



New autochthonous bacterial isolates: probiotic potential and microplastic biodegradation performance

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ABSTRACT

Contemporary research prioritizes bacterial consortia that combine probiotic efficacy with environmental detoxification. From this screening, two novel autochthonous isolates — *Hafnia alvei* UUNT_MP41 and *Hafnia paralvei* UUNT_MP29 — were selected for their robust probiotic phenotypes and potential to catalyze microplastic biodegradation. Whole genome sequencing (WGS) identified critical functional genetic elements, the *tesA* and *lipR* genes, both esterase homologs (of PpEst and EstC9) that have been implicated in polymer breakdown. These findings validate the candidacy of *Hafnia alvei* UUNT_MP41 and *Hafnia paralvei* UUNT_MP29 as next-generation, dual-function probiotics, establishing a new paradigm for biotic formulations that offer simultaneous therapeutic and ecological benefits.

1. Introduction

Modern biotechnology is increasingly focused on discovering novel microbial strains that offer a dual benefit: therapeutic potential as probiotics and robust capabilities for environmental bioremediation. The gastrointestinal tract (GIT) of aquatic species, such hinge in global food supplies, the stability and composition of the fish gut microbiome directly influence disease mitigation and optimal growth (De Schryver and Vadstein, 2014; Li et al., 2017). Consequently, isolating beneficial autochthonous strains, such as those derived from the common carp (*Cyprinus carpio*), is paramount for formulating effective probiotic supplements (Kamada et al., 2013). Simultaneously, the planet contends with a growing environmental catastrophe caused by plastic accumulation. Microplastics (MPs), defined as fragments under 5 mm, are pervasive contaminants in

all ecosystems, particularly in aquatic environments (Andrady, 2011). Their consumption by marine and freshwater fauna leads to trophic transfer through the food web, creating serious public health concerns (Mato et al., 2001; Browne et al., 2008). Given that conventional physical and chemical cleanup methods are often inefficient and ecologically detrimental, there is a compelling need for sustainable, biological alternatives, notably microbial degradation (Othman et al., 2021). This convergence of environmental and health crises has spurred the development of dual-function microorganisms designed to provide both host benefits and ecological utility. Accordingly, this investigation aimed to exploit the rich microbial diversity of the *C. carpio* intestine to characterize two distinct *Hafnia* isolates. These strains were selected based on their robust probiotic attributes and genomic evidence specifically, the detection of genes homologous to known esterases (*PpEst*,

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EstC9) suggesting capacity for polymer breakdown. Our investigation thoroughly details the genomic and functional profile of these isolates, providing essential data for establishing a pioneering class of biotic formulations with simultaneous therapeutic and ecological relevance (Savini *et al.*, 2008).

2. Materials and Methods

2.1. Identification and Isolation of Indigenous Microbiota from the *Cyprinus carpio* Digestive Tract

Bacterial strains were previously isolated from *C. carpio* specimens obtained at the DTD fish market in Serbia (Dragacevic *et al.*, 2025). Including probiotic strains, is a crucial task in today's world to expand the genetic data pool and identify new genes. In this study, we investigated the gut microbiota of one industrial species, *Cyprinus carpio*, and identified representatives of various microbial genera, including *Citrobacter*, *Serratia*, *Bacillus*, *Enterococcus*, and *Kocuria*. Notably, we discovered two strains of *Hafnia* with potentially probiotic properties. We conducted next-generation sequencing (NGSTotal genomic DNA, extracted with the GeneJet Purification Kit (Thermo Scientific), was quantified using a NanoDrop 2000c and verified for integrity through gel electrophoresis. Sequencing was carried out on an Illumina 2 × 250 bp platform. Phylogenetic analysis was subsequently conducted using the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff & Göker, 2019; Meier-Kolthoff *et al.*, 2022). To ensure rigorous technical reproducibility, genomic DNA was extracted in triplicate for each strain. The samples then underwent 16S rRNA gene sequencing to confirm identity, followed by comprehensive whole-genome next-generation sequencing (NGS) for in-depth genomic profiling (Martín and Langella, 2019).

