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Development of a synbiotic dietary supplement containing potential Next Generation Probiotics for modulation of the gut microbiome and metabolome

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ABSTRACT

The term Next Generation Probiotics (NGPs) refers to microbial strains positively impacting on human health, but do not belong to common probiotic species (e.g., lactic acid bacteria, LAB). We characterized genomically and phenotypically 14 strains isolated from the gut microbiome of healthy individuals, to evaluate their ability to produce urolithins, equol and short-chain fatty acids (SCFA). The 4 most promising strains (namely *Bacteroides uniformis* A4, *Bacteroides thetaiotaomicron* A14, unclassified *Bacteroidaceae* A26 and unclassified *Lachnospiraceae* A49) were used for the production of a synbiotic formulation, containing the strains and the precursors of health-promoting molecules. This dietary supplement was administered for 2 weeks to a continuous mucosal-Simulator of the Human Intestinal Microbial Ecosystem (mSHIME) model inoculated with a faecal sample from a low fiber-consuming donor. We performed Shotgun Metagenome Sequencing on a total of 204 samples collected from lumen and mucosa compartments, and determined the concentration of SCFA, equol and urolithin. Our results highlighted that the potential NGP strains contained in the supplement persisted in the gut ecosystem during 2 weeks of washout (Wilcoxon's rank sum test, p-value <0.05). In addition, the treatment led to an enrichment in beneficial taxa and to an increase in the production of SCFAs (p-value <0.05). This study demonstrated that feeding the gut microbiota with NGPs and dietary prebiotics can modulate both the gut microbiome and metabolome, suggesting a potential beneficial impact on human health. However, further *in vivo* studies are needed to confirm these results.

1. Introduction

Even though a consensus statement on Next Generation Probiotics (NGPs) is currently lacking, the term usually applies to microbial strains that can have a positive effect on human health, but do not belong to common probiotic species (e.g., lactic acid bacteria, *Bifidobacterium* (O'Toole et al., 2017)). Indeed, large metagenomics surveys of the human gut microbiome carried out in the past years led to the discovery of a number of previously unexplored microorganisms and highlighted their potential role in promoting health. These strains typically exert their effects through unique metabolic capabilities, such as the production of bioactive metabolites (e.g., SCFAs, urolithins, equol) or the degradation of otherwise indigestible substrates. Among the most interesting species proposed as potential NGPs there are *Roseburia intestinalis*, *Eubacterium* spp., *Ruminococcus bromii*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Clostridium* spp., *Bacteroides* spp. And *Dysosmobacter welbionis* (De Filippis et al., 2022; Le Roy et al., 2022). However, in most cases, the available evidence comes from studies lacking interventions, where positive associations between these taxa and health-related parameters have been identified in gut metagenomes. To assess the actual production of beneficial metabolites and ultimately accelerate the development of NGPs for market application, additional *in vitro* and *in vivo* studies are needed. Another issue is related to the huge strain-level diversity existing within these species: most of our knowledge is limited to the species-level resolution, while wide genomic studies have highlighted that different strains within these species may have potentially divergent activities and possibly a contrasting behavior in relationship with human health, such as the genus *Ruminococcus* that

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is dominant in the human gut but higher levels of some species, such as *R. gnavus*, *R. torques*, and *R. bromii*, have been linked to health or disease. *R. torques* and *R. gnavus* have been linked with gut inflammation, inflammatory bowel disease (IBD), and irritable bowel syndrome, allergy, and early stages of colorectal cancer (CRC), whereas *Ruminococcus bromii* has been identified as a promising next-generation probiotic thanks to its ability to hydrolyze resistant starch (RS) (Valentino et al., 2024).

NGPs may exert their beneficial impact on human health through the production of bioactive metabolites. Among these, short-chain fatty acids (SCFAs) produced from the fermentation of dietary fibre are the most extensively studied. Evidence suggests that SCFAs support immune homeostasis and intestinal health and exhibit anti-inflammatory, anti-obesity, anti-diabetic, anticancer, cardioprotective, hepatoprotective, and neuroprotective properties (Ríos-Covián et al., 2016). However, other bioactive compounds may also be produced, including vitamins, neuroactive molecules (e.g., serotonin, indoles), and metabolites derived from polyphenols catabolism (e.g., urolithins, equol), all of which play key roles in the regulating host physiology.

Since these taxa do not have a history of safe use, they are likely to fall under the drug regulatory framework, although current legislation in Europe remains unclear. NGPs must go through extensive research and testing in carefully designed preclinical and clinical trials to make sure they are safe for human use and effective in providing the desired health benefits (Singh and Natraj, 2021).

However, a significant milestone was reached a few years ago when *Akkermansia muciniphila* was firstly approved for the use in its pasteurized form as a novel food under Regulation (EU) 2015/2283. This approval has further stimulated both industrial and academic interest in NGPs. Indeed, there is growing interest in the development of gut microbial strain collections for future commercial use, pending clearer regulatory guidance from the EU.

Beyond regulatory uncertainties, working with these taxa presents technical challenges due to their nutritional requirements, high sensitivity to oxygen, and, in many cases, instability to gastric conditions encountered after ingestion (Jan et al., 2024). For these reasons, further research is needed to optimize their cultivation, and to ensure their viability during typical preparation, storage and delivery processes. Moreover, a symbiotic supplement containing both the strains and the precursors of beneficial metabolites need to be evaluated, since they may be more effective than the strains alone in the modulation of the gut microbiome and metabolome.

In this study, we characterized strains isolated from faecal samples of healthy high fiber-consuming adult donors for their ability to produce beneficial metabolites. Four of our strains qualified as potential NGPs due to their ability to produce *in vitro* key metabolites relevant to host physiology, such as urolithins, equol and SCFAs. We formulated a synbiotic formulation including the four strains and precursors of the microbial-derived metabolites *ex vivo* on the human gut microbiome using the Simulator of Human Intestinal Microbial Ecosystem (SHIME) model to estimate the strains persistence in the gut environment and the release of targeted health-promoting metabolites in a complex ecosystem.

2. Materials and methods

2.1. Strains and cultivation conditions

In this study, 14 strains of human gut origin were characterized (Table S1). Strains were previously isolated from fecal samples of 3 healthy vegan or vegetarian donors (two male and one female participants, aged 25–35). Strains were grown at 37 °C in an anaerobic chamber (A45 Don Whitley Scientific, Bingley, West Yorkshire, United Kingdom) filled with a mixture of 10 % H_2, 10 % CO_2 and 80 % N_2. All strains were cultivated in YCFAGSC broth modified (Lopez-Siles et al., 2012) (Bacto casitone 10 g/L; yeast extract 2.5 g/L; $NaHCO_3$ 4 g/L; soluble starch 2 g/L; cellobiose 2 g/L; mineral solution 1150 mL/L; mineral solution 2150 mL/L; SCFA solution 3.1 mL/L; haemin solution 10 mL/L; vitamin solution 1 1 mL/L; vitamin solution 2 1 mL/L; resazurin (0.1 % in water), 1 mL/L; Cysteine-HCl 1 g/L. Mineral Solution 1: K_2HPO_4 3 g/L; Mineral Solution 2: KH_2PO_4 3 g/L, $(NH_4)_2$ 6 g/L, NaCl 6 g/L, $MgSO_4$ 0.6 g/L, $CaCl_2$ 0.6 g/L; SCFA solution: acetic acid 17 mL/L, propionic acid 6 mL/L, iso-butyric acid 1 mL/L, n-valeric acid 1 mL/L, iso-valeric acid 1 mL/L; Vitamin solution 1: biotin 1 mg/100 mL, folic acid 5 mg/100 mL, pyridoxine-HCl 15 mg/100 mL, vitamin B12 1 mg/100 mL, p-aminobenzoic acid 3 mg/100 mL; Vitamin solution 2: thiamine 5 mg/100 mL, riboflavin 5 mg/100 mL; haemin solution: KOH 0.28 g/100 mL, ethanol 95 % 25 mL/100 mL, haemin 100mg/100 mL; resazurin solution: 0.1 g/100 mL); all purchased from Thermo Fisher Scientific (Waltham, Massachusetts, United States), except for haemin, purchased from Sigma Aldrich (Saint Louis, Missouri, United States). The medium, the mineral solutions and SCFA were sterilized in autoclave at 121 °C for 15 min, while vitamins and haemin solutions were filter-sterilized using filters with 0.22 μm pores (Sartorius, Göttingen, Germany).

2.2. Genome sequencing, taxonomic identification and functional analysis

Microbial strains were firstly identified by MALDI-TOF Biotyper (Bruker, Billerica, Massachusetts, United States), preparing the fresh colony by with 1 μL of α-Cyano-4-hydroxycinnamic acid (HCCA) as reported by the manufacturer.

In order to confirm the identification, we also carried out Whole Genome Sequencing (WGS) of the isolates. DNA was extracted from pure cultures using DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). DNA libraries were prepared according to Illumina protocol and WGS was carried out on Illumina NovaSeq platform, leading to 2 × 150bp, paired-end reads. Reads were quality-filtered using PRINSEQ 0.20.4 (Schmieder and Edwards, 2012), reads with bases having a Phred score <15 were trimmed and those <75 bp were discarded. High-quality reads were assembled using megahit (k = 21,33,55,71,81,91) (Li et al., 2015) and contigs <1000 bp were discarded. Genomes were clustered with a publicly available database and using a pipeline previously developed (Pasolli et al., 2019). Briefly, pairwise genetic distances between genomes were calculated using Mash (version 2.0; option "-s 10, 000" for sketching (Ondov et al., 2016); and genomes were assigned to a species if Mash distance was ≤5 %. When a genome showed >5 % distance from any of the reference genomes, it was considered a novel species. In this case, the taxonomic assignment was made at genus (>5 and < 15 % distance), family (>15 and <25 % distance), or phylum (>25 % distance) level, using thresholds previously reported (Li et al., 2015). Final taxonomic identification achieved with MALDI-TOF and

Table 1
Taxonomic identification of the bacterial strains characterized in this study.

