films under lab conditions. The biodegradation assays showed that *Bacillus* sp. and *Vreelandella* sp. isolates slowly degrade LLDPE films and to the best of our knowledge, this is the first report demonstrating this. This sheds light and addresses a knowledge gap in the current scientific literature on the fate of LLDPE in seawater. Indeed, existing literature predominantly focuses on biotic attack of commercial, pre-treated, or naturally weathered LDPE or HDPE.

2. Materials and methods

2.1. Bacterial isolation

Bacteria were isolated from marine samples (sea water and microplastics) collected from three different sampling sites in the Tyrrhenian Sea. The following cultivation media were used: Tryptic Soy broth supplied with 30 g/L NaCl (mTSB), Bacto Marine 2216 (BM) and modified mineral salt medium (mMSM) having the following composition: KH_2PO_4 0.7 g/L; Na_2HPO_4 0.9 g/L; $NaNO_3$ 2.0 g/L; $MgSO_4{\cdot}7H_2O$ 0.4 g/L; $CaCl_2$ 0.1 g/L; NaCl 30 g/L; trace element solution 2.0 mL/L; pH 6.7; trace element solution: $FeSO_4{\cdot}7H_2O$ 2.0 g/L; $MnSO_4{\cdot}4H_2O$ 1.5 g/L; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.6 g/L (Giacomucci et al., 2019). Five milliliters of each sample were supplemented with 5 mL of medium and the flasks were incubated at 30 °C on a rotary shaker (150 rpm). After incubation for 72 h, bacterial isolation was performed by spreading serial dilutions in sterile saline solution (30 g/L NaCl in distilled water) on plates (agar: 15 g/L) of mMSM, mTSB and BM. Agar plates were incubated at 30 °C and morphologically distinct colonies were purified after three successive streakings on the same medium and preserved at −80 °C in the respective broth of selection supplemented with 20 % (v/v) glycerol until use.

All reagents were purchased from Sigma Aldrich Merck (Darmstadt, Germany).

2.2. Bacterial identification

Bacterial isolates were identified by partial 16S rRNA and *gyrB* genes amplification using primers and conditions according to (Romano et al., 2020; Zlatković et al., 2020) and sequencing. The similarity search was performed *in-silico* by BLASTn and the evolutionary analyses were conducted in MEGA11 using the Neighbor-Joining method (Saitou and Nei, 1987; Tamura et al., 2021). Bootstrap with 1000 replicates was done to achieve the dendrogram for sequence homology with the nearest species. The evolutionary distances were computed using the Maximum Composite Likelihood method involving 45 nucleotide sequences. All ambiguous positions were checked for each sequence pair.

2.3. LLDPE film

Virgin and plasticizers-free LLDPE films, with a thickness of 0.057 mm, were used. They were cut into small pieces (approximately 1.5 cm × 1.5 cm), cleaned with sterile distilled water, and sterilized by two successive cycles of 45 min immersion in 70 % (v/v) ethanol solution followed by 3 rinses with sterile distilled water before use (Giacomucci et al., 2019, 2020).

2.4. LLDPE biodegradation assays

Biodegradation assays by marine single bacterial isolates and artificial consortia were set up in mMSM at pH 7 supplemented with LLDPE film and yeast extract 0.5 g/L (ye). The artificial consortia were constructed based on pure isolate's ability to coexist together without inhibiting each other. For this purpose, the antimicrobial activity of their cell free supernatants (CFS) obtained after bacterial growth in mTSB was evaluated using the well-diffusion method (Sathiyaraj et al., 2021).

The bacterial pre-inoculum was prepared by transferring single colonies from agar plates to 100 mL flasks containing 20 mL of mTSB medium. The bacteria were grown overnight (at 30 °C, 150 rpm) and the biomass was recovered by centrifugation (Thermo Scientific SL 16R; 6000 rpm, 10 min, 15 °C). The bacterial cells were washed twice with sterile saline solution (9 g/L NaCl) and then inoculated at a final concentration of 10^6–10^8 CFU/mL.

The pre-inoculum of artificial consortia was prepared by combining the washed cells of individually cultivated pure isolates. Consortia formed by two isolates were combined at a ratio of 50 %, while those formed by three isolates were combined at a ratio of 33 %. Subsequently, they were inoculated at the same final concentration as the culture of LLDPE-pure isolates.

Diverse control conditions were concurrently established to assess the microbiological degradation of the target polymer (Giacomucci et al., 2019). Initially, mMSM added with ye was set up to evaluate the contribution of the ye to bacterial growth. Subsequently, mMSM with ye and glucose was analysed to investigate planktonic growth when an easily available carbon source was used. In addition, a condition without ye but with glucose was introduced to understand the contribution of ye and glucose. All the biotic conditions were inoculated following the same procedure as described for the condition with ye and LLDPE for both pure strains and artificial consortia. Finally, a set of abiotic controls were incubated under the same conditions for the entire duration of the experiments.

Biodegradation experiments were conducted at 30 °C and 150 rpm for 60 days for pure bacterial isolates and for up to 120 days in the case of artificial consortia.

2.5. Analytical methods

2.5.1. Evaluation of bacterial growth

Planktonic bacterial growth was monitored at various time intervals by cell counts using the drop plate method (De Giorgi et al., 2018). The growth curves were established by plotting the $\log_{10}$ CFU/mL as a function of time (days).

2.5.2. Evaluation of microbial colonization of the LLDPE film surface

Biofilm formation was assessed by quantifying the proteins adhered to the surface of LLDPE film at the end of experiments. For this purpose, to detach the proteins, LLDPE films were incubated overnight with 4 mL of 6 M urea solution at 4 °C on a rotating shaker (150 rpm). Proteins quantification was then performed on the resulting solutions according to Lowry method (Lowry et al., 1951) and 6 M urea solution was used as blank.

Furthermore, light microscopy (Zeiss, Germany) has been used for a preliminary observation of biofilm formations on LLDPE films.

