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Shrimp by-product-derived astaxanthin modulates gut microbiota composition and proteolytic metabolism: valorization of a sustainable bioactive ingredient

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ABSTRACT

Astaxanthin is a bioactive carotenoid of growing interest as a functional food ingredient, yet its recovery from sustainable sources and its interactions with the gut microbiota remain insufficiently characterized. In this study, astaxanthin-rich extracts were recovered from shrimp by-products (*Parapenaeus longirostris*) through ultrasound-assisted extraction and subjected to detailed chemical characterization by HPLC and UPLC-ESI-TQ-MS, confirming astaxanthin as the predominant carotenoid (80.4% of the quantified fraction) alongside a defined isomeric profile. To assess the biological activity of this ingredient at the colonic level, physiologically relevant concentrations were tested in *in vitro* colonic fermentations inoculated with fecal microbiota from three healthy human donors, enabling the capture of interindividual variability. Microbial dynamics were assessed by 16S rRNA gene sequencing and culture-based methods, and functional responses were determined through short-chain fatty acid and ammonium production. Astaxanthin supplementation induced donor-dependent responses, with health-associated genera including *Bifidobacterium*, *Faecalibacterium*, *Roseburia*, and *Coprococcus* increasing in responsive donors, while potentially pro-inflammatory taxa such as *Escherichia–Shigella*, *Bilophila*, and *Desulfovibrio* decreased. Functionally, astaxanthin attenuated proteolytic metabolism, evidenced by reduced ammonium and branched-chain fatty acid levels, suggesting a selective shift toward a more saccharolytic and metabolically favorable microbial profile. Overall, shrimp-derived astaxanthin acts as a selective modulator of gut microbial metabolism in a microbiota-dependent manner, and these findings support the valorization of crustacean by-products as a sustainable source of bioactive astaxanthin with promising applications in gut health-oriented ingredients.

1. Introduction

Astaxanthin, a highly bioactive xanthophyll carotenoid naturally found in microalgae, crustaceans, and salmonid fish, is well known for its potent antioxidant and anti-inflammatory properties. As a fat-soluble compound with a unique polar-nonpolar-polar molecular structure, astaxanthin can span the lipid bilayer of the cell membrane, where stabilizes membrane integrity and provides antioxidant protection at both the membrane surface and within the lipid core. Through this positioning, astaxanthin efficiently neutralizes reactive oxygen species (ROS) and reactive nitrogen species (RNS), thereby protecting cells from oxidative damage (Pereira et al., 2021; Sztretye et al., 2019). This specific structure is the reason for its broad biological activity, including anti-inflammatory effects, and support of mitochondrial and cellular function (Joubert et al., 2025; Wolf et al., 2010; Youssef et al., 2025). Importantly, *in vivo* and *in vitro* studies comparing astaxanthin with closely related carotenoids—such as β-carotene, α-carotene, lutein, and lycopene—have shown that astaxanthin exhibits higher antioxidant activity (Donoso et al., 2021).

Astaxanthin has been associated with multiple physiological effects, including immunomodulation, protection against lipid oxidation, and potential benefits in cardiovascular, metabolic, and neurodegenerative

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conditions (Hashemi et al., 2025; Wang et al., 2022; Yamashita, 2021). Clinical studies further support its safety and bioactivity in humans (Bjørklund et al., 2022; Donoso et al., 2021). Natural astaxanthin occurs as a mixture of geometric (e.g., all-trans and cis forms) and optical isomers (Xue et al., 2025; Yu & Liu, 2020), whose distribution varies depending on the biological source and processing conditions (Elbahnaswy & Elshopakey, 2024; Tan et al., 2021). These structural features, together with differences in esterification have been shown to directly influence its biological activity and functional properties (Xue et al., 2025).