Strain ID	MALDI-TOF Taxonomy	Whole Genome Sequencing Taxonomy
A8BL	*Collinsella aerofaciens*	*Collinsella aerofaciens*
A8D22′	*Bacteroides uniformis*	*Bacteroides uniformis*
A8N_3	*Faecalicatena contorta*	*Faecalicatena contorta*
A4	*Bacteroides uniformis*	*Bacteroides uniformis*
A10	*Parabacteroides distasonis*	*Parabacteroides chinchillae*
A14	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron*
A17	*Collinsella aerofaciens*	*Collinsella* sp.
A19	*Faecalicatena contorta*	*Clostridium* sp.
A24B	*Collinsella aerofaciens*	*Collinsella* sp.
A26	*Bacteroides salyersiae*	unclassified *Bacteroidaceae*
A28	*Pseudoruminococcus massiliensis*	unclassified *Oscillospiraceae*
A39	*Phocaeicola vulgatus*	*Phocaeicola* sp.
A49	*Dorea longicatena*	unclassified *Lachnospiraceae*
A88	*Bacteroides stercoris*	unclassified *Bacteroidota*

genome analyses is reported in Table 1, while Mash distances and closest references are reported in Table 2. Coding sequences (CDS) for each genome were predicted using Prokka (version v1.11) (Seemann, 2014). Predicted genes were aligned using DIAMOND blastx (v. 2.0.4; e-value cutoff of $1e^{-5}$, >90 % of identity over at least 75 % of the query length) against known enzymes involved in the metabolism of polyphenols downloaded from NCBI (a list of the genes and their accession numbers are reported in Table S2). In addition, we focused on Carbohydrates-Active genes, aligning predicted genes against the CAZy database (non-redundant at 90 % identity) by using DIAMOND v. 2.0.4 (e-value cutoff of $1e^{-5}$, >50 % of identity over at least 50 % of the query length). Furthermore, we screened the genomes for genes linked to virulence factors and antimicrobial resistance through abricate (version 1.0.1) and the ResFinder (Florensa et al., 2022) and Virulence Factor (Liu et al., 2022) databases (Table S3). Only matches showing a percentage of identity ≥70 % over at least 80 % of the query length were considered, as suggested by the European Food Safety Authority (EFSA) guidelines (https://www.efsa.europa.eu/sites/default/files/2021-03/EFSA-statement-EFSA-Q-2019-00434.pdf).

2.3. Growth curves in aerobic and anaerobic conditions

To evaluate the ability of the strains to grow in aerobic conditions, an overnight culture of the strain (grown in anaerobic conditions at 37 °C) was inoculated in YCFAGSC broth (1 %). The suspension was aliquoted in a 96-well microplate (4 replicate wells for each strain), that was incubated at 37 °C for 24 h both in aerobic and anaerobic conditions. Optical Density (OD) at 560 nm was measured each 30 min using a microplate reader (Spark Multimode Microplate Reader, Tecan). Growth curves were drawn in Excel.

2.4. Ability to use different polysaccharidic sources

The analysis was carried out as previously described by Lopez-Siles et al. (2012) (Lopez-Siles et al., 2012). Briefly, different carbohydrates were added to a minimal YCFAGSC broth, prepared with a reduced concentration of casitone (0.2 %). Different broths were prepared adding individually one of the following polysaccharides at a final concentration of 0.5 % (wt/vol): pectin from apple (galacturonic acid ≥65 %; degree of esterification 50–75 %), pectin from citrus peel (galacturonic acid ≥74.0 %; methoxy groups ≥6.7 %), glucuronic acid (>98 %), amylopectin from potato, fructo-oligosaccharides (≥90 %), inulin from dahlia tubers, arabinoxylan, polygalacturonic acid (≥90 %), glucan from oat, xyloglucan (β-D-Glucan from barley ≥95 %; all purchased from Sigma Aldrich; Saint Louis, Missouri, United States).

An overnight culture of the strain was inoculated (1 %) in the different broths. The suspension was aliquoted in a 96-well microplate (4 replicate wells for each strain), that was incubated at 37 °C for 24 h in anaerobic conditions. For each broth, blanks (uninoculated broths) were also incubated in the same conditions and OD subtracted to the respective curve. Growth curves were obtained in the microplate reader as reported above.

Table 2
Taxonomic identification of the NGP strains genomes. For each genome, the closest MAG (i.e., showing the lowest MASH distance) and its taxonomy are reported.

Strain	Closest MAG	MASH distance	MAG taxonomy
A4	TC2_L_25_10.bin.26	0.035	*B. uniformis*
A14	TC1_L_18_10.bin.32	0.021	*B. thetaiotaomicron*
A26	DC2_L_13_11.bin.24	0.047	*B. salyersiae*
A49	DC2_M_23_10.bin.40	0.114	Unclassified *Lachnospiraceae*

2.5. SCFA analysis in pure culture supernatant and mSHIME samples

To evaluate the production of SCFAs, the strains were inoculated (1 %) in YCFAGSC broth added with different carbohydrates and incubated in anaerobic chamber for 48 h at 37 °C. SCFA production was evaluated only for those combinations of strains and carbohydrates where a microbial growth was observed (as reported in section 2.4). Ten mL of the overnight broth culture or mSHIME lumen content were centrifuged at 12,000×*g* for 4 min and the concentrations of SCFAs (isobutyric, hexanoic, butyric, propionic, acetic, 2-methylbutyric, isovaleric, valeric, 2-hydroxybutyrate, L-lactic acids) were analyzed in the supernatant. SCFA were extracted using IPA (cold isopropanol) and derivatization with 3-nitrophenylhydrazine. The resulting solution were then analyzed using an Orbitrap Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS), equipped with a HPLC Vanquish system (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and a Kinetex Evo C18 column (150 mm × 2.1 mm), with a particle size of 2.6 μm and a pore size of 130 Å (Phenomenex, Torrance, California, United States). Quantification of all SCFAs was performed using a calibration curve based on 10 points, each analyzed in triplicate. Values were reported as mg/L.

2.6. Equol and urolithin analyses in pure culture supernatants and mSHIME samples

Daidzein (final concentration of 50 μM; Sigma Aldrich; Saint Louis, Missouri, United States) and ellagic acid (final concentration of 30 μM; Sigma Aldrich; Saint Louis, Missouri, United States) were filter-sterilized and added to YCFAGSC broth. Strains were inoculated (1 %) and incubated in anaerobic chamber for 48 h at 37 °C. Ten mL of the overnight broth culture or mSHIME lumen content were centrifuged at 12.000×*g* for 4 min and the supernatant was used for equol and urolithin analyses. Samples (2 mL) were extracted with 2 mL of ethyl acetate acidified with 1.5 % formic acid and centrifuged at 3500×*g* for 10 min (García-Villalba et al., 2022). The organic phase was separated and evaporated under nitrogen. The dry samples were then resuspended in methanol prior to LC-HRMS/MS analysis. Sample analysis was performed using a Vanquish Core LC system coupled to a high-resolution quadrupole Orbitrap mass spectrometer (Exploris 120, Thermo Fisher Scientific, Waltham, Massachusetts, United States). Separation of equol and urolothins was performed on a Gemini C18-110 Å 5 μm column (150 mm × 2.0 mm) (Phenomenex, Torrance, California, United States) thermostated at 30 °C using the solvent gradient as previously shown (Meslier et al., 2020). In negative ion mode, the H-ESI source was set to a spray voltage of −3.0 kV, with the ion transfer tube and vaporizer temperatures at 320 °C and 300 °C, respectively. Sheath gas and auxiliary gas flow rates were set to 35 and 15 arbitrary units. Data were acquired in positive and negative data-dependent scanning mode with a targeted compound filter applied specifically for urolithins and equol. Normalized collision energy was set at 30 %, 40 % and, 50 % with Orbitrap resolution at 45,000, and quadrupole isolation window width at 1.2. Calibration curves for Equol and Urolithin B were build in the same MS conditions. Urolithins were expressed as urolithin B equivalents. The limit of detection and quantitation are detailed in Table S4.

2.7. Twin mSHIME experiment

According to previous tests, we identified 4 best performing strains. Strain A4 (*Bacteroides uniformis)* was selected due to its ability to grow under aerobic conditions, as reported in Fig. S1A. Strain A49 (unclassified *Lachnospiraceae)* was selected for its capacity to metabolize different polysaccharides, its high SCFA production, and its ability to produce both equol and urolithins from the degradation of daidzein and ellagic acid. Strain A26 (unclassified *Bacteroidaceae*) was chosen for its high equol production, and strain A14 (*Bacteroides thetaiotaomicron)* for its ability to use different polysaccharides sources and to produce SCFAs

and equol.