2.5.3. Evaluation of LLDPE films biodegradation

2.5.3.1. Gravimetric weight loss. Measurement of the dry weight of LLDPE film was determined at the beginning (initial weight) and at the end of experiments (after biofilm removal). The LLDPE films were dried under vacuum at room temperature until the weight remained constant. Weight was measured using a balance with five digits accuracy and the gravimetric weight loss percentage (%) was calculated as follows:

$$\text{Weight loss}\ (\%) = (\text{Initial weight} - \text{Final weight})/\text{Initial weight}*100$$

2.5.3.2. Fourier-Transform InfraRed spectroscopy (FTIR) of polyethylene. FTIR spectra of three types of LLDPE films were analysed: bacterial-treated LLDPE, medium-treated LLDPE (abiotic) and an untreated LLDPE (virgin LLDPE). The spectra of the biofilm-cleaned and dried LLDPE films were recorded over the wavelength range of 650–4000 cm^{-1} using an Agilent's Cary 630 spectrometer operating in diamond ATR mode. A total of 100 scans were taken for each spectrum.

2.5.3.3. Scanning electron microscopy (SEM) of LLDPE films. The biofilm-free LLDPE films which showed statistically significant gravimetric weight loss % ($p < 0.05$) compared to the abiotic control as well as biotic induced alteration of the LLDPE FTIR-spectra were analysed by SEM to explore modifications in the morphological structure of the film due to microbial attack. The biofilm-free films were coated with a fine layer of gold of approximately 9.8 nm to increase conductivity and the surface was observed at diverse degree of magnifications from 5.0 K× to 100.0 K×.

2.5.4. Statistical analyses

Results of gravimetric weight loss (%) and proteins quantification were statistically evaluated using one-way ANOVA. Post-hoc Tukey test was applied to determine whether weight loss (%) and proteins quantification showed significant different extents compared to their abiotic controls. Statistically significant results were depicted by p-values < 0.05. All the analyses were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). All experiments were performed in triplicate.

3. Results and discussion

3.1. Identification of the bacterial isolates

Forty bacterial isolates were obtained from marine samples collected from three different sites in the Tyrrhenian Sea of the Italian region of Tuscany. Among them, seven isolates (MT1, MT8, MT9, MT11, MT18, MT22, and MT35) were selected based on their phenotypic differences, sampling sites and growth media. Isolates MT8, MT11, MT22 and MT1 had between 99.36 % and 99.86 % sequence similarities with their closest relative type strains in the NCBI database based on their partial 16S rRNA gene and between 99.74 % and 100 % based on *gyrB* gene sequences (Table S1). *Vreelandella titanicae* BH1 (previously *Halomonas titanicae* BH1 (de la Haba et al., 2023; Oren and Göker, 2024)), isolated from the rusticles of the RMS Titanic wreck (Sánchez-Porro et al., 2010), was the closest relative species of the MT8, MT11 and MT22 isolates. *Vreelandella venusta* strain Hven7 and *Vreelandella venusta* DSM 4743 were the closest relative of MT1 isolate based on *gyrB* and partial 16S rRNA genes, respectively (previously *Halomonas venusta* (de la Haba et al., 2023; Oren and Göker, 2024)). The isolates MT9 and MT35 exhibited 16S rRNA gene sequence similarities of 99.64 % with *Bacillus velezensis* strains D103 and NN-FX52, respectively. Furthermore, their *gyrB* gene sequences showed similarities of 99.83 % and 99.91 % with *Bacillus velezensis* strain B268, respectively. The *gyrB* gene analysis can support species-level determination within the *Bacillus* sp. genus when similarities are >95.5 % (Adeniji et al., 2019; Wang et al., 2007, 2008).

For isolate MT18, the partial 16S rRNA gene sequence showed a 99.57 % similarity with *Stenotrophomonas maltophilia* SKK55, while *gyrB* gene showed a 99.65 % similarity with the same strain. The *gyrB* gene sequences provide higher species resolution for bacteria in the *Stenotrophomonas* genus compared to 16S rRNA gene sequences (Svensson-Stadler et al., 2012). The phylogenetic three based on *gyrB* gene sequences is shown in Fig. 1.

3.2. LLDPE film biodegradation assays by pure isolates

The seven isolates (MT8, MT11, MT22, MT1, MT9, MT35 and MT18) were evaluated for their potential ability to degrade LLDPE films under shaken flasks aerobic conditions for 60 days. Among them, five isolates denoted as MT8, MT11, MT1, MT9 and MT35 exhibited gravimetric weight loss (%) of the recovered films at the end of the experiments of 1.790 ± 0.230, 2.132 ± 0.911, 2.023 ± 0.191, 2.597 ± 0.971, 2.031 ± 0.479 %, respectively (Fig. 2). Conversely, films treated with MT18 and MT22 showed lower mass loss compared to the films exposed to the five most active isolates (data not shown). A mass depletion of 0.167 ± 0.117 % was observed under the same conditions for the abiotic control.

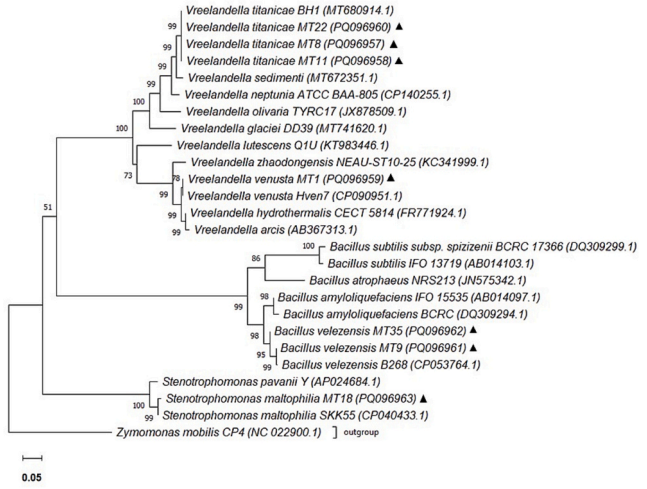

Fig. 1. Phylogenetic tree of isolates MT8, MT11, MT1, MT22, MT9, MT35, MT18 and their closest relative species based on *gyrB* gene sequences. *Zymomonas mobilis* CP4 was included as an outgroup. The tree was constructed using the neighbour-joining method. Bootstrap values expressed as 1000 replications are shown at branch points. Bars, 0.05 substitution per nucleotide position.