Despite its wide applicability in nutraceutical, cosmetic, aquaculture, and pharmaceutical sectors, the large-scale production of natural astaxanthin remains limited by cost and yield constraints (Peng et al., 2025; Zhu et al., 2023). While it is mainly obtained from microalgae and yeast, alternative and more sustainable sources are being explored (Abdelazim et al., 2023; Basiony et al., 2022). Marine animals such as salmon, trout, and shrimp cannot synthesize astaxanthin *de novo* and instead accumulate it through their diet. For instance, shrimp processing, particularly of the genera *Penaeus* and *Litopenaeus,* generates large quantities of by-products, including heads, shells, viscera, and exoskeleton (Gulzar et al., 2020; Putra et al., 2024; Šimat et al., 2022). These wastes represent an inexpensive and sustainable raw material for the recovery of high-value astaxanthin using environmentally friendly extraction technologies (Panagiotakopoulos & Nasopoulou, 2024).

In addition to its systemic effects, current evidence suggests that gut microbiota may influence the bioavailability and biological activity of astaxanthin (Li et al., 2022), while astaxanthin itself may modulate microbial composition and metabolic outputs. Specifically, some animal studies have shown that carotenoids, including astaxanthin, promote taxa associated with beneficial functions, as well as influence the production of bioactive metabolites (Pratap et al., 2022; Ren et al., 2025a). However, direct evidence of astaxanthin-microbiota interactions in human-derived systems remains limited. *In vitro* colonic fermentation models provide a controlled framework to investigate these interactions, enabling the assessment of microbial composition and metabolic activity in response to dietary compounds (Cueva et al., 2015; Tamargo et al., 2018). Although previous studies have highlighted inter-individual variability as a key factor influencing microbial responses to bioactive compounds (Huan et al., 2025), this aspect has not been systematically addressed for astaxanthin. Across *in vitro* studies conducted to date involving astaxanthin directly, pooled fecal samples have been consistently used (Dai et al., 2022; Ren et al., 2025a, 2025b), thereby limiting the assessment of donor-specific responses. Moreover, most available studies on astaxanthin-microbiota interactions exclusively used astaxanthin derived from microalgae, fungi or commercial sources (Huan et al., 2024; Rong et al., 2026; Wang et al., 2019), with no attention to alternative sources such as marine by-products.

The present study aimed to investigate, for the first time, the effect of a chemically characterized astaxanthin extract obtained from the by-products of deep-water rose shrimp (*Parapenaeus longirostris*) on the gut microbiota using *in vitro* colonic fermentations inoculated with fecal samples from three healthy donors. We combined microbial community profiling through 16S rRNA gene sequencing with functional analyses (short-chain fatty acids and ammonium production) to gain insights into both ecological and metabolic responses, with a particular focus on donor-specific variability.

2. Materials and methods

2.1. Extraction and preliminary characterization of astaxanthin from shrimp by-products

Shrimp (*Parapenaeus longirostris*) by-products were collected from a local company, Centaurus Ltd., Mravince, Croatia. The shrimps were not treated for melanosis with any substance. After shrimp processing, the by-products (exoskeletons, heads) were delivered to the laboratory in ice at a temperature of 2 ± 1 °C and homogenized in a MultiDrive control mill (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 10,000 rpm. The crude extract was prepared using ultrasound-assisted extraction (UAE) (DU-100 Digital Ultrasonic Cleaner, Giorgio Bormac, Carpi, Italy) at room temperature for 30 min in ethanol, centrifuged (Rotafix 32A, Hettich, Tuttlingen, Germany), and the upper part was decanted. The concentration of astaxanthin was measured using spectrophotometric analysis (UV-1900i, Shimadzu, Tokyo, Japan) at 479 nm against the standard curve.

