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Original Scientific Paper

Novel autochthonous strains from *Cyprinus carpio* as candidates for probiotic use and microplastic-degrading properties

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ABSTRACT

In the modern era, identifying and characterizing novel bacterial strains with possible probiotic potential and environmental bioremediation capabilities is an emerging focus in microbiology and biotechnology. This study analysed the cultivable gut microbiota of the freshwater fish, *Cyprinus carpio*, and identified six different bacterial genera, including *Citrobacter*, *Serratia*, *Bacillus*, *Enterococcus*, and *Kocuria*. Among these, two novel autochthonous strains—*Hafnia alvei* UUNT_MP41 and *Hafnia paralvei* UUNT_MP29—were isolated and selected for further investigation due to their promising probiotic traits and potential to degrade microplastics in aquatic ecosystems. Both strains were evaluated for antibacterial activity against pathogens and susceptibility to a broad spectrum of antibiotics. Whole-genome analysis using next-generation sequencing (NGS) revealed the presence of genes potentially associated with probiotic properties, such as *ClpB*, as well as genes potentially involved in the biodegradation of common microplastics, including the *tesA* gene, a homolog of the *PpEst* gene from the genome of *Pseudomonas pseudoalcaligenes*, and the *lipR* gene, a homolog of the *EstC9* gene from the genome of *Acidocella* sp. Here, we performed a more in-depth analysis of the similarity between the genes/proteins we identified as potentially involved in plastic biodegradation and previously described ones. Notably, the identified strains' potential to degrade microplastics under conditions relevant to the human gastrointestinal system positions them as candidates for a new generation of dual-function probiotics, supporting both human health and microplastic detoxification. These findings lay the groundwork for future development of multifunctional probiotic formulations with environmental and therapeutic benefits.

1. Introduction

The microbiota plays a crucial role in maintaining health by supporting digestion, providing essential molecules, modulating the immune system, and protecting against certain diseases. Probiotic strains, such as *Lactobacillus* species and *Bifidobacterium*, can enhance health status by eliminating harmful substances. On the other hand, exposure to various pollutants, including water, soil, and air contamination,

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can lead to serious health issues. Plastic, as one of the primary materials in use, pollutes the complete environment, contaminating water, soil, and air. The mechanisms by which microbes degrade plastic are currently under active investigation *(Cai et al.*, 2023; *Payanthoth et al*., 2024). Microbial plastic biodegradation likely occurs through biofilm formation and enzymatic degradation. Studying plastic biodegradation by microorganisms from aquatic ecosystems is essential for developing effective strategies. The discovery of bacterial strains with probiotic and plastic-degrading properties could be a game-changer in biotechnology and the healthcare sector.

2. Materials and methods

2.1 Isolation of autochthonous species from the gastrointestinal tract of Cyprinus carpio

Bacterial strains from *Cyprinus carpio* were identified from fish samples collected at the DTD fish market in Serbia. The fish samples were thoroughly cleaned and dissected, and samples of gut were homogenized before being plated onto de Man Rogosa Sharpe (MRS) agar plates. The bacteria isolated were grown overnight in MRS broth at 37°C, and glycerol stocks were created. Details of the procedure are published in our previous paper (*Dragacevic et al*., 2025).

2.2 Genomic DNA extraction from the bacteria strains isolated from Cyprinus carpio and NGS Sequencing and Bioinformatics Analysis

Following the manufacturer's protocol, DNA was extracted using the GeneJet Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). The purity and concentration of the extracted DNA were assessed with the Nanodrop 2000c spectrophotometer (Thermo Scientific), and finally, samples were analysed for genomic DNA integrity using agarose gel electrophoresis. The extracted genomic DNA was sequenced on the Illumina Short Reads platform (2 × 250 bp) with 30X target coverage. The phylogenetic tree was constructed using the Type (Strain) Genome Server (TYGS) bioinformatics platform *(Meier-Kolthoff et al., 2022; Meier-Kolthoff & Göker*, 2019).

2.3 Antibiotic susceptibility

The strains were assessed using the disk diffusion method (Kirby-Bauer test) on the Mueller-Hinton agar (MHA) reference medium to determine their antibiotic susceptibility. Overnight cultures were inoculated aerobically into Mueller-Hinton broth at 37°C. Antibiotic discs were placed onto MHA, incubated at 37°C for 48 hours, and inhibition zone diameters were measured in millimetres.