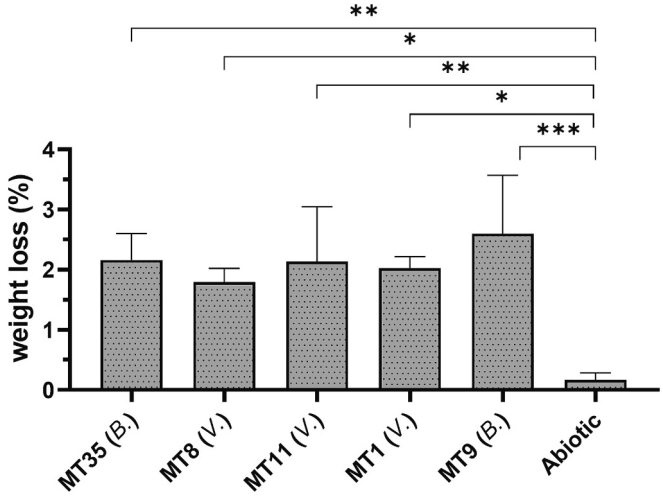

Fig. 2. Highest gravimetric weight loss (%) of biofilm-free LLDPE films recovered after 60 days incubation with the marine isolates MT35, MT8, MT11, MT1 and MT9 and under abiotic condition (ANOVA: $p < 0.0014$, F:7.039, R square: 0.7012). The Tukey's test was carried out to assess post hoc differences among the treatments. Statistically differences were represented as: $0.013 < p < 0.05$ (*); $0.006 < p < 0.045$ (**); $0.0002 < p < 0.006$ (***). Abbreviations: (*B.*): *Bacillus* sp.; (*V.*): *Vreelandella* sp.

This is one of very few evidences of plasticizers-free unpretreated LLDPE film biodegradation under laboratory-simulated marine conditions. Harshvardhan and colleague(s) reported weight losses of 1.0 %, 1.5 % and 1.75 % of LDPE film after 30 days incubation in the presence of marine isolates *Kocuria palustris*, *Bacillus pumilus* and *Bacillus subtilis*, respectively (Harshvardhan and Jha, 2013). The films used in their experiments were autoclave sterilized pieces of commercial LDPE. Moreover, Khandare et al. (2021) reported the biodegradation potential of new marine isolates of *Cobetia* sp., *Halomonas* sp., *Exigobacterium* sp. and *Alcanivorax* sp., which demonstrated a maximum weight loss of LDPE film of 1.72 % after 90 days aerobic incubation. *Halomonas* sp. strains are known for their salt tolerance and ability to produce interesting enzymes, biosurfactants, and exopolysaccharides (Ventosa et al., 2011). However, their potential for plastic degradation remains relatively unexplored (Martinez-Abarca et al., 2021). In addition, the bacteria isolated by Khandare et al. (2022) from various sites of Gujarat coast, e.g. two *Marinobacter* sp. (H-244 and H-246) and one *Bacillus subtilis* (H-248), were found to reduce the weight of plasticizer-free LDPE film (with 0.015 mm thickness) up to 1.68 % by H-246 after 90 days aerobic incubation. Liu and colleague(s) reported the isolation and identification of *Bacillus velezensis* isolate C5 according to 16S rRNA sequence from landfills soil samples (Liu et al., 2022). The commercial and briefly ultraviolet irradiated LDPE film exposed to C5 strain exhibited a mass loss of 1.00 ± 0.47, -3.93 ± 1.46 and 8.01 ± 1.70 % after 30, 60 and 90 days of aerobic incubation, respectively. The negative value recorded at 60th days incubation was linked to the dense attachment of the bacteria to the LDPE film surface.

Overall, the strains described in our study are among the few marine PE degrading microorganisms described so far under laboratory conditions mimicking those found in the marine environment. On the other hand, under real marine conditions, very few *in-situ* LLDPE biodegradation studies have been carried out. Under such conditions, the degradation is influenced by a variety of environmental factors that are not fully replicable under controlled laboratory settings. These factors include exposure to ultraviolet (UV) radiation, temperature fluctuations, wave currents, humidity, and the presence of a diverse microbial community, all of which can impact the biodegradation process. However, this does not imply that biodegradation rates in real marine environment, where the conditions are not optimized for the degradation of the polymer, would be much higher or that the biodegradation would be quicker compared to what we observed in our study under lab scenario (with optimal conditions for bacterial growth and activity). Indeed, a deep literature review was performed and the findings are that all studies have been performed using commercial/additive or PE blends and that a low biodegradation was observed under *in-situ* conditions. Nauendorf et al. (2016) carried out an *in-situ* study where commercial polyethylene carrier bags were incubated for 98 days in natural oxic and anoxic sediments from the Western Baltic Sea. The study revealed no evidence of plastic biodegradation and the lack of microbial attack was attributed to the presence of TiO_2 in the plastic bag (a compound with antimicrobial effect which hampered microbial colonization). Sudhakar et al. (2007) observed, after 6 months *in-situ* incubation in ocean waters, a maximum gravimetric weight loss of up to 2.5 % and 0.8 % for commercial LDPE and HDPE respectively. Rutkowska et al. (2002) found no evidence of polyethylene-starch blends biodegradation after 20 months incubation at a depth of 2 m in the Baltic Sea. Zettler et al. (2013) documented a microbial community colonizing plastic debris including PE sampled from North Atlantic surface waters. They observed pits on the plastic surfaces that resembled bacterial size and shape, suggesting potential biodegradation.

Also fungi have been reported as able to attack and degrade PE films (Ghatge et al., 2020). In particular, *Alternaria alternata* FB1 was able to efficiently degrade PE films by inducing the occurrence of carbonyl functional groups on the surface of the polymer, an evident reduction of the crystallinity degree (from 62.79 % to 52.02 %) of PE and the release of diglycolamine as the highest degradation product detected using GC–MS (Gao et al., 2022). According to a transcriptomic analysis performed on the strain, 153 enzymes are potentially involved in the PE biodegradation processes. Among them, two overexpressed oxidative enzymes in *E. coli*, i.e., laccase and glutathione peroxidase demonstrated a synergic degradation effect on PE film supported by reduction of its molecular weight in the fungus-treated PE compared to those of control.

The gravimetric weight losses reported are preliminary indications of PE biodegradation, but they need to be supported by other data such as the bacterial growth increment in the presence of the plastic film as well as biofilm formation, chemical (ATR-FTIR) and morphological (SEM) changes of the biomass-free film surface.