These strains were selected for testing in a twin mucosal-Simulator of the Human Intestinal Microbial Ecosystem (twin mSHIME; Prodigest, Gent, Belgium), consisting of two parallel SHIME systems, each comprising five double-wall biovessels simulating the stomach (ST), small intestine (SI), ascending colon (AC), transverse colon (TC), and descending colon (DC). The system was kept under anaerobic conditions using nitrogen flushing and maintained at 37 °C by a circulating water bath. The pH of AC, TC, and DC was continuously controlled and adjusted to mimic physiological conditions using 0.5 M HCl and 0.5 M NaOH, maintaining the pH ranges of 5.7–5.9 for the AC, 6.15–6.4 for the TC, and 6.6–6.9 for the DC. The performance of pH probes was monitored daily by measuring the pH of an aliquot from each vessel with an external pH meter. The pH was not controlled in stomach and small intestine. In each colon vessel, 4 bags of 15 mucin-coated carriers (60 carriers/vessel) were added. During the whole experiment, 50 % of the carriers were replaced 3 times/week to simulate the renewal of the mucus layer. The parallel systems were inoculated with a fecal sample provided by an adult (age 35 y. o.) healthy man, obese (BMI = 32 kg/m^2) who did not assumed antibiotics or probiotics for 6 months before the experiment, selected because usually consuming a diet poor in fiber (fruit/vegetables <3 servings/day). The fecal sample was self-collected at home using the GutAlive kit (Microviable Therapeutics, Spain) and transported to the laboratory within 2 h. The study was approved by the Ethics Committee of the University of Naples Federico II – Campania 3 (Protocol number: 271/21). Fifty g of fresh fecal material were diluted and homogenized with 250 mL of sterile anaerobic phosphate buffer (K_2HPO_4 8.8 g/L, KH_2PO_4 6.8 g/L, sodium thioglycolate 0.1 g/L, sodium dithionite 0.015 g/L, pH 7; all purchased from Sigma Aldrich; Saint Louis, Missouri, United States). The solution was centrifuged at 500×*g* for 2 min. Then the supernatant was inoculated (5 %) in the colon vessels. System set-up and stabilization was carried out following the manufacturer's instruction. The stabilization period (2 weeks) was followed by a control period (2 weeks), a treatment period (2 weeks) and wash-out period (2 weeks). The control period started when similar SCFA concentrations and ratios were obtained for the two parallel vessels (similarity ≥90 %). During the whole experiment, both the twin SHIME systems received 3 times/day 140 mL of the standard nutritional medium provided by Prodigest (Gent, Belgium), with the following composition: arabinogalactan (1.2 g/L), pectin (2 g/L), xylan (0.5 g/L), glucose (0.4 g/L), yeast extract (3 g/L), special peptone (1 g/L), mucin (2 g/L), L-cystein-HCl (0.5 g/L), starch (4 g/L) all purchased from Sigma Aldrich (Saint Louis, Missouri, United States). The average retention times simulated the *in vivo* conditions, and were 20, 32 and 24 h in the ascending, transverse and descending sections, respectively.

During the treatment period, both the twin SHIME parallel lines received a supplement containing polysaccharides, ellagic acid and daidzein, that were added in the stomach vessels once a day (Fig. S2). The mix included 25 mL of pectin from apple (15 mg/mL), 25 mL of pectin from citrus peel (15 mg/mL), 5 mL of FOS (200 mg/mL), 5 mL of glucuronic acid (200 mg/mL), 5 mL of daidzein (0.6 mg/mL) and 3 mL of ellagic acid (0.33 mg/mL); all purchased from Sigma Aldrich (Saint Louis, Missouri, United States). In addition, only one line received also the 4 strains from the overnight fresh culture, that were added in the AC vessel once a day at a final dose of 10^7 CFU/each. The concentration of ellagic acid was calculated according to the average content in 30 g of nuts, that is the recommended consumption in a Mediterranean diet (Casas et al., 2016) (30 g nuts/day contains about 100 mg of ellagic acid). For daidzein, the concentration in the supplement was calculated considering the average quantity present in 60 g of soy, that are considered having a preventive activity against cancer (60 g of soy contains about 3 mg of daidzein; (Guha et al., 2009). For fibres, it was considered the maximum amount that can be added daily according to SHIME manufacturers (max 3 g/day of total fibres), as well as the solubility of the different compounds. After 2 weeks of treatment, we carried out 2 weeks of wash-out. In the first week of wash-out period, we stopped the administration of the strains, but kept administering the fibre/polyphenols mix once a day. During the second wash-out week, we stopped any administrations and reported the mSHIME to the same conditions of the control period. Every two days, we collected 20 mL of lumen content from each colon vessel (AC, TC, DC), that were used for the analyses (SCFA, urolithins, equol and DNA extraction for microbiome analysis). In addition, mucus was collected from the carriers at every replacement (see above). Carriers were washed twice in anaerobic phosphate buffer and centrifuged at 12,000×*g* for 3 min. Pellet was used for DNA extraction.

2.8. DNA extraction and microbiome analysis of mSHIME samples

A total of 12, 21, 9 and 9 lumen samples for the stabilization, treatment and wash out (first and second week) periods were analyzed for each parallel mSHIME, collected every 2 days, together with mucin-coated carriers replacement. Similarly, 12, 21, 9 and 9 mucosa samples were also analyzed for each parallel mSHIME. Samples were stored at −80 °C until DNA extraction, that was performed according to the International Human Microbiome Standards (IHMS) SOP 007, version 2. Whole Metagenome Sequencing was performed, and DNA libraries were prepared according to Illumina protocol on an Illumina NovaSeq platform, leading to 2 × 150bp, paired-end reads. Quality of the raw reads was investigated using FastQC (version 0.12.1) using default options. Reads were quality-filtered using PRINSEQ 0.20.4 (Schmieder and Edwards, 2012), trimming bases having a Phred score <15 and discarding reads <75 bp. Taxonomic profiling of samples was performed through MetaPhlAn (version 4.0.2; database version mpa_vJun23_-CHOCOPhlAnSGB_202,307), with default settings. Filtered reads were assembled into contigs using MegaHIT (k = 21,33,55,71,81,91) (Li et al., 2015), and only contigs >1000 bp were retained for further analysis. Reads quality and assembly statistics are reported in Table S5. Metagenemark (Zhu et al., 2010) performed gene calling on the contigs. Reads were aligned to the contigs sample-wise through BowTie 2 (2.2.9, "very-sensitive-mode" method) (Langmead and Salzberg, 2012), and the resulting alignment files were provided to MetaBAT in order to bin contigs into Metagenome-Assembled Genomes (MAGs). Percent completeness and contamination of each MAG were assessed through CheckM (version 1.0.13) (Parks et al., 2015). Only MAGs showing completeness ≥50 % and contamination <5 % (i.e., medium-high quality MAGs) (Pasolli et al., 2019) were retained. A phylogenetic tree based on a Multiple Sequence Alignment (MSA) of genes predicted from all the MAGs to a set of 120 bacterial marker genes was performed through the commands 'classify_wf' and 'infer' from GTDB-Tk using the database version 'reference 95' (program version, citation). The phylogenetic tree was visualized in iTol (Letunic and Bork, 2007). EggNOG mapper (version 2.1.12) (Cantalapiedra et al., 2021) functionally annotated metagenomes and MAGs. The completeness of KEGG pathways in each metagenome was estimated using KEGGaNOG (version 0.7.2; https://github.com/iliapopov17/KEGGaNOG, accessed in February 2025).

2.9. Statistical analysis

Statistical analysis of microbiome data was performed in R environment (https://www.r-project.org). Relative abundances of species or metabolites were compared between the groups using the Wilcoxon's rank sum test ('wilcox.test (.)' function from R 'base' package), setting a p-value of 0.05 as significance threshold. The False Discovery Rate approach was employed to address for multiple testing through the function 'p.adjust (.)' and the option "method = fdr" from the R 'stats' package. The Cohen's d effect size of metabolites variations in the SHIME samples was calculated with the 'cohens_d (.)' function from the 'effectsize' R package. Co-occurrence and co-exclusion pattern among species was estimated with the Spearman's rank correlation coefficient through the function 'correlate (.)' (option 'method = "spearman"') from

the 'corrr' R package. Principal Coordinates Analysis (PCoA) was performed with the 'cmdscale' from the R 'base' package on pairwise Bray-Curtis dissimilarity matrices, unless otherwise stated. To test whether the clustering of samples according to the Bray-Curtis dissimilarity matrix was linked with categorical variables, we applied the Permutational Multivariate ANOVA (PERMANOVA) with 999 permutations using the 'adonis 2' function from the 'vegan' R package. Microbial diversity of SHIME samples was estimated with the Shannon's and Simpson's diversity indices, computed through the 'diversity (.)' function from the 'vegan' R package. All the plots were produced using the functions from the 'ggplot 2', 'ggpubr' and 'ggsci' R packages.