For HPLC analysis, the shrimp by-product extract was saponified. The 10% KOH–ethanol solution was added to the crude extract to adjust the pH to 10, shaken evenly, and saponified in the refrigerator at 4 °C for 2.5 h. The pH was adjusted to 7 with hydrochloric acid, then filtered, and the filtrate was evaporated under vacuum at 40 °C (Hu et al., 2019). The sample was filtered through a 0.22 μm PVDF (Polyvinylidene Fluoride) filter prior to analysis and analyzed by HPLC (Thermo Fisher Scientific UltiMate 3000) on a HALO C30 column (150 × 3.0 mm i.d., 2.7 μm). The column was maintained at 35 °C. The mobile phases consisted of (A) 1% (v/v) acetic acid in water, (B) acetonitrile, and (C) methanol. The flow rate was 1.00 mL min^{-1}, and detection was performed at 450 nm. The gradient program was as follows: 0.00 - 0.20 min, 70% B/0% C; 0.20 - 8.00 min, linear gradient to 100% B/0% C; 8.00 - 9.00 min, linear gradient to 50% B/50% C; 9.00 - 17.00 min, hold at 50% B/50% C; 17.00 - 17.10 min, return to 70% B/0% C; 17.10 - 22.00 min, re-equilibration at initial conditions. Total run time was 22.0 min. The injection volume was 10 μL. Astaxanthin was identified by comparing retention time and absorption spectra with a commercial standard (Astaxanthin ≥95% (HPLC), CAS 472-61-7, Sigma Aldrich).

2.2. Targeted UPLC-ESI-TQ-MS analysis of astaxanthin in shrimp by-product extract

The astaxanthin shrimp by-product extract was characterized prior to colonic fermentation experiments. The determination was carried out using an UPLC-ESI-TQ MS method optimized in this study to achieve selective quantification and structural confirmation in a complex lipid-rich matrix. Prior chromatographic analysis, AST extraction was performed following a previously reported protocol with minor modifications (Diao et al., 2025). Briefly, samples were subjected to ultrasound-assisted extraction in ethanol at 50 °C for 20 min, followed by alkaline saponification with 10% KOH. After neutralization to pH 7, astaxanthin was recovered by liquid–liquid extraction with ethyl acetate. The organic phase was evaporated under reduced pressure, and the residue was reconstituted in methanol and filtered through a 0.22 μm membrane. Chromatographic analyses were performed on a Waters Acquity UHPLC system (Waters, Milford, USA) equipped with a binary solvent manager, an autosampler maintained at 10 °C, and a thermostated column compartment set at 40 °C. Separation was achieved on a BEH-C18 column (2.1 × 50 mm, 1.7 μm particle size; Waters, Milford, USA). The mobile phase consisted of 3 mM ammonium acetate with 0.1% formic acid in water (Phase A) and methanol (Phase B), delivered at a constant flow rate of 0.30 mL/min over a total run time of 10 min, as previously reported with minor modifications (Hu et al., 2019). Initial conditions were 10% A and 90% B, maintained isocratically for 4 min, followed by a linear increase to 95% B between 4.0 and 5.0 min. This composition was held until 6.0 min, after which the organic content was increased to 99.9% B between 6.0 and 7.0 min and maintained until 8.0 min to ensure complete elution of highly hydrophobic carotenoid species and adequate column cleaning. Initial conditions were restored at 9.0 min and maintained until the end of the run to allow column re-equilibration.

Detection was performed using a triple quadrupole mass spectrometer operating in positive electrospray ionization mode. The precursor ion corresponding to astaxanthin was detected at m/z 597.6 under full scan conditions using a cone voltage of 30 V. Structural confirmation was achieved through product ion scan experiments applying a collision

energy of 25 eV, which generated a characteristic fragment ion at m/z 147.2. Quantification was carried out in MRM mode using the transition m/z 597.6 → 147.2, ensuring high analytical selectivity against potential matrix interferences inherent to shrimp-derived lipid fractions. Data acquisition and processing were conducted using MassLynx software version 4.1 (Waters, Milford, USA).