The most promising isolates, e.g. MT8, MT11, MT1, MT9 and MT35, displayed increase of planktonic cells concentration in the presence of LLDPE film and this suggests that the film components sustained their growth. Moreover, the bacterial growth was concurrently established for

control incubation conditions in the absence of LLDPE and with the addition of ye and/or glu as carbon sources. As reported in Fig. 3A, the initial *V. titanicae* isolate MT11 cell counts (of 7.71 ± 0.24 $\log_{10}$ CFU/mL in the mMSM + ye + LLDPE) remain constant until 40 days to then slightly decrease to 6.27 ± 0.80 $\log_{10}$ CFU/mL after 60 days incubation. Without LLDPE (mMSM + ye), cell counts marked fall (from 7.24 ± 0.12 to 5.25 ± 0.36 $\log_{10}$ CFU/mL) at the 14th day incubation to remain constant thereafter. These findings indicate that LLDPE film sustained bacterial growth probably acting as a slowly available additional carbon source as compared to ye. We supplemented 0.5 g/L of ye to provide additional carbon, nitrogen and vitamins sources for the microorganisms. In mMSM + ye supplemented with only 5 g/L of glucose (e.g., without LLDPE film), the bacterial cell concentration decreased dramatically after 10 days incubation, suggesting cell death due to nutrient depletion. The same trend was found with mMSM and the sole glucose. The *V. venusta* isolates MT1 and *V. titanicae* isolate MT8 showed very similar growth trends under the same mentioned conditions (data not shown).

Focusing on the growth of *B. velezensis* isolate MT9 (Fig. 3B), the conditions mMSM + ye + LLDPE, mMSM + ye + glu and mMSM + ye did not show significant differences and the same trend was observed under the different experimental conditions for *B. velezensis* isolate MT35 (data not shown). This *Bacillus* sp. growth trend may be attributed to its ability to form endospores (Biermann and Beutel, 2023; Hussey, 2013), but more probably to biopolymer accumulation, acting as carbon reservoir during nutrient depletion (Mohapatra et al., 2017).

The biodegradation of polymers normally begins with an effective colonization of the film surfaces followed by the excretion of extracellular polymer degrading enzymes (Gao and Sun, 2021; Syranidou et al., 2017). Light microscopy observation evidenced dense cell colonization of LLDPE films surface inoculated with isolates MT9, MT35 and MT11 (Fig. 1S).

The occurrence of biofilms on the inoculated and incubated LLDPE films is also confirmed by the significantly higher protein concentration ($p < 0.05$) quantified on the surface of these films with respect to the one on the same films incubated under abiotic conditions at the end of the experiments (60 days) (Fig. 4). Indeed, extractable protein content and the abundance of cells within biofilms follows a direct correlation as has been proposed previously by Ragusa et al. (2004) and Wilson et al. (2017). Isolate MT1 exhibited the highest protein concentration (16.83 ± 2.60 μg/cm^2) over twice higher than that displayed by MT11 and MT9 isolates (with values of 6.84 ± 4.11 ($p < 0.0075$) and 8.10 ± 2.10 μg/cm^2 ($p < 0.0328$), respectively). Similar results were observed on commercial LDPE films exposed to *Pseudomonas* sp. isolate AKS2 (Tribedi and Sil, 2013). During the initial phase of the 5-day incubation, extractable protein levels reached up to 5.0 μg/cm^2 LDPE, gradually decreasing to 4.0 μg/cm^2 LDPE by the end of the 45-day incubation period in a medium containing mineral oil. Lower extractable protein content was observed for conditions without mineral oil suggesting that it promotes the biofilm formation and then LDPE degradation. Conversely, a negative effect on degradation level was observed supplementing only tween 80 indicating that it prevents biofilm formation (Tribedi and Sil, 2013). Gilan and colleagues found that the protein content on UV-irradiated PE treated with *Rhodococcus ruber* C208 increased rapidly (to ~2.8 μg protein/cm^2 PE) over 2–3 days of incubation, decreasing then to minimal levels (to ~0.8 μg protein/cm^2 PE) after 8 days, in a mineral medium with mineral oil as a co-inducer (Gilan et al., 2004).

3.3. Surface analysis of LLDPE film using scanning electron microscopy

Surface morphological changes of biofilm-free LLDPE films incubated in the presence of the most active LLDPE degraders or under abiotic conditions were assessed by SEM analysis at the end of incubation (60 days) (Fig. 5). In contrast to the control films, which displayed a smooth surface, the LLDPE films exposed to the isolates that ensured higher film weight losses revealed variable degree of surface deterioration, damaged layers, fractures and abrasions. The isolate *B. velezensis* MT9 showed the most important surface alteration followed by *V. venusta* MT1 and *V. titanicae* MT11 (Fig. 5). Similar findings were also reported in literature for PE films exposed to active PE-degrading microbes (Gao et al., 2022; Khandare et al., 2021, 2022; Kumari et al., 2019; Z. Li et al., 2020).

3.4. FTIR analysis

The impact of the microbial colonization of the LLDPE films was assessed via FTIR analyses, which were carried out on the films incubated with the most promising isolates i.e., *B. velezensis* MT9 and *V. venusta* MT1 and *V. titanicae* MT11. In the case of the films incubated with *B. velezensis* MT9 (Fig. 6A), a decrease of two characteristic peaks (2915 and 2848 cm^{-1}) that fall in the region of the C—H stretching (Jung et al., 2018) were observed when compared with the untreated LLDPE. A similar result was highlighted by Khandare and colleagues who observed a decrease in peak intensity at 2800–3000 cm^{-1} region for PE films incubated with *Marinobacter* and *Bacillus* strains (Khandare et al., 2022). Moreover, the same authors observed a similar trend for other strains belonging to *Cobetia* sp., *Exiguobacterium* sp. and *Alkanivorax* sp. genera (Khandare et al., 2021). Another peak was observed at 3309 cm^{-1} and falls in the spectrum region (3200–3600 cm^{-1}) of hydroxyl group (-OH). The presence of hydroxyl group in the bacterial treated polymer was also reported by other researchers (Gao and Sun,