3. Results

3.1 Identification of autochthonous strains using 16S rRNA gene sequencing, isolated from C. carpio

Thirty-nine bacterial strains were isolated from the gut of healthy *C. carpio* and were identified using 16S rRNA gene amplification and sequencing. The bacterial strains that were identified at the species level using standard/universal 16S rRNA gene primers (27F-AGAGTTTGATCCTGGCTCAG and 1492R-ACGGYTACCTTGTTACGACTT) are presented in Table 1.

Table 1. Identified bacteria species isolated from the gastrointestinal tract of *Cyprinus carpio* using 16S rRNA sequencing

Identified genus	Identified species	No of strains
Citrobacter	*Citrobacter freundii*	13
Serratia	*Serratia liquefaciens*	10
	Serratia plymuthica	1
	Serratia marcescens	1
Bacillus	*Bacillus pumilus*	3
Hafnia	*Hafnia alvei*	1
	Hafnia paralvei	1
Enterococcus	*Enterococcus pseudoavium*	1
Kocuria	*Kocuria rhizophila*	8

Among 39 strains, six genera were identified in the gastrointestinal tract of *C. carpio* (Table 1). Genome sequences of two novel strains, denoted as *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29, were deposited in the National Library of Medicine (NIH), https://www.ncbi.nlm.nih.gov/biosample/SAMN44705350, and https://www.ncbi.nlm.nih.gov/biosample/SAMN44705351. These strains were then selected for further research due to their potential classification as next-generation probiotics (NGP), making them particularly appealing now. Genomic DNA was extracted from these

strains in three independent replicates, and 16S rRNA gene sequencing was carried out, followed by whole-genome next-generation sequencing (NGS). The assembled genomes have been deposited in the NCBI database (*Sayers et al*., 2022) under BioProject numbers PRJNA1185451 for strain UUNT_MP41 and PRJNA1185452 for strain UUNT_MP29, using NGS sequencing of both *Hafnia* strains. Based on the Kraken analysis results, strain UUNT_MP29 is closely related to *H. paralvei*, while strain UUNT_MP41 is most closely associated with *H. alvei.* The phylogenetic tree was published in our previous paper (*Dragacevic et al.*, 2025).

3.2 Antibiotic susceptibility of H. alvei UUNT_MP41 and H. paralvei UUNT_MP29

The study found that *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29 exhibited the highest sensitivity (among the isolated bacteria) to imipenem, ciprofloxacin, enrofloxacin, ampicillin, ceftazidime, and five other medications, suggesting potential probiotic activity. *H. alvei* UUNT_MP41 demonstrated greater sensitivity than *H. paralvei* UUNT_MP29. A figure depicting the disc diffusion method for assessing antibiotic susceptibility was published in our previous paper (*Dragacevic et al*., 2025).

3.3 Search and analysis of potential candidate genes for microplastic degradation

To identify genes responsible for plastic biodegradation in both *Hafnia* species, a local sequence alignment was performed using the BLAST tool (blastp) (*Altschul et al*., 1990), comparing annotated proteins from *Hafnia* genomes against protein sequences from the PMBD database. Two proteins, encoded by the *tesA* and *lipR* genes, showed the highest percentage of identity to the proteins PpEst (UniProt ID: AMW89397.1) and EstC9 (UniProt ID: AOR05752.1), respectively (*Uniprot Consortium*, 2025). PpEst is an esterase capable of hydrolysing the co-aromatic-aliphatic polyester poly(1,4-butylene adipate-co-terephthalate) (PBAT), characterized initially in *Pseudomonas pseudoalcaligenes* (*Wallace et al*., 2017). EstC9 was isolated from the