2.3. In vitro colonic fermentation

Three independent static colonic fermentation assays were performed in the simgi® model system using fecal samples from three healthy adult donors: V1 (male, 32 years old, BMI = 23.39), V2 (male, 28 years old, BMI = 23.1), and V3 (female, 28 years old, BMI = 19.8). All volunteers were healthy, with no history of gastrointestinal disorders or other diagnosed chronic diseases, and did not receive antibiotics or pro-/prebiotics for at least six months prior to the study. Fecal inocula were prepared by homogenizing 1 g of feces in 10 mL of phosphate-buffered saline (PBS, 0.1 M, pH 7) in a stomacher for 2 min under anaerobic conditions. Fermentation vessels were filled with 135 mL of prereduced sterile colon nutrient medium (Tamargo et al., 2019) and 15 mL of fecal inoculum. In the treatment condition, 40 mg of astaxanthin extract was added, whereas the control contained only medium and fecal inoculum. Fermentations were carried out under controlled conditions (pH 6.8, 37 °C, 150 rpm) for 48 h, simulating the distal colon environment. At 0, 24, and 48 h, 9 mL samples were collected, centrifuged (10,000 rpm, 4 °C, 10 min), and divided into aliquots. Supernatants were stored at −20 °C for the determination of carotenoid and phenolic profiles, ammonium ion, and short-chain fatty acids. Pellets were stored at −80 °C for DNA extraction and metataxonomic analysis.

The experimental protocol involving human fecal samples was approved by the Ethics Committee of the Spanish National Research Council (CSIC), registered under the internal code PID2019-108851RB-C21, and conducted in accordance with the Declaration of Helsinki. Samples were collected on site on the day of the assay and used immediately.

2.4. Microbial community analyses

2.4.1. Microbial plate counting

Microbial populations were determined by plate counting in the digested samples collected at 0, 24 and 48 h. Microbial counts were performed following standard culture-based procedures as described by Tamargo et al. (2018). Results were expressed as logarithmic colony-forming units per milliliter (log CFU/mL). All plate counts were performed in triplicate.

2.4.2. DNA extraction, 16S ribosomal DNA sequencing and data processing

Pellets of 2 mL from each colonic fermentation flask were used for DNA extraction, using the QIAmp® Fast DNA Stool MiniKit (Qiagen, Germany), following the manufacturer's recommendations, with some modifications (Jiménez-Arroyo et al., 2023. The V3-V4 region of the 16S ribosomal RNA gene was amplified using the following primers: forward 5′- CCTACGGGNGGCWGCAG-3′ and reverse 5′-GACTACHVGGGTATCTAATCC-3′. The Illumina® two-step PCR protocol was followed for library preparation, and sequencing was performed on an Illumina® MiSeq instrument (Illumina®, USA) performing 2 × 275 bp paired-end reads.

RStudio 2024.09.0 software was used to process the files with raw reads. Taxonomic assignment was performed using the naïve Bayesian classifier implemented in DADA2 using Silva v.138.2 as a reference database (Quast et al., 2013). A total of 713 ASVs were detected. Biodiversity, expressed in terms of α-diversity, was estimated using the ASVs by calculating the Observed, Shannon and Simpson indices through the "Phyloseq" package. The β-diversity was assessed by employing a Principal Coordinate Analysis (PCoA) based on weighted unifrac distances. Relative abundances of each taxon were calculated for each sample at the phylum, family and genus level.

2.5. Microbial functionality analysis

2.5.1. Ammonium ion production

To determine the proteolytic activity of the microbiota, the ammonium concentrations in colonic fermentation samples were determined using the photometric Spectroquant® ammonium reagent test (Merck, USA). The results, measured at 690 nm, were obtained from a linear regression plot constructed using ammonium standard solution (Sigma-Aldrich, Merck, USA) whose linear range of interest was between 2 and 75 mg $NH4^{+}$/L. Analyses were performed in duplicate.