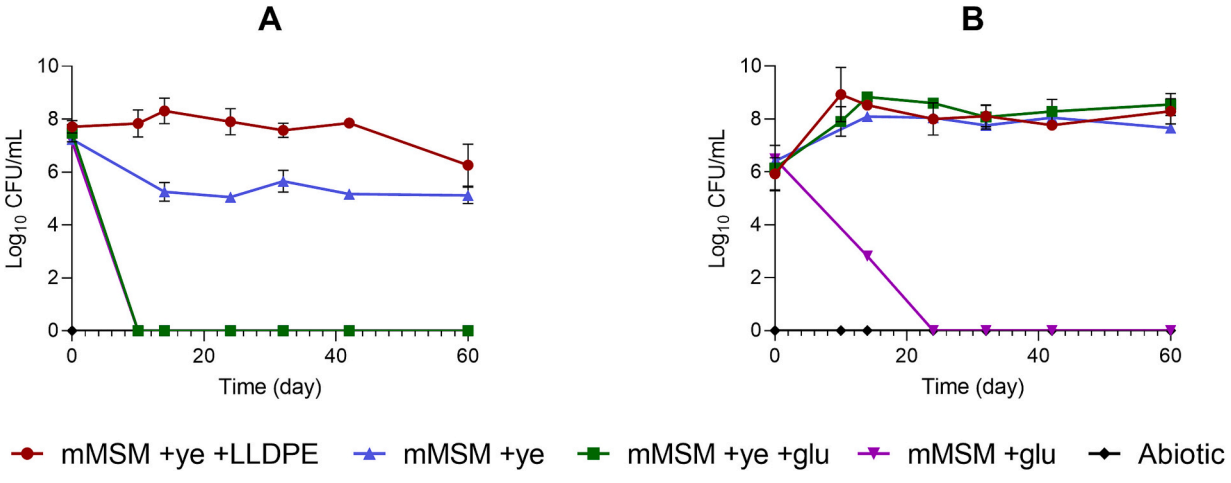

Fig. 3. (a, b). Growth curves of *V. titanicae* isolate MT11 (A) and *B. velezensis* isolate MT9 (B) in mMSM supplemented with different carbon sources.

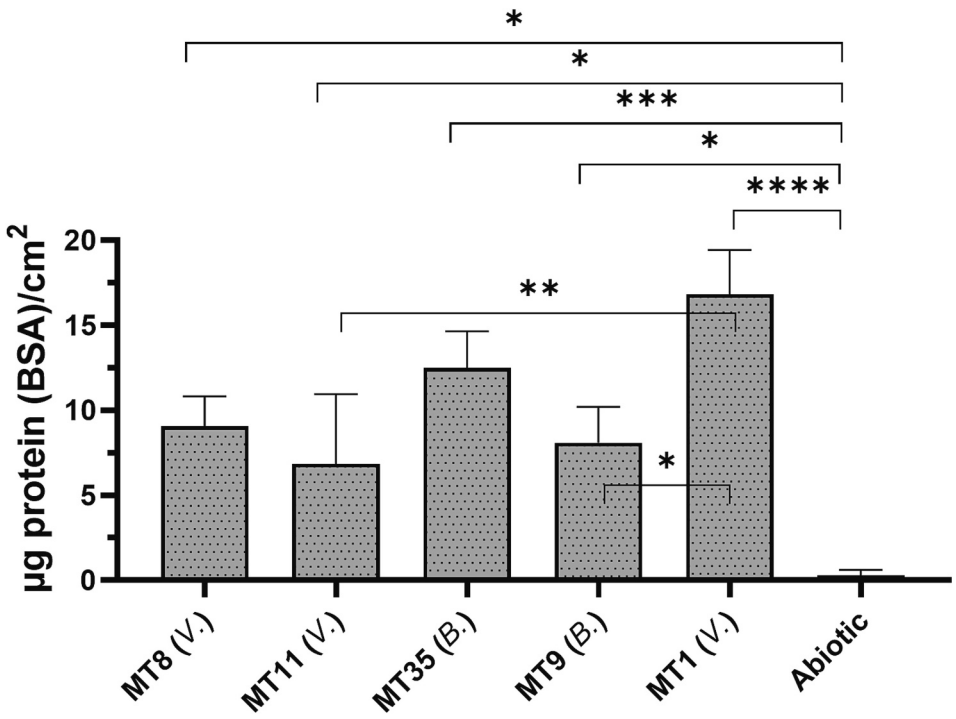

Fig. 4. Quantification of adherent proteins for the LLDPE films that had been incubated with selected pure marine isolates (MT35, MT8, MT11, MT1, and MT9) and under abiotic conditions for a 60-day period (ANOVA: $p < 0.0001$, F: 16.98, R square: 0.8853). (*B.*): *Bacillus* sp.; (*V.*): *Vreelandella* sp. The Tukey's test was carried out to assess post hoc differences among the treatments. Statistical differences were represented as: $0.013 < p < 0.05$ (*); $0.006 < p < 0.045$ (**); $0.0002 < p < 0.006$ (***); $p < 0.0001$ (****).

2021; Z. Li et al., 2020). We observed another peak at 1650 cm^{-1} that was not present under abiotic conditions and in the virgin polymer and we attributed it to terminal double bond formation (C=C). A similar result was also reported by Harshvardhan and Jha (2013) who noticed an increment in the vinyl bond index (VBI) compared to the abiotic control in PE film exposed to specialized microorganisms. Moreover, at 908 cm^{-1} a slight increment in intensity compared to controls was observed; this shift in intensity has been attributed to an internal C=C that falls in the region of 905–915 cm^{-1}. The formation of terminal double bonds due to biodegradation has been described by other scientists (Albertsson et al., 1987; Harshvardhan and Jha, 2013; Syranidou et al., 2019) with three different isolates belonging to *Kocuria* and *Bacillus* genera. They proposed that the formation of double bonds was possible due to Norrish type II reaction or by the synthesis of esters, but the connection of this with the PE biodegradation needs further clarification. However, the unexpected C=C peak could indicate a high degradation specificity on C=C which can be more easily attacked/more reactive than C—C bond. Several low-intensity bands were also found in the LLDPE film incubated with MT9 located in the region of 1275–1025 cm^{-1} that is characteristic of the stretching vibrations of C—O groups due to the -OH group. Similar peaks in that region were also observed by others (Gao et al., 2022; Khandare et al., 2021, 2022; Satlewal et al., 2008).

The *Vreelandella* sp. treated film displayed a spectrum similar to the one of the non inoculated polymers (Fig. 6B). This may indicate that bacterial cells attacked/used the outer layers (surface biodegradation) of LLDPE. Similar findings were reported for weathered LDPE by Syranidou et al. (2017).