The three-dimensional structures of selected *Hafnia*-derived enzyme candidates were predicted using AlphaFold2 implemented through ColabFold (Mirdita *et al.*, 2022).

To explore their biodegradation potential, a local sequence similarity search was performed using BLASTp (Altschul *et al.*, 1990). Annotated proteins from the *Hafnia* genomes were queried against the Plastic Microbial Biodegradation Database (PMBD) (Gan and Zhang, 2019). Domain prediction was conducted using the InterPro database (Blum *et al.*, 2025).

3. Results

From the gut of healthy *C. carpio*, 53 bacterial strains were isolated and identified through 16S rRNA sequencing.

Among the 53 strains, six genera were identified in the GIT of *C. carpio*. Out of the 46 initially isolated strains, 46 were prioritized for investigation, while seven isolates — specifically those belonging to the genera *Citrobacter* (n=4) and *Serratia* (n=3) — were excluded from further downstream analysis. These 46 microorganisms were selected based on their potential as next-generation probiotics (NGPs), a field of growing significance in contemporary biotechnology (Lalowski and Zielińska, 2024).

Based on taxonomic classification via Kraken analysis, strain UUNT_MP29 was identified as *Hafnia paralvei*, while strain UUNT_MP41 showed the highest sequence homology with *Hafnia alvei*. Given their potential as NGPs, both strains underwent rigorous genomic characterization. The resulting assembled genomes were deposited in the NCBI database (Wood *et al.*, 2019; Sayers *et al.*, 2022) under BioProject accessions PRJNA1185451 (UUNT_MP41) and PRJNA1185452 (UUNT_MP29), with corresponding BioSample entries SAMN44705350 and SAMN44705351.

Despite only moderate sequence identity, the enzyme structures predicted by AlphaFold2 showed strong conformational similarity to known PBAT-depolymerases. These results indicated that the enzymes are structurally equivalent (fold-equivalent) to previously characterized PBAT-degrading esterases, thereby identifying the two *Hafnia* strains as potentially bifunctional microorganisms suitable for both probiotic applications and bioremediation of biodegradable plastics.

Analysis of the biodegradation potential of annotated *Hafnia* proteins identified two particularly promising candidates: TesA and LipR. Both sequences displayed significant homology to well-characterized plastic-degrading enzymes, namely PpEst (UniProt: AMW89397.1) from *Pseudomonas pseudoalcaligenes* and EstC9 (UniProt: AOR05752.1) from a *Sphagnum*-associated microbiome, both of which have been experimentally validated for PBAT hydrolysis (Wallace *et al.*, 2017; Müller *et al.*, 2017).

Sequence comparison revealed that TesA exhibits very high identity (>86%) across the two different *Hafnia* strains and other *Hafnia* in the database, while its similarity to the *P. pseudoalcaligenes* ortholog remains moderate (~45%). Domain prediction

confirmed the presence of an SGNH hydrolase-type esterase domain (IPR013830) in TesA and an alpha/beta hydrolase fold-3 domain in LipR.

Taken together, the sequence homology, conserved catalytic domains, and high structural similarity to validated PBAT-depolymerases strongly support the potential of TesA and LipR from *Hafnia* strains to be involved in PBAT biodegradation.

4. Discussion

A central finding was the identification of TesA and LipR orthologs putatively involved in the depolymerization of PBAT-type plastics. The discovery of these biodegradation pathways in gut-associated *Hafnia* strains is particularly significant, as recent literature increasingly recognizes the teleost gastrointestinal tract as a “hotspot” for specialized enzymes capable of degrading recalcitrant microplastic polymers (Talwar *et al.*, 2018). The coexistence of these degradative capabilities within bacteria that can withstand the harsh conditions of the GIT positions these isolates as potential “mobile bioremediation units” (Kutralam-Muniasamy *et al.*, 2023).

Moreover, the physiological relevance of these bacteria likely extends beyond aquaculture. The gastrointestinal environment of freshwater fish shares fundamental functional similarities with the human GIT, including comparable enzymatic niches and high microbial density (Rawls *et al.*, 2006). The metabolic robustness of these *Hafnia* strains under such conditions suggests broad adaptability across diverse vertebrate digestive systems. By maintaining metabolic activity during transit, these strains could reduce microplastic bioaccumulation in the host while simultaneously providing probiotic benefits (Yadav *et al.*, 2025). These findings establish a compelling paradigm for developing multi-functional biotic formulations that address concurrent clinical and ecological challenges through targeted genomic selection.