3. Results

3.1. Genome analyses and in vitro screening

Genomes of the 14 strains were firstly identified through clustering to a published genome catalogue, including 80,990 isolate genomes and 154,723 genomes reconstructed from human metagenomes (Pasolli et al., 2019), in order to confirm the taxonomic identification obtained by MALDI-TOF. Results are reported in Table 1. Indeed, in several cases the genome-based identification revealed that our isolates showed >5 % distance with all the genomes included in the database, possibly suggesting the presence of putative new species. In three cases (strains A26, A28, A49) we only reached the family level assignment, while in one case we reached only the phylum identification. We screened the genomes for the presence of genes involved in degradation of polyphenols and in the production of beneficial metabolites. Indeed, strains A49 (unclassified *Lachnospiraceae*) and A17 (*Collinsella* sp.) showed higher occurrence of these genomic traits (Fig. 1A). We also explored the presence of CAZy genes. We observed that *Faecalicatena contorta* (A8N3), *Parabacteroides chinchillae* (A10), *Clostridium* sp. (A19) and *Collinsella aerofaciens* (A8BL) showed a high number of genes related to the GH family, while in the genomes of *Bacteroides thetaiotaomicron* (A14), *Phocaeicola* sp. (A39) and *Bacteroides uniformis* (A4) the CAZy GT family was particularly prevalent (Fig. 1B). In addition, high occurrence of both GH and GT family genes was found in the strain A49 (unclassified *Lachnospiraceae;* Fig. 1B).

3.2. Growth in aerobic and anaerobic conditions

We explored the ability of the isolated strains to grow in both aerobic and anaerobic conditions. For each strain, growth during 24 h was monitored. We highlighted that the strains showed different growth behavior in aerobic or anaerobic conditions. All of them presented shorter lag phases and reached higher OD values at the end of the exponential phase when cultivated in anaerobic conditions. In particular, the strains *Bacteroides uniformis* A4 (Fig. S1A), *Parabacteroides chinchillae* A10 (Fig. S1H), *Bacteroides* sp. A8D22' (Fig. S1E), *Phocaeicola* sp. A39 (Fig. S1O), unclassified *Bacteroidota* A88 (Fig. S1P), *Collinsella aerofaciens* A24B (Fig. S1M) were able to grow in aerobic condition, although at lower levels. On the contrary, the strains *Bacteroides thetaiotaomicron* A14 (Fig. S1B), *Faecalicatena contorta* A8N_3 (Fig. S1G), unclassified *Lachnospiraceae* A49 (Fig. S1D), unclassified *Oscillospiraceae* A28 (Fig. S1N), unclassified *Bacteroidaceae* A26 (Fig. S1C), *Collinsella aerofaciens* A8BL (Fig. S1F), *Clostridium* sp. A19 (Fig. S1L) and *Collinsella* sp. A17 (Fig. S1I) did not show growth in aerobiosis.

3.3. Dietary polysaccharides and fiber fermentation

We further explored the ability of the strains to use complex polysaccharides, preparing 10 different minimal broths with the addition of specific carbohydrates as only carbon source and monitoring growth dynamics. Indeed, different utilization patterns were identified. *Bacteroides uniformis* A4 (Figure S3 A), *Bacteroides thetaiotaomicron* A14 (Figure S3 B), unclassified *Bacteroidaceae* A26 (Figure S3 C), unclassified *Lachnospiraceae* A49 (Figure S3 D), *Bacteroides* sp. 8D22' (Figure S3 G), *Phocaeicola* sp. A39 (Figure S3 O), *Parabacteroides chinchillae* A10 (Figure S3 I), *Collinsella sp.* A24B (Figure S3 M), were able to use different polysaccharides to grow, reaching the exponential phase in few hours. We observed that the most used fibers among the strains tested were fructo-oligosaccharides (FOS), amylopectin and arabinoxylan. The FOS were used by the strains *Bacteroides* sp. A8D22' (Figure S3 G), *Clostridium* sp. A19 (Figure S3 L), *Parabacteroides chinchilla* A10 (Figure S3 I), unclassified *Bacteroidaceae* A26 (Figure S3 C), unclassified *Lachnospiraceae* A49 (Figure S3 D), followed by amylopectin that was used by three strains, i.e., *Bacteroides thetaiotaomicron* (A14), unclassified *Bacteroidaceae* (A26) and *Phocaeicola* sp. (A39). Finally, arabinoxylan was used by *Bacteroides uniformis* (A4), *Collinsella* sp. (A24B) and

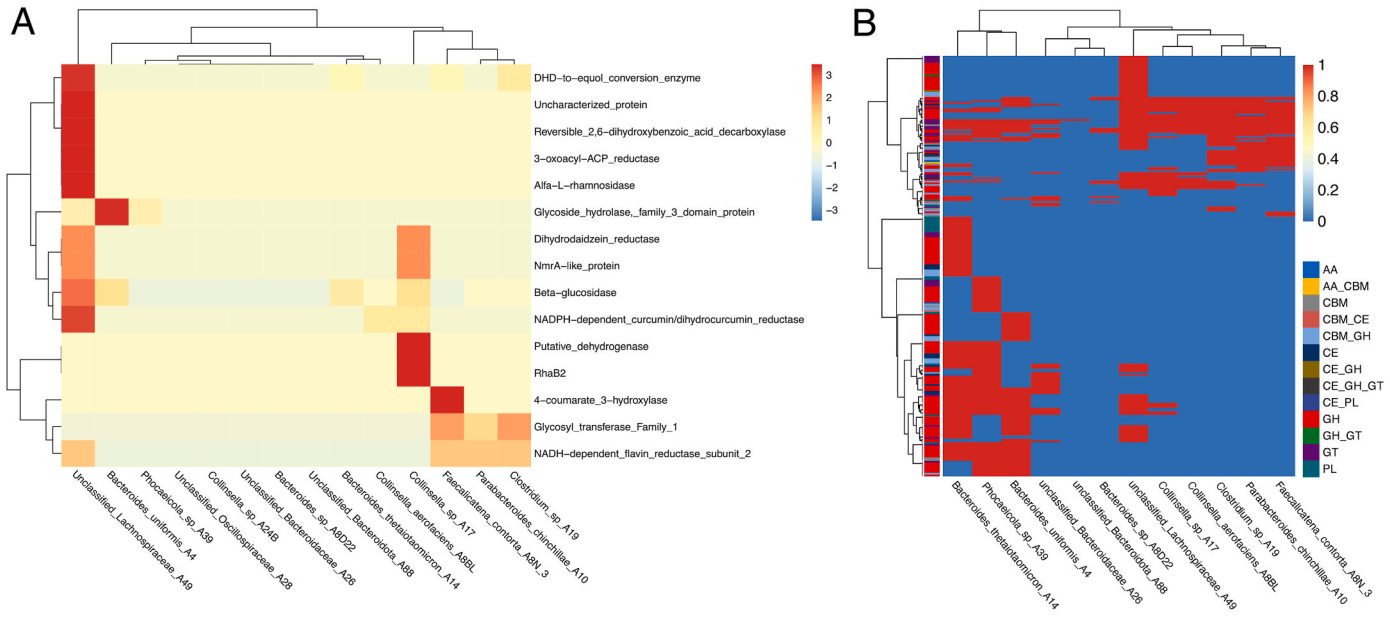

Fig. 1. A. Heatmap representing the number of genes associated with degradation of polyphenols found in the genomes analyzed. **B.** Heatmap representing the presence-absence pattern of different CAZy families found in the genomes analyzed.

unclassified *Lachnospiraceae* (A49). On the contrary, unclassified *Bacteroidota* (A88), *Faecalicatena contorta* (A8N_3), *Collinsella* sp. (A17), *Collinsella aerofaciens* (A8BL) and unclassified *Oscillospiraceae* (A28) were not able to use any of the carbohydrates tested. Finally, glucan from oat and xyloglucan were not used by any of the strains tested.

3.4. In-vitro production of beneficial metabolites

Considering the ability of the strains to use different carbohydrate sources, we further tested their capacity to produce SCFAs from those carbohydrates where a good growth performance was observed. The concentration of the main SCFAs produced in the different broths is reported in Fig. 2A. Indeed, we confirmed that several strains were able to produce SCFAs from carbohydrates fermentation. *Bacteroides uniformis* (A4 and A8D22') produced high concentration of butyric acid from glucuronic acid and FOS, unclassified *Lachnospiraceae* (A49) and *Clostridium* sp. (A19) produced butyrate from FOS, while unclassified *Bacteroidaceae* (A26) was able to produce SCFAs from pectin of different origin (citrus and apple) and, to a lower extent, from FOS, amylopectin and polygacturonic acid (Fig. 2A).

We also evaluated the ability of the strains to produce beneficial metabolites such as equol and urolithins in broth containing the related precursors (i.e., daidzein and ellagic acid; Table S6). The strains *Faecalicatena contorta* (A8N_3), *Bacteroides thetaiotaomicron* (A14), *Clostridium* sp. (A19), *Bacteroides uniformis* (A4), *Collinsella* sp. (A17) and unclassified *Bacteroidaceae* (A26) were able to convert daidzein into equol *in vitro*. The highest amount was observed for *Collinsella* sp. (A17), followed by unclassified *Lachnospiraceae* (A49), consistently with genomic data, that highlighted the presence of a wide pattern of polyphenol-active genes in these strains. Considering urolithins production, 6 strains (unclassified *Lachnospiraceae* A49, *Bacteroides uniformis* A4, *Collinsella* sp. A17, *Collinsella* sp. A24B, unclassified *Bacteroidota* A88, *Bacteroides* sp. A8D22') produced both urolithin C and D, while *Collinsella aerofaciens* (A8BL) and *Parabacteroides chinchillae* (A10) only produced urolithin C and D, respectively. Unclassified *Lachnospiraceae* (A49), *Collinsella aerofaciens* (A8BL) and *Bacteroides uniformis* (A4) produced the highest amount of these metabolites. Conversely, the remaining isolates were not able to produce these beneficial metabolites (Fig. 2B).