The different chemical alterations found on the film polymer surface seem to indicate that *B. velezensis* degrades PE via a pathway different from that used by *Vreelandella* sp. The mechanism of PE degradation is not fully understood. Based on the available literature reports (Gao et al., 2022; Li et al., 2024; Rong et al., 2024a,b; Zadjelovic et al., 2022), the mechanism for PE biodegradation might involve in a first step, the non-specific oxidation of the polymer through the production of extracellular reactive oxygen species. Then enzymes like peroxidases, oxygenases and laccases break PE into smaller fragments that are then transported into the microbial cells and further degraded by enzymes like hydroxylases, monooxygenases and lipases, following pathways similar to those for alkanes biodegradation. The intermediates are converted into alcohols (monooxygenase), then oxidized to aldehydes (alcohol dehydrogenases) and finally to fatty acids (aldehyde dehydrogenases) that enter the β-oxidation cycle. These water-soluble intermediates are used in microbial metabolism, leading to the complete mineralization of PE into energy source, CO_2 and H_2O (Gao et al., 2022; Li et al., 2024; Rong et al., 2024a,b; Zadjelovic et al., 2022).

With regard to *B. velezensis* isolate MT9, a hypothetical biodegradation mechanism may be proposed based on the few data available from FTIR analysis which showed C=C and -OH groups in the residual PE, on the fact that *B. velezensis* strains are known for their ability to produce peroxidases (Al-Dhabi et al., 2020; Chen et al., 2023) and laccases (T. Li et al., 2020) and on reports available in literature on PE biodegradation mechanisms (Gao et al., 2022; Li et al., 2024; Rong et al., 2024a,b; Zadjelovic et al., 2022). This degradation mechanism, described in Fig. 7, might include the production of extracellular enzymes and ROS, the ROS attack leading to the formation of C=C bonds and -OH groups. Then different enzymes participate to the degradation by breaking the C—C bonds and the release of short chain products that are then up-taken by the cells, further oxidized and mineralized through beta-oxidation and TCA cycle. However, further analyses need to be performed in order to better determine the mechanism of LLDPE biodegradation by *B. velezensis* isolate MT9.

3.5. LLDPE film biodegradation by selected artificial consortia

Four different artificial consortia were constructed (as reported in the Materials and methods section) and then their ability to biodegrade unpretreated additive-free LLDPE films were evaluated (consortia

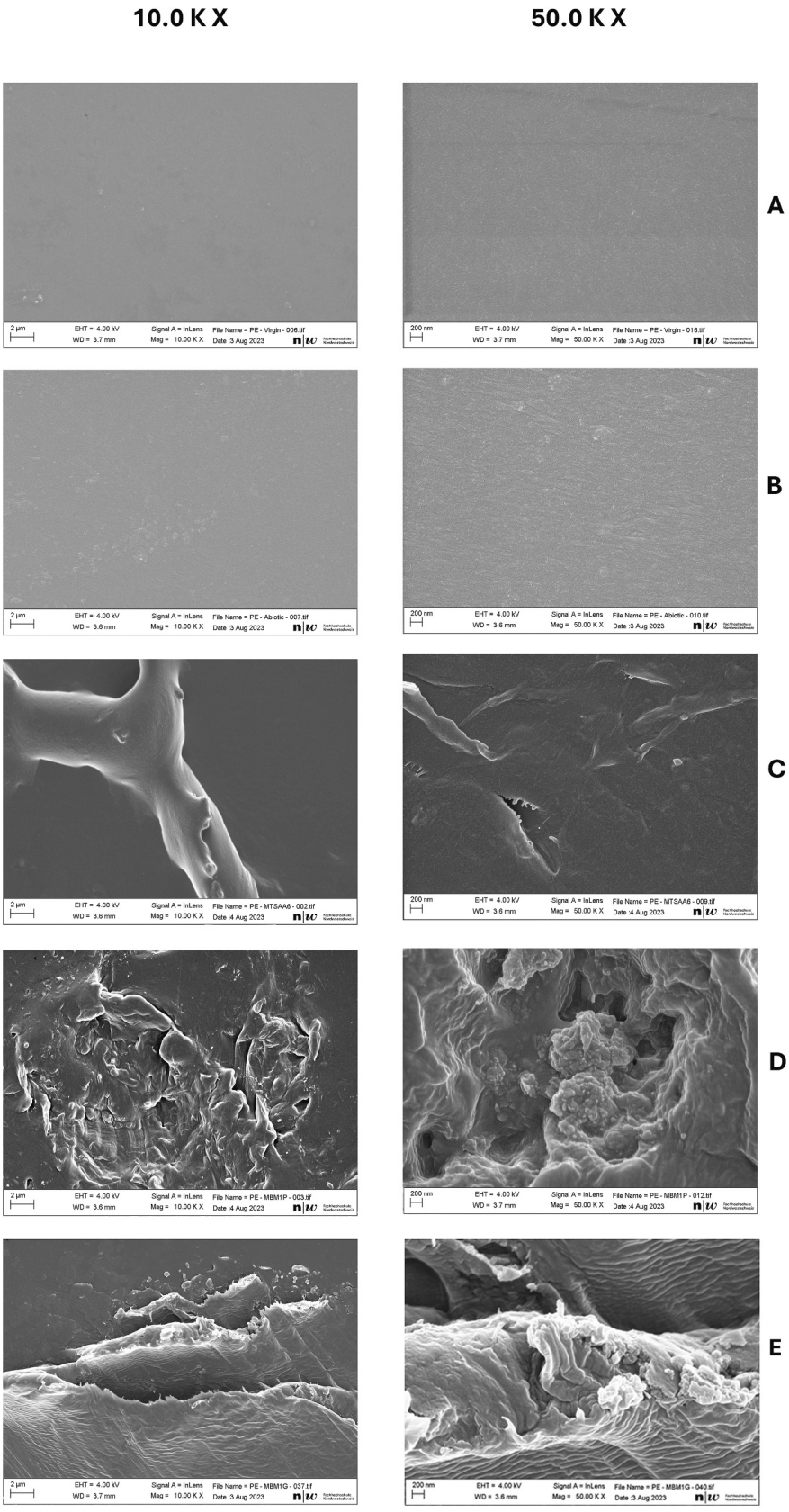

Fig. 5. SEM images at 10.0 K× and 50 K× magnifications of biofilm-free LLDPE films incubated with *B. velezensis* isolate MT9 (E), *V. venusta* isolate MT1 (D) and *V. titanicae* MT11 (C) or abiotic conditions (B) for 60 days and untreated-LLDPE (A).