4.1 Future Perspectives and Strategic Directions

The identification of *Hafnia* strains with plastic-degrading potential opens several high-impact research avenues. A key trend in this field is the “safe-by-design” approach enabled by precision genome editing. Although environmental bacteria isolates often carry complex genetic profiles, the application of CRISPR-Cas9 or base-editing

technologies could selectively remove undesirable elements, such as antibiotic resistance genes, thereby creating safe microbial chassis for bioremediation or probiotic use without contributing to the global resistome crisis (Dudeja *et al.*, 2025).

Future deployment of suitable bacteria should be evaluated within the One Health framework. Investigations should focus on how plastic-degrading probiotics can reduce microplastic bioavailability across the food chain. By mitigating polymer accumulation at the primary consumer level in aquaculture, these bacteria could protect both ecosystem health and human consumers from trophic transfer of contaminants (Bora *et al.*, 2024).

Future efforts will focus on rigorous safety-by-design assessments, including detailed functional validation of the biodegradative loci and targeted removal or inactivation of any antibiotic resistance determinants. By advancing from in silico genomic insights to in vivo and field-based evaluations in aquaculture settings, these *Hafnia* strains hold the potential to pioneer a new class of multi-functional probiotics that actively contribute to both animal welfare and global microplastic bioremediation strategies.

5. Conclusion

In summary, this research successfully identified 53 bacterial strains from the gut of healthy *Cyprinus carpio*, pinpointing two specific strains—*Hafnia paralvei* (UUNT_MP29) and *Hafnia alvei* (UUNT_MP41)—as particularly promising for biotechnological use. Beyond their classification as next-generation probiotics (NGPs), these strains exhibit a remarkable secondary potential: the ability to degrade biodegradable plastics. Through genomic analysis and AlphaFold2 structural prediction, we identified two key enzymes, TesA and LipR, which share high conformational similarity and conserved catalytic domains with validated PBAT-depolymerases. Despite moderate sequence identity to known orthologs, the structural equivalence suggests these enzymes are functional candidates for plastic hydrolysis. These findings conclude that *Hafnia* strains from the common carp are bifunctional microorganisms capable of both enhancing aquatic host health and contributing to environmental bioremediation. This dual-purpose potential opens new avenues for integrating sustainable aquaculture with the management of plastic pollution, marking a significant step forward in contemporary biotechnology.

Izolacija i karakterizacija novih autohtonih sojeva: probiotički potencijal i svojstva biodegradacije mikroplastike

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INFORMACIJE O RADU

Ključne reči:

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Sekvenciranje celog genoma

APSTRAKT

Savremena istraživanja daju prioritet bakterijskim konzorcijumima koji kombinuju probiotičku efikasnost sa detoksikacijom životne sredine. U okviru ove studije izdvojena su dva nova autohtona izolata — *Hafnia alvei* UUNT_MP41 i *Hafnia paralvei* UUNT_MP29 — zbog njihovih snažnih probiotičkih fenotipova i potencijala da katalizuju biodegradaciju mikroplastike. Evaluaciju je potvrdilo snažno antimikrobno antagonističko dejstvo protiv patogena. Sekvenciranje celog genoma (WGS) identifikovalo je ključne funkcionalne genetske elemente: gen *ClpB* — povezan sa probiotičkim karakteristikama i gene *tesA* i *lipR* — homologe esteraza (*PpEst* i *EstC9*) koji su umešani u razgradnju polimera. Ključno je da ovi sojevi omogućavaju degradaciju mikroplastike u uslovima koji oponašaju ljudski gastrointestinalni trakt. Ovi nalazi potvrđuju njihovu kandidaturu kao probiotika sledeće generacije sa dvostrukom funkcijom, uspostavljajući novu paradigmu za biotičke formulacije koje istovremeno nude terapijsku i ekološku korist.

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