3.5. Evaluating the impact of a synbiotic supplement on the gut microbiome in mSHIME model

Four strains were considered more promising for the production of beneficial metabolites (SCFA, urolithins, equol), i.e., *Bacteroides uniformis* A4, *Bacteroides thetaiotaomicron* A14, unclassified *Bacteroidaceae* A26 and unclassified *Lachnospiraceae* A49. Genomes of these strains were screened for antimicrobial resistance and virulence genes, and no matches to critically important antibiotics (according to the World Health Organization; https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf?sfvrsn=3320dd3d_2) or hazardous virulence factors were identified (Table S3). As a control, a supplement containing all components except the strains was used. After 2 weeks of treatment, we carried out a wash out phase where both the SHIME lines received only the polyphenols/carbohydrate mixture (without strain addition; washout_w1). Finally, a second week of wash out where both the lines received only the standard mSHIME medium was carried out (washout_w2). The concentration of SCFAs in the lumen in the different phases (end of the stabilization period: 1–7 days; end of the treatment period: 8–23 days; after 1 week of washout; after 2 weeks of washout) is reported in Fig. 3. The concentration of butyrate and acetate was significantly higher in the SHIME receiving the strains compared with the control, both in AC (95 % CI -1.32 – 0.78 and −1.27 – 0.83 for acetate and butyrate, respectively) and DC (95 % CI -0.46 – 1.69 and −0.69 – 1.42 for acetate and butyrate, respectively) at the end of the experiment ($p < 0.05$; Fig. 3). Notably, this difference was still evident at the end of the wash out periods, suggesting a possible persistence of the inoculated strains up to 2 weeks after the end of the administration. The presence of urolithins and equol was also evaluated in lumen content. However, these metabolites were not detected in any of the samples.

We analyzed shotgun metagenomes of lumen and mucosa samples collected from the SHIME vessels to evaluate the impact of the treatment on gut microbiome. Genomes of the 4 strains used in the treatment were placed in a phylogenetic tree with all the 6027 medium/high quality Metagenome-Assembled Genomes (MAGs; showing completeness ≥50 % and contamination <5 %) (Pasolli et al., 2019) reconstructed from 204 mSHIME samples. Indeed, strains A4, A14 and A26 fell within the *Bacteroidaceae* clade, while strain A49 showed a phylogenetic similarity with *Lachnospiraceae* (Fig. 4 and Table 2). The MAGs clustered in 168 Species-level Genome Bins (SGBs; Table S7). The most prevalent SGBs in the samples were taxonomically linked to *Acidaminococcus*

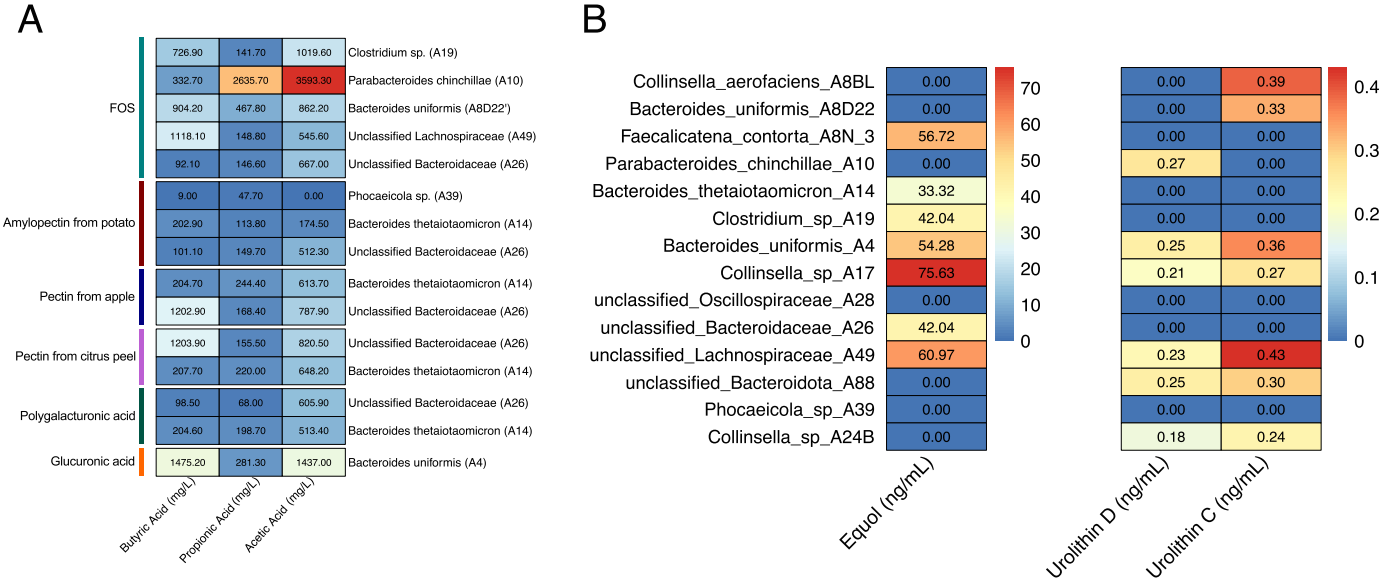

Fig. 2. A. Short-Chain Fatty Acids (SCFA) production by the strains tested from the degradation of different polysaccharides. Values are reported as average of 3 tests (mg/L). **B.** Equol and urolithins production from degradation of daidzein and ellagic acid by the strains tested. Values are reported as average of 3 tests (ng/mL).

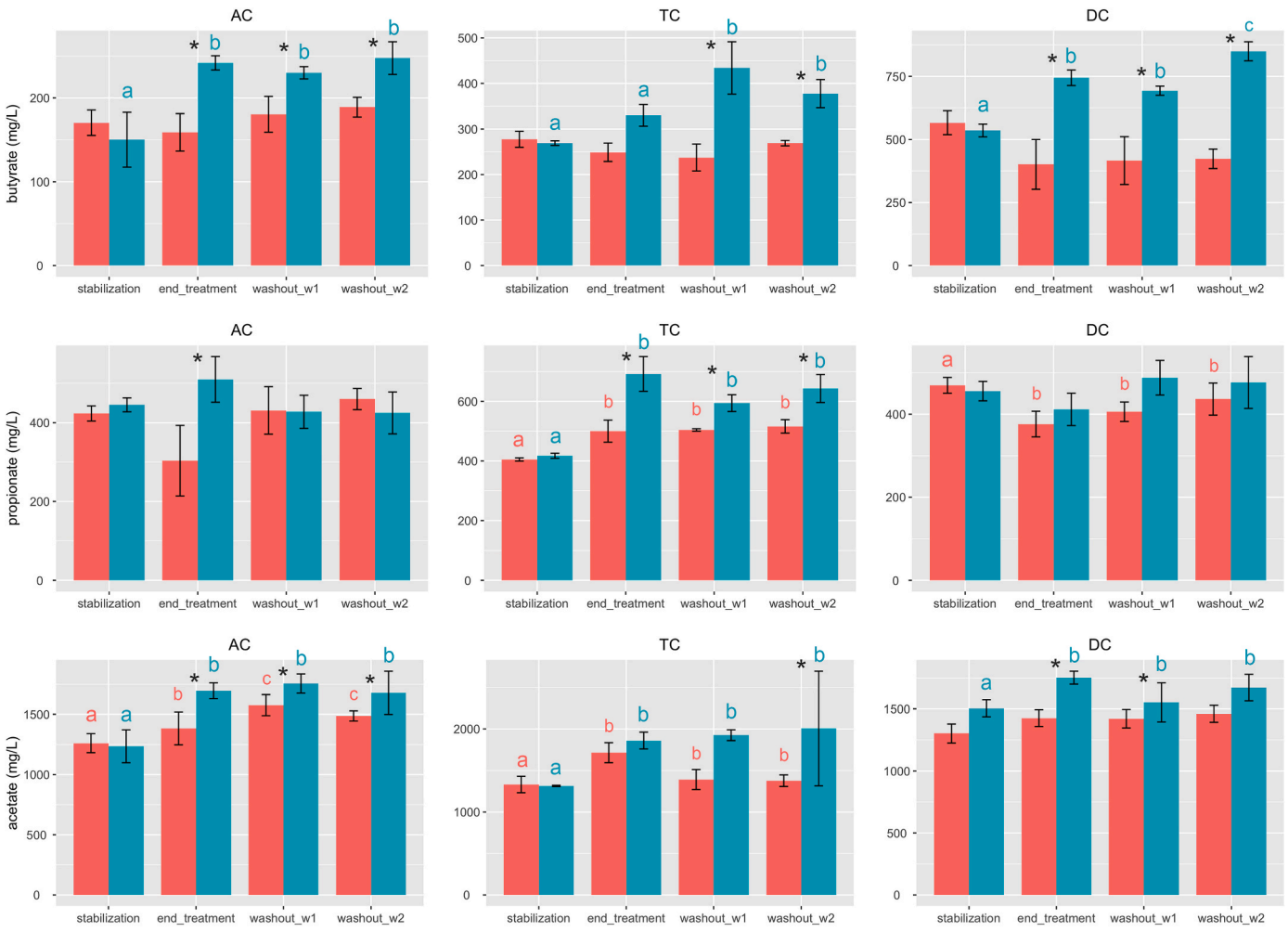

Fig. 3. SCFA amount (mg/L) produced in the mSHIME lumen during the experiment. P ≤ 0.05; * indicates different concentrations between the treatment and the control at each timepoint; different letters indicate significantly different concentrations over time; treatment; control.

intestini (SGB 19) and *Anaeroglobus massiliensis* (SGB 17), with 203 and 198 MAGs respectively, followed by *Bilophila wadsworthia* (SGB 4, n = 184) and *Bifidobacterium adolescentis* (SGB 1 and SGB 40, with 1 and 171 MAGs, respectively). The SGB taxonomically assigned to *B. thetaiotaomicron* (SGB 31) and *B. uniformis* (SGB 365) included 81 and 134 MAGs, respectively. As expected, the reconstruction of MAGs from these species was enriched in the Treatment group (chi-squared test p-value <0.05) and was higher in the treatment group compared to control (chi-squared test p-value <0.05; Fig. S4A and S4B). Notably, this result was not influenced neither by the number of reads, nor by the number of MAGs detected in each sample (Fig. S4C and S4D).