2.5.2. Analysis of short chain fatty acids

The analysis of short-chain fatty acids (SCFAs) in *in vitro* colonic fermentation samples was performed using UPLC-ESI-TQ MS, following a previously reported methodology (Cueva et al., 2015; Jiménez-Arroyo et al., 2024) with modifications proposed by Han et al. (2015). SCFAs were quantified using calibration curves of their corresponding commercially available standards from a single supplier (Sigma Aldrich). Data acquisition and processing were performed using MassLynx version 4.1 software. The analysis was carried out using the supernatants from the colonic fermentation assays. All analyses were performed in duplicate for each experiment. An *in situ* internal standard was synthesized for each molecule (acetic acid, propionic acid, butyric acid, valeric acid, caproic acid) using a modified isotopic derivatizing agent with 6 carbon atoms of Carbon-13 (13C6-3-NPH).

2.6. Carotenoids (astaxanthin) monitoring

Astaxanthin stability and potential biotransformation were monitored in colonic fermentation supernatants at 0, 24, and 48 h. Samples were analyzed by direct injection after dilution in methanol using the UPLC-ESI-TQ-MS system and methodology described in Section 2.2, monitoring the MRM transition m/z 597.6 → 147.2. The method provided a LOD and LOQ of 0.010 and 0.031 ppm, respectively, ensuring sufficient analytical sensitivity for the detection of astaxanthin and its potential transformation products in the fermentation matrix.

2.7. Statistical analysis

Statistical analysis was performed using R software v. 4.3.0 and XLSTAT Static for Microsoft Excel. 2024.3.0. A two-way ANOVA was used to evaluate the effect of treatment (baseline/control microbiota vs. astaxanthin extract and fermentation time (0 h, 24 h, 48 h) on all microbial parameters measured for each volunteer's dataset. This factorial analysis was applied independently at each taxonomic level to microbial counts, 16S rRNA gene profiling (phylum and genus levels), and SCFA production. Pairwise comparisons were performed using the Games-Howell post hoc test to resolve differences detected in the two-way ANOVA. To complement the statistical analysis and explore global patterns in microbial community structure, a Principal Component Analysis (PCA) was conducted at the genus level using samples collected at 24 h and 48 h. Spearman's correlation analysis between relative abundance of bacteria at genus level (>0.5%) and SCFAs and ammonium production was applied. All correlation analyses performed were conducted using RStudio v.1.3.1093 and *stats*, *utils* and *qdapTools* packages. A two-way ANOVA with Tukey post hoc correction was used to evaluate differences in ammonium production. Statistical significance was established at an adjusted $p\text{-value} < 0.05$ for all comparisons.

3. Results and discussion

3.1. Characterization of astaxanthin extract and analytical monitoring

The concentration of astaxanthin, determined

spectrophotometrically, was 8.06 ± 0.01 μg/mL. The extract was subjected to saponification and subsequently analyzed by HPLC, which confirmed the presence of astaxanthin as the main carotenoid (Fig. S1). To further ensure analytical selectivity within the complex lipid matrix of shrimp exoskeletons, the extract was additionally evaluated by UPLC–ESI–TQ MS to quantify astaxanthin and assess its isomeric profile. This complementary approach strengthened the chemical characterization and minimized potential interferences from co-extracted carotenoid species that may not be resolved by single-detection techniques. Quantitative analysis revealed that the predominant astaxanthin peak accounted for 80.4% of the total quantified carotenoid fraction (112.55 ± 5.99 μg/g), while two minor peaks represented 7.6% (10.59 ± 0.73 μg/g) and 12.0% (16.81 ± 0.18 μg/g), respectively (Fig. 1). A wide range of extraction protocols for astaxanthin recovery from shrimp by-products has been reported, including supercritical CO_2 extraction (Cabanillas et al., 2021; Mirzaee et al., 2026), microwave-assisted extraction (Nunes et al., 2021), and biotechnological approaches involving enzymatic or microbial-assisted processes (Abd El-Ghany et al., 2023). In the present study, an ultrasound-assisted extraction method was applied, yielding astaxanthin levels within the range reported in the literature. For example, Hu et al. (2019) reported astaxanthin contents ranging from 1.86 to 239.96 μg/g across different crustacean by-products, with optimized ultrasound-assisted extraction conditions yielding approximately 43.7 μg/g in *Pandalus borealis* shells. Similarly, Tran et al. (2024) obtained approximately 48 μg/g using deep eutectic solvents combined with ultrasound. These values are lower than those observed in the present study, suggesting that the extraction protocol and/or raw material used here enabled efficient recovery of astaxanthin. Notably, concentrations within lower ranges have been associated with biological activity in previous studies (Vargas et al., 2025), supporting the suitability of the extract used in the present work. Variability in astaxanthin content across studies is well documented and depends strongly on species, tissue type, and extraction conditions (Hu et al., 2019; Šimat et al., 2022).