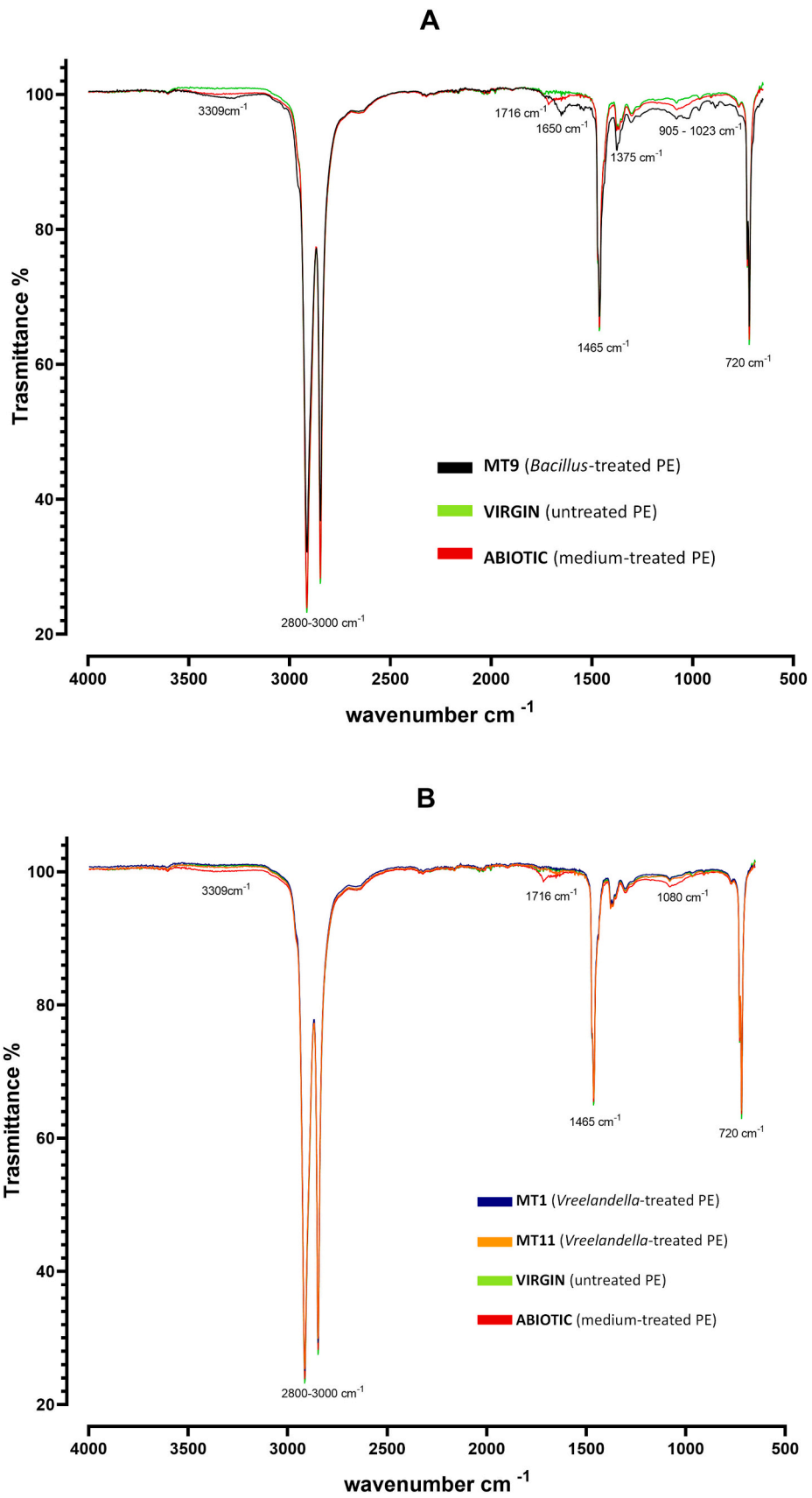

Fig. 6. (a, b). A) FTIR spectra of *Bacillus*-treated PE (MT9), medium-treated PE (abiotic) after 60 days incubation and untreated-PE (virgin polymer). B) FTIR spectra of *Vreelandella*-treated LLDPE (MT1 and MT11), medium-treated PE (abiotic) after 60 days incubation and untreated-PE (virgin polymer).

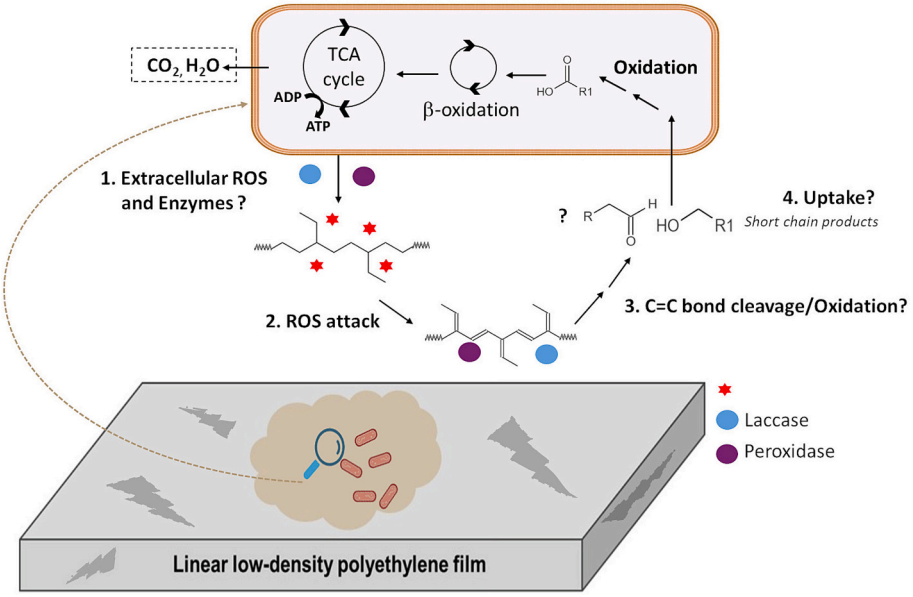

Fig. 7. Hypothetical degradation pathway of LLDPE by marine *B. velezensis* isolate MT9. The degradation process might include the production of extracellular enzymes and ROS, the ROS attack leading to the formation of C═C bonds and -OH groups. Then different enzymes participate to the degradation by breaking the C—C bonds and the release of short chain products that are then up-taken by the cells, further oxidized and mineralized through beta-oxidation and TCA cycle.

combination in Table 1). The general cell growth trends showed that the isolates can coexist for the whole duration of the experiment, but the concentration of living cells constantly decreases in time (from 7.83 ± 0.49 $\log_{10}$ CFU/mL to 6.80 ± 0.74 and 5.47 ± 1.08 $\log_{10}$ CFU/mL after 60 and 120 days incubation, respectively).