However, the phylogenetic trees of *B. thetaiotaomicron* and *B. uniformis* built on species-specific genes and including both MAGs and isolates did not highlight a strain level stratification according to treatment and period (Figs. S5 and S6).

A total of 331 species were detected in mSHIME taxonomic profiles. Of these, 16 were dominant, occurring in at least 95 % of the samples with an average relative abundance >0.1, including *Faecalibacterium prausnitzii, Bifidobacterium adolescentis*, *Bacteroides ovatus* and *Parabacteroides merdae*. Principal Coordinates Analysis based on the pairwise Bray-Curtis distance of the taxonomic profiles highlighted that samples collected from lumen and mucosa clustered separately (Fig. S7; PERMANOVA p-value <0.05), although we did not observe a separation according to the treatment (PERMANOVA p-value >0.05). In addition, we observed a very strong similarity between communities detected in transverse and descending traits (Fig. S7; PERMANOVA p-value >0.05), therefore we pooled samples from these two vessels in subsequent analyses. Although the treatment did not increase the microbial diversity of the communities in the vessels (Fig. S8; Wilcoxon rank sum test p-value >0.05), the abundance of the species added during the experiment was higher in the treatment compared with the control. Interestingly, *Bacteroides uniformis* (species of the strain A4) was also found with a higher relative abundance in the mucosal compartment of the ascending section in the treatment group compared to control, whereas *Bacteroides salyersiae* (the closest match of the strain A26; Table 2) enriched in both lumen and mucosa of the transverse + descending sections in the treatment group. In addition, the abundance of *B. salyersiae* was significantly higher in treatment also during the two wash out weeks, suggesting that these strains possibly established in the mucosa of the vessels (Fig. 5A and B). This was not observed for *B. thetaiotaomicron* (species of the strain A14, Fig. 5C). Noteworthily, the added strains beneficially impacted on the microbiome composition and functionality. Indeed, we observed that the relative abundance of *B. thetaiotaomicron* positively correlated with *Akkermansia muciniphila* (Spearman's rho = 0.80; corrected p-value <0.05) and *Phocaeicola vulgatus* (Spearman's rho = 0.72; corrected p-value <0.05), whereas *B. uniformis* co-occurred with *A. muciniphila* (Spearman's rho = 0.75; p-value <0.05) and *B. thetaiotaomicron* (Spearman's rho = 0.83; p-value.< 0.05; Fig. S9). In addition, we calculated Gut Microbiome Wellness Index 2 (GMWI) (Chang et al., 2024), an index that predicts the health status according to the taxonomic profiling of the gut microbiome. We observed a higher GMWI in the mucosa of treatment samples compared

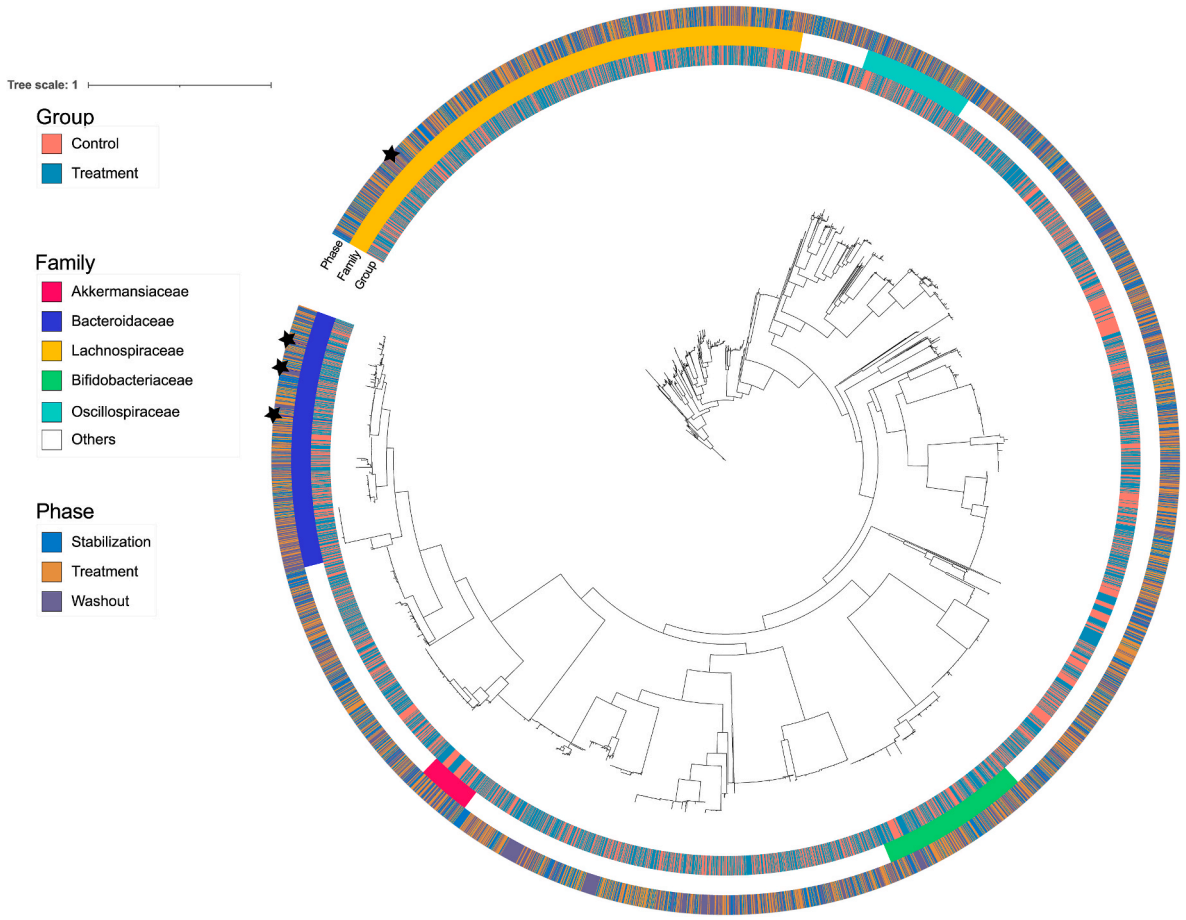

Fig. 4. Phylogenetic tree based on a Multiple Sequence Alignment of all the 6027 MAGs and NGP genomes (marked with a star) to a set of 120 bacterial marker genes from the GTDB-Tk database. Nodes are color-coded according to treatment (inner ring), family (middle ring) and experimental phase (outer ring).

with the control, suggesting that the treatment enriched the community with beneficial species that are considered as marker of eubiosis (Fig. 6).

Furthermore, the supplement containing the putative NGP strains impacted on the metabolic potential of metagenomes. We evaluated the completeness of KEGG pathways using KEGGaNOG (https://github.com/iliapopov17/KEGGaNOG). Our results show that the completeness of pathways related to nitrite and nitric oxide reduction was depleted by the treatment with NGP, while the completeness of "mevalonate pathway", related to the production of vitamin K, increased (Fig. 7A). These changes led to a clustering of samples of lumen according to the treatment and the phase (Fig. 7B). Consistently, a Principal Coordinates Analysis (PCoA) based on KEGG pathways completeness highlighted a separation of samples from NGP-receiving SHIME (PERMANOVA p-value <0.05; Fig. 7C).

4. Discussion

The attention of the scientific and industrial community towards the identification of putative NGP strains is increasing. Indeed, accruing evidence suggests that these strains may have higher chance to reach, proliferate and persist in the gut, thus producing beneficial metabolites *in situ* (De Filippis et al., 2022). In this study, we firstly screened several strains of gut origin for the production of beneficial metabolites *in vitro*. Subsequently, the most promising strains were administered in a mSHIME model, to evaluate their behavior in the complex gut microbiome community. Putative NGP strains were tested for their ability to produce beneficial metabolites, such as SCFA, urolithins and equol. Indeed, we confirmed that *Bacteroides* strains are able to grow using a wide range of complex carbohydrates, including pectin (Lalowski and Zielińska, 2024). This versatility may help to explain the prevalence of *Bacteroides* spp. As dominant species in the colon (Lalowski and Zielińska, 2024). Moreover, we identified several new strains that are able to use a diverse pattern of polysaccharides, suggesting that a complementary activity may be necessary in the gut, in order to break-down complex matrices ingested through the diet. Among them, we also found some strains that were not identified previously, belonging to *Bacteroidota* class, *Oscillospiraceae* and *Lachnospiraceae* family, highlighting the importance of the gut microbiome as a still unexplored reservoir of interesting strains, with potentially probiotic activity. The break-down of plant polysaccharides is the necessary step for the fermentation of sugars by gut microbiome, with production of SCFAs. SCFAs have been widely studied for their beneficial role on host health, such as providing energy to colonocytes, maintaining intestinal barrier integrity, and supporting the immune functions (Duncan and Sabater, 2025). In addition, dietary fibre utilization in the colon helps maintaining eubiosis, preventing pathogen growth, and promoting the absorption of nutrients (Duncan and Sabater, 2025). Most of the strains tested in this study were able to produce the three major SCFA *in vitro* using different carbohydrates and the administration of four of them in the SHIME model was able to boost the production of SCFA in the gut.