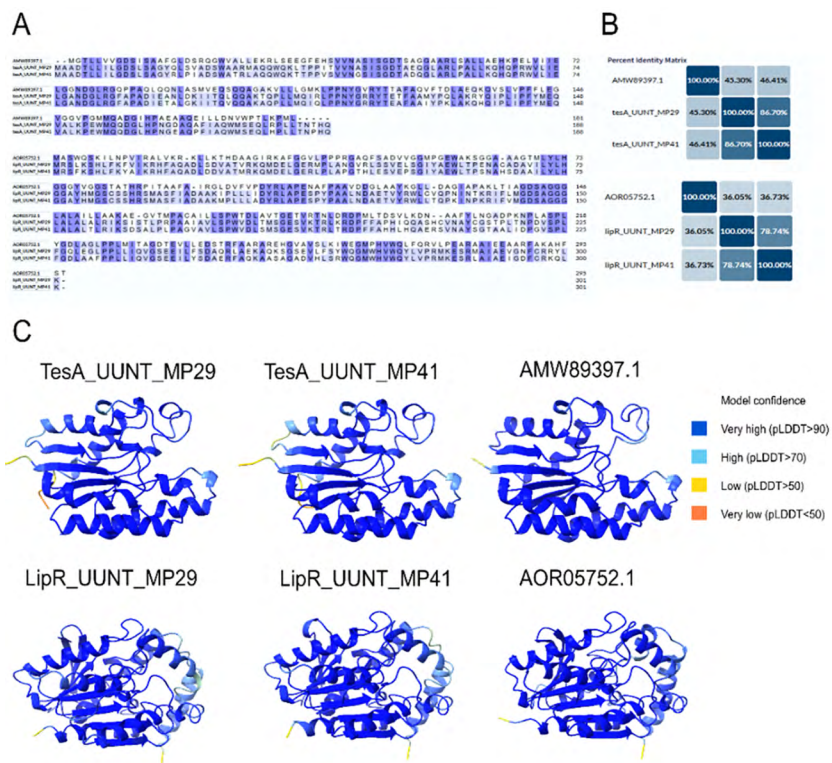

Figure 2. Comparison of proteins that may be involved in plastic biodegradation from *Hafnia* genomes with the characterized proteins AMW89397.1 and AOR05752.1. A – Alignment of the primary amino acid sequences. B – Percent identity matrix, with color intensity showing the degree of sequence identity. C – AlphaFold2 structural prediction of the compared proteins.

microbiome of the moss *Sphagnum magellanicum* and is presumed to be encoded in the genome of an *Acidocella* species (*Müller et al.*, 2017). It has also been shown to hydrolyse PBAT (*Müller et al.*, 2017). We analysed the sequences of these proteins (Figure 2) and found that for TesA, the percent identity between the *Hafnia* strains exceeded 86%, while the identity between *Hafnia* TesA and the protein from *P. pseudoalcaligenes* was just over 45%. All three proteins were predicted to contain an SGNH hydrolase-type esterase domain (InterPro ID: IPR013830) (*Blum et al.*, 2025). Esterases in this group are known to share a similar fold with flavoproteins—specifically, a three-layer α/β/α structure where the β-sheet consists of five parallel strands. Enzymes containing this domain function as esterases and lipases but exhibit low sequence homology to true lipases (*Akoh et al.*, 2004; *Mølgaard et al.*, 2000). We predicted the protein structures using AlphaFold2 via ColabFold (*Mirdita et al.*, 2022), and structurally, the proteins were nearly identical. For the LipR protein, the percent identity between *Hafnia* strains was around 78%, and between *Hafnia* LipR and EstC9, slightly over 36%. All three proteins were predicted to contain the Alpha/beta hydrolase fold-3 domain (InterPro ID: IPR013830), and the structures predicted by AlphaFold2 also appeared highly similar in all three cases. Based on these findings, *Hafnia* strains may prove to be promising candidates for use as microorganisms in plastic biodegradation, particularly for plastics like PBAT.

4. Discussion

The *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29 strains, which potentially possess probiotic properties, underwent a more in-depth analysis. A genomic study was conducted to identify antibiotic resistance genes, and antibiogram tests were performed and analysed. Bioinformatics analysis revealed that the genomes of *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29 contain numerous genes responsible for antibiotic resistance. Additionally, genes encoding products that may degrade plastics, primarily PBAT-type plastic, were identified and characterized through bioinformatic approaches. Therefore, the described *Hafnia* strains represent interesting and promising candidates for further investigation.

5. Conclusion

The results showed that various antibiotics significantly resist *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29. Taking into consideration the results obtained in this study, we suggest that these two novel bacteria strains could be considered as a candidate for a potential new generation of probiotics that could also have role in microplastic biodegradation. Further investigation will focus on a complete analysis of the safe use of these strains and potential genes for plastic biodegradation.

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