This may indicate that consortia components are in a state of stress due to scarce availability of carbon sources, or they might have adhered to the LLDPE surface forming biofilm resulting in a lower planktonic cell density. However, no significant increase in the concentration of proteins associated with biofilm was observed on the recovered films between 60- and 120-days incubation (data not shown).

The highest gravimetric weight losses were recorded for the LLDPE films incubated with the consortia MT11 + MT9 (1.356 ± 0.093 %) and MT11 + MT1 + MT22 (1.912 ± 0.287 %) after 60- and 120-days incubation, respectively. While negligible LLDPE weight losses were recorded for medium treated incubations (Table 1). Even with an extended incubation period, the highest mass loss for artificial consortia was relatively lower than those recorded for LLDPE films incubated with pure isolates. Nevertheless, the gravimetric weight losses suggest that the consortia are attacking LLDPE, albeit at a lower extent than the pure isolates. Thus, no significant improvement in biodegradation was observed with the use of consortia. Some of the lower extents of biodegradation observed could be due to possible phenomena of bacterial inhibition in biofilm formation due to polymer surface competition. Moreover, the ATR-FTIR spectra of the LLDPE films incubated with the artificial consortia that exhibit highest weight loss % at 60 and 120 days showed no enhancement in biodegradation. Specifically, the formation of characteristic peaks due to microbial attack was not observed but a spectrum similar to the virgin polymer was obtained (data not shown).

Table 1
Gravimetric weight loss (%) of LLDPE films after 60- and 120-days incubation with different artificial consortia MT11 + MT9, MT11 + MT1, MT11 + MT1 + MT18 and MT11 + MT1 + MT22 or under abiotic conditions. *Abbreviations: V.*: *Vreelandella* sp.; *B.*: *B. velezensis*; *S.*: *S. maltophilia.*

Artificial consortia	Gravimetric weight loss (%)	
	60 days	120 days
MT11 (*V.*) + MT9 (*B.*)	1.356 ± 0.093	1.179 ± 0.236
MT11 (*V.*) + MT1 (*V.*)	1.313 ± 0.207	1.496 ± 0.224
MT11 (*V.*) + MT1 (*V.*) + MT18 (*S.*)	0.557 ± 0.102	0.741 ± 0.111
MT11 (*V.*) + MT1 (*V.*) + MT22 (*V.*)	1.106 ± 0.609	1.912 ± 0.287
Abiotic	0.167 ± 0.117	0.031 ± 0.044

The construction of PE degrading consortia composed of PE degraders using a bottom-up approach has been poorly reported and mainly in terrestrial site (Zhang et al., 2023). Park and Kim (2019) reported that a mixed bacterial consortium mainly consisting of *Bacillus* sp. and *Paenibacillus* sp. isolated from a landfill site could attack PE microplastics (MPs-PE, CAS number 9002-88-4). The MPs-PE mass loss was 14.7 % after a 60-days incubation period in basal medium, while mass loss around 5 % under abiotic conditions was recorded, suggesting that recovering all the PE granules for weight loss determination could be challenging due to their very small size.

4. Conclusions

In this work aerobic marine bacteria from sea water and MPs samples collected from the Tyrrhenian Sea were isolated, characterized, and investigated for their ability to biodegrade virgin plasticizers-free LLDPE film. Five pure isolates, that belong mainly to the genera *Bacillus* and *Vreelandella* showed the ability to grow on and biodegrade LLDPE film in 60 days of aerobic incubation. Specifically, the most active isolate was shown to be *B. velezensis* MT9 which exhibited the maximum LLDPE films mass loss of 2.597 ± 0.971 %, along with chemical (FTIR) and morphological changes (SEM) of the biomass-free LLDPE film recovered at the end of the incubation. Additionally, four artificial consortia were constructed, and their biodegradation abilities were assessed under the same biodegradation assay of pure isolates over a period of up to 120 days. The consortia exhibited a maximum weight loss of 1.912 ± 0.287 % by the end of the experiment, particularly in the consortium formed by three *Vreelandella* strains. This result indicates that no significant improvement was observed with mixed consortia. Although cross-inhibition tests were performed for the selection of the isolates to be used in the artificial consortia reconstruction, we cannot exclude that the presence of the polymer in the growth medium has led to the production of inhibitory substances by some of the isolates towards each-other or to the production of compounds that have been exploited as carbon source leading to the decrease of the efficiency of degradation.

Moreover, as we reported in discussion session, another possible reason could be due to the fact the adhesion to the polymer film surface and biofilm formation for some isolates was inhibited as a result of bacterial competition.

Hence, the results of this study highlight how marine *B. velezensis* isolate MT9 followed by *V. venusta* MT1 and *V. titanicae* MT11 can slowly attack virgin and plasticizers-free LLDPE under laboratory conditions. This sheds light and addresses a knowledge gap in the current scientific literature on the potential fate of LLDPE in seawater. Indeed, existing literature predominantly focuses on biotic attack of commercial, pre-treated, or naturally weathered LDPE or HDPE. Further experiments are necessary to evaluate their biomineralization activities using a stable isotope tracing assay with ^{13}C-labelled polyethylene to obtain unambiguous proof of $^{13}CO_2$ production from ^{13}C-PE.

CRediT authorship contribution statement

Kejvin Bajo: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Roberta Romano:** Writing – original draft, Visualization, Investigation. **Boris Kolvenbach:** Writing – original draft, Visualization. **Seyed Amirabbas Nazemi:** Visualization, Investigation. **Patrick Shahgaldian:** Writing – review & editing. **Philippe F.-X. Corvini:** Writing – review & editing. **Fabio Fava:** Writing – review & editing, Resources. **Noura Raddadi:** Writing – review & editing, Visualization, Supervision, Data curation, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.117115.

Data availability

Data will be made available on request.

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