Although SCFAs are the most studied among the beneficial metabolites produced by gut bacteria, other important compounds emerged more recently, including those derived from polyphenols degradation. Urolithins are formed through the gut microbial metabolization of ellagic acid. These compounds are reported to have several health-promoting activities, including anti-inflammatory and anti-carcinogenic roles (García-Villalba et al., 2022; Makki et al., 2018; Gandhi et al., 2022). The ability to produce urolithins varies widely in

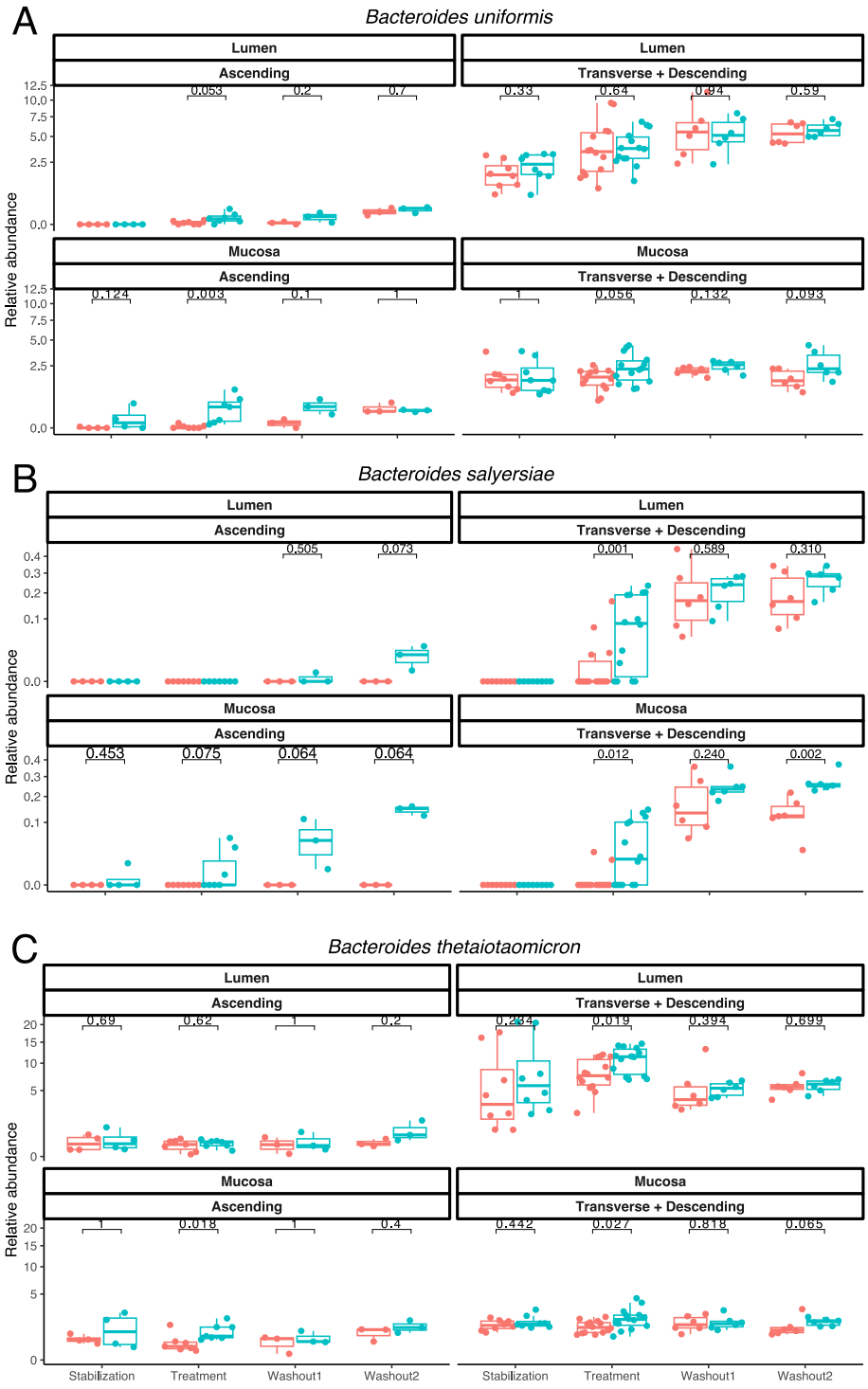

Fig. 5. A. Box plots comparing the relative abundance of *B. uniformis* between treatment and control samples in all the phases, grouped by lumen or mucosa and SHIME vessel (ascending, transverse or descending). **B.** Box plots comparing the relative abundance of *B. salyersiae* between treatment and control samples in all the phases, grouped by lumen or mucosa and SHIME vessel (ascending, transverse or descending). **C.** Box plots comparing the relative abundance of *B. thetaiotaomicron* between treatment and control samples in all the phases, grouped by lumen or mucosa and SHIME vessel (ascending, transverse or descending). The Wilcoxon's rank sum test was used to compare the mean values between the groups; ■ treatment; ■ control.

the population, since it depends on the composition of the individual gut microbiome (Gandhi et al., 2022; Iglesias-Aguirre et al., 2023a). Indeed, several people lack the necessary bacteria in the gut and are not able to produce urolithins, even if consuming ellagic acid-rich foods, such as pomegranate, berries and nuts (Meslier et al., 2020; Meroño et al., 2022). The number of urolithin-producing bacteria reported in the literature is limited, including *Eggerthella*, *Gordonibacter*, *Ellagibacter* spp., and *Bifidobacterium* spp. (Iglesias-Aguirre et al., 2023b; Selma et al., 2014; He et al., 2024). Another health-related metabolite coming from microbial metabolism of polyphenols is equol, produced by gut bacteria metabolizing daidzein, a typical isoflavone present in soy. Equol has been suggested to have a protective role against cardiovascular and neurodegenerative diseases, and an estrogenic and anticancer activity (Mayo et al., 2019; Lv et al., 2024). It has been estimated that

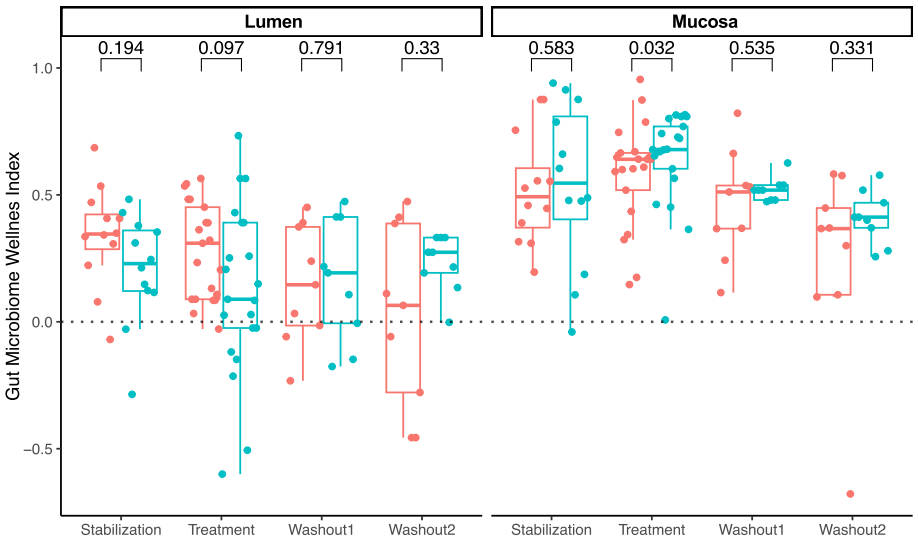

Fig. 6. Box plot comparing the microbiome Gut Microbiome Wellness Index 2 between treatment and control at all the phases. Samples are split by lumen or mucosa. The Wilcoxon's rank sum test was used to compare the mean values between the groups; treatment; control.