Astaxanthin exists in multiple geometric configurations due to its extended conjugated double-bond system. The all-trans (all-E) configuration is generally reported as the predominant form in natural sources, whereas cis-isomers, particularly 9-cis and 13-cis, may occur in smaller proportions and can be generated during processing steps such as heat exposure, light, solvent interactions, or alkaline saponification (Zou et al., 2023). These isomers are typically resolved as distinct peaks under reversed-phase chromatographic conditions. In agreement with previous reports, the isomeric distribution observed in the present extract falls within the ranges described for processed crustacean-derived astaxanthin (Li et al., 2024; Yang et al., 2015), supporting the chemical plausibility and consistency of the detected profile. This compositional characterization provides a robust basis for interpreting the subsequent microbiota modulation results.

Although the extract was obtained by ethanol-based ultrasound-assisted extraction, condition under which major structural components of the crustacean exoskeleton such as chitin and calcium carbonate are not expected to be present at significant levels in the final extract, the potential contribution of minor co-extracted lipophilic compounds cannot be entirely excluded. The chemical characterization confirmed astaxanthin as the predominant component (80.4% of the total quantified carotenoid fraction), and the observed effects on microbial composition and fermentative activity should therefore be interpreted primarily as reflecting the biological activity of the astaxanthin-rich carotenoid fraction, consistent with the concept of marine by-product valorization.

Astaxanthin was monitored in colonic fermentation supernatants throughout the 48 h incubation period. No signal exceeding donor background was detected under the targeted MRM transition (data not shown), with chromatographic profiles comparable to those of the corresponding controls. Rather than reflecting a methodological limitation, this observation is consistent with the known susceptibility of astaxanthin to microbial transformation in colonic environments. Notably, Dai et al. (2022) reported that astaxanthin exhibited the highest colonic degradation rate among four carotenoids tested under *in vitro* fermentation conditions, with retention markedly lower in the presence of gut microbiota than in abiotic controls. At the physiologically relevant concentration used in the present study (0.037 μg/mL), intact