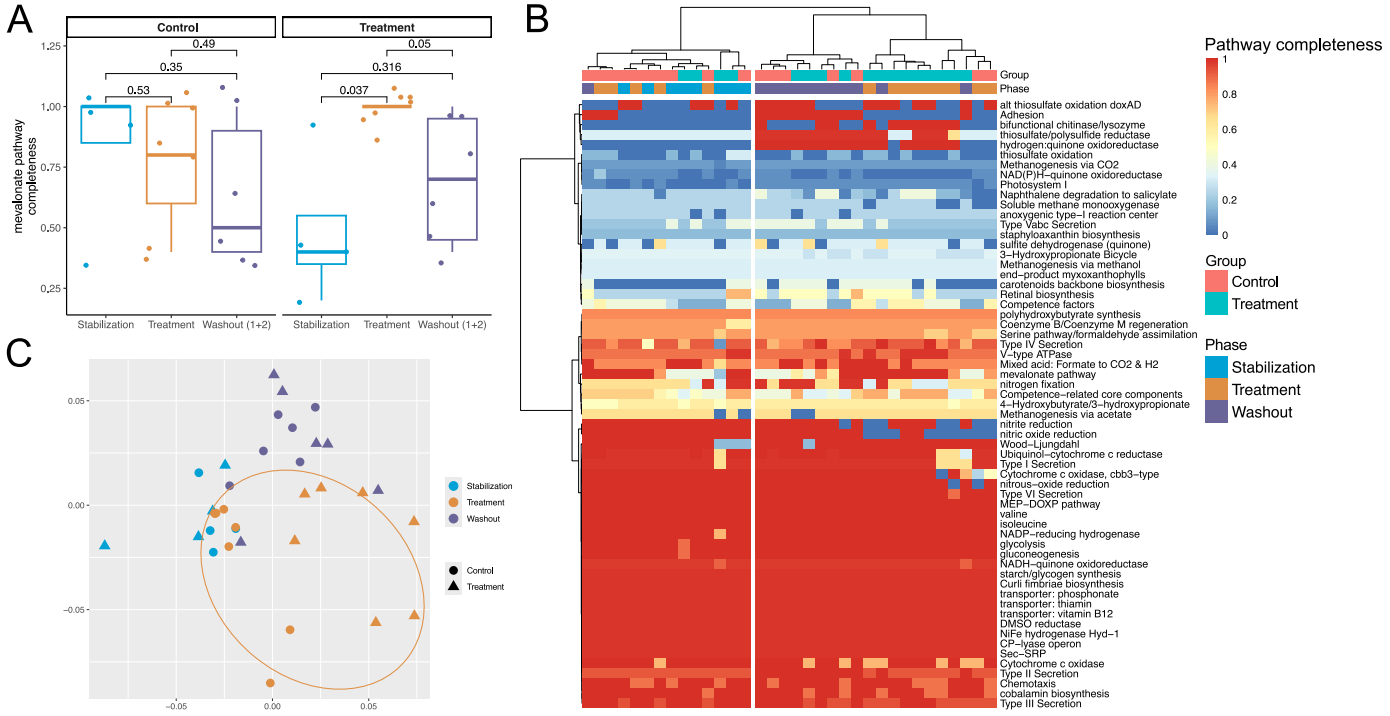

Fig. 7. A. Box plot comparing the completeness of the "mevalonate pathway" between samples collected during different experimental phases. **B.** Ward's linkage clustering of the KEGG pathway completeness profiles of lumen samples (0 = pathway absent; 1 = pathway complete). Columns (i.e., samples) are colored according to group (treatment or control) and phase (stabilization, treatment, washout). **C.** Principal Coordinate Analysis of KEGG pathways completeness profiles of lumen samples based on the Bray-Curtis distance.

only 30 % of Western population is able to produce equol, regardless the consumption of the dietary precursor (Magee, 2011; Wu et al., 2016). Indeed, also in this case only few taxa have been shown to be equol-producers, such as members of the *Coriobacteriaceae* and *Eggerthellaceae* families, and some strains of lactic acid bacteria (Sánchez-Calvo et al., 2013; de la Bastida et al., 2021; Dufault-Thompson et al., 2022; Wang et al., 2023). Therefore, the administration of polyphenol-degrading NGP may overcome the restrictions linked with the limited widespread of polyphenol-metabolizing potential in the gut microbiome. Interestingly, we described the ability of producing urolithins C and D *in vitro* in some *Bacteroides* strains and in an unclassified strain from *Lachnospiraceae* family, while some strains of *Collinsella*, *Bacteroides* and *Lachnospiraceae* also produced equol, consistently with the genomes screening, that showed a high prevalence of genes involved in the conversion of daidzein to equol (e.g., NADPH-dependent curcumin/dihydrocurcumin reductase, DHD-to-equol conversion enzyme and dihydrodaidzein reductase) in the strains. Nevertheless, none of the strains tested was able to produce these metabolites when inoculated in a complex microbiome community in the SHIME ecosystem. This may be explained by the competition with the resident microbiome and/or the shift towards different metabolisms. This activity may be boosted increasing the concentrations of the strains and/or the precursors added

and therefore, the use of these strains as NGPs needs further optimization. Indeed, the quantities of ellagic acid (urolithin precursor) and daidzein (equol precursor) were chosen based on those that can be introduced through nuts and soy in healthy dietary patterns (e.g., Mediterranean diet, cancer-preventive diet) according to previous studies. At the same time, the use of these strains as postbiotics (e.g., inanimate preparations containing the dead cells and the growth supernatant) may also be evaluated.

However, our results suggested that the administration of a synbiotic supplement containing NGPs, fibre and polyphenols may positively change gut microbiome composition, boosting the development of beneficial taxa and increasing SCFA production. Synbiotic formulations (i.e., mixture of probiotics and prebiotics (Swanson et al., 2020)), have proved to outperform probiotics and prebiotics alone in restoring the gut microbiome eubiosis and in ameliorating diseases (Wang et al., 2023; Khursheed et al., 2022; Zhang et al., 2023; Batista et al., 2020). Indeed, our results showed that the administration of the selected strains to the SHIME reactors impacted on the microbiome composition and functionality more efficiently than prebiotics alone (fiber + polyphenols) *ex vivo*. Although SHIME microbiome taxonomic profiling stopped at species level, our results suggested that the strains added were able to engraft in the mucosal layer and to persist up to 2 weeks of wash out, indicating that long-term effects from the regular intake of the putative NGP strains might be expected. *Bacteroides* spp. Strains contained in the supplement mainly persisted in transverse and descending traits (i.e., the most anoxic parts of the gut), since their sensibility even to low oxygen concentrations (Boyanova et al., 2024). In addition, the supplement also might boost the enrichment in health-promoting species such as *Akkermansia muciniphila*, which plays a pivotal role in maintaining the gut barrier integrity and in defending the host from pathogens (De Filippis et al., 2022), and *Parabacteroides distasonis,* a species encompassing candidate probiotics producing neuroactive compounds and beneficial metabolites (Duan et al., 2024). Both species have been previously reported as positively affected by polyphenols presence in the gut. Indeed, *A. muciniphila* seems to be selectively promoted by different polyphenol classes (Rodríguez-Daza et al., 2021; Rodríguez-Daza and de Vos, 2022), while both *P. distasonis* and *A. muciniphila* were enriched in mice receiving a supplement containing pomegranate fruit pulp polyphenols (Song et al., 2021). Moreover, a positive correlation of *A. muciniphila/P. distasonis* with *B. thetaiotaomicron/B. uniformis* was found, possibly indicating a cross-feeding between them. The synbiotic treatment also led to a significant increase of the Gut Microbiome Wellness Index in the arm receiving fiber, polyphenols and strains compared to the control, confirming the switch towards a health-promoting gut community. The intervention also had effects on the metabolic potential of the microbiome in the vessels. We observed a reduction in the nitrite and nitric oxide reduction pathways, used by several gut pathogens to overcome the toxicity of reactive nitrogen species (Leclerc et al., 2021; Alam et al., 2002). Their reduction in the lumen might indicate a depletion of species linked with pro-inflammation and pathogenesis. On the contrary, the enrichment in the completeness of the mevalonate pathway might be linked with enhanced production of vitamin K precursors by the gut microbiome (Liao et al., 2016), further contributing to keep microbial diversity and prevent inflammation (Chatterjee et al., 2023).

5. Conclusions

Our study followed a comprehensive pipeline to test and develop novel NGPs aimed at beneficially modulating the gut microbiome. The potential food supplement tested here may serve as a promising dietary aid, particularly for individuals who cannot consistently adhere to a healthy diet. It contains bioactive compounds, such as polysaccharides, dietary fibers and polyphenols—common in fruits, vegetables, and legumes, key components of the Mediterranean diet—which, together with metabolically active strains, effectively modulated the gut microbiome *ex vivo*. NGPs that produce beneficial metabolites like SCFAs, urolithins, and equol show promise for gut health. SHIME trial indicates these strains can survive, engraft, and promote beneficial microbes in the gut, such as *Akkermansia muciniphila*. However, we have to point out that the results observed have been obtained in a simulator of the gastrointestinal ecosystem, that only partly reproduce the human gut microbiome. In addition, donor-specific traits in the gut microbiome may influence strain engraftment and their behavior in the gut ecosystem. Indeed, before human use, rigorous safety and regulatory assessments, including antibiotic resistance and toxicology analyses, followed by clinical trials on large human cohorts are needed. However, these preliminary studies may pose important scientific bases to speed up the regulatory approval of NGP. Next steps should integrate mechanistic studies to understand microbial interactions and contribution in metabolites production, and human trials to evaluate safety, tolerability and *in vivo* efficacy.

CRediT authorship contribution statement

A.E., V.V. investigation, formal analysis, visualization, writing – original draft; S.T., G.S. formal analysis, writing – review & editing; P.V. resources, supervision, writing – review & editing; D.E., conceptualization, resources, writing – review & editing; F.D.F., conceptualization, resources, supervision, writing – original draft.

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.

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The graphical abstract was created in BioRender. Valentino, V. (2026) https://BioRender.com/h2bbfg1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2025.101289.

Data availability

The sequence data that support the findings of this study (both Whole Genome Sequencing and shotgun metagenomes) are openly available in Sequences Read Archive (SRA).

Database of the NCBI at https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1203439PRJNA1203439https://github.com/enzovalentino/NGP-paper-data-analysis

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