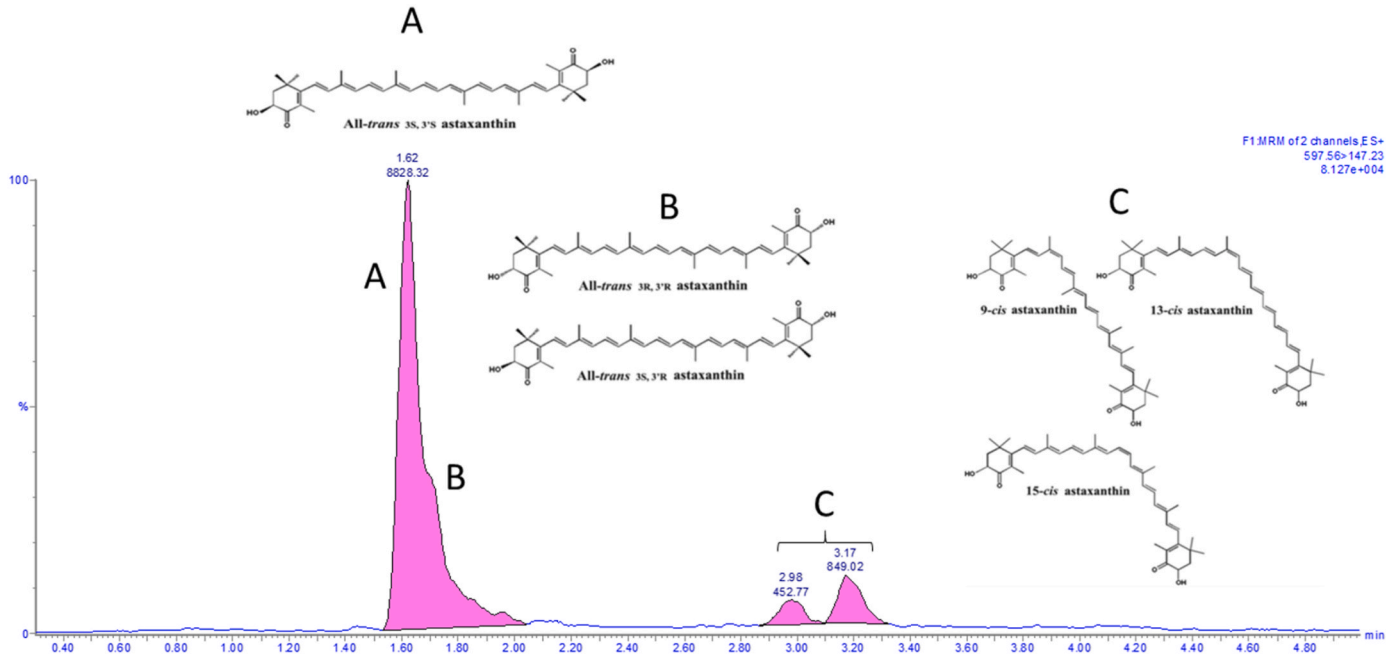

Fig. 1. UPLC–ESI–TQ MS chromatographic profile of astaxanthin extracted from shrimp exoskeleton under the monitored MRM transition m/z 597.6 → 147.2. The figure shows a magnified view of the chromatographic region corresponding to the initial isocratic elution segment (10% A/90% B). The predominant peak (RT 1.62 min) corresponds to the major astaxanthin configuration, accounting for 80.4% of the total quantified fraction. Two additional minor peaks detected at RT 2.98 and 3.17 min represent geometric isomers (7.6% and 12.0%, respectively), tentatively assigned to cis-astaxanthin forms. Structural representations of all-trans and selected cis isomers are included for reference.

astaxanthin would not be expected to persist in detectable amounts following microbial exposure. Biotransformation products, including Z-isomers generated by microbial isomerization (Li et al., 2022) and potential apocarotenoid derivatives arising from oxidative cleavage of the polyene chain (Ahrazem et al., 2016), would yield molecular species with altered fragmentation patterns beyond the scope of the targeted approach employed here. Characterizing the full spectrum of astaxanthin-derived colonic metabolites therefore warrants further investigation, particularly given that direct carotenoid-microbiota contact, as in the present model, may produce transformation profiles distinct from those arising after systemic absorption.

3.2. Colonic fermentation performance and microbial viability

Previous *in vitro* colonic fermentation studies with astaxanthin have generally used pooled fecal inocula and relatively high astaxanthin final concentrations (at least 0.59 μM of free-astaxanthin, 1.68, 16.8 and 838 μM, as reported by Zhang et al. (2026), Ren et al. (2025a), Ren, Qi, et al. (2025), and Dai et al. (2022), respectively). However, some of these concentrations largely exceed those expected under physiological conditions. Human intervention studies typically report daily astaxanthin intakes ranging from 1.8 to 40 mg, with most commonly used doses between 3 and 6 mg/day (Donoso et al., 2021), while EFSA has established a maximum permitted intake of 8 mg/day for adults and adolescents (EFSA, 2020). Moreover, available evidence suggests that a substantial fraction of astaxanthin reaches the colon in bioaccessible form, thereby supporting the physiological plausibility of investigating its direct interactions with the colonic microbiota (Ma et al., 2026). Based on typical intake levels (3–8 mg/day) and estimated colonic conditions, theoretical astaxanthin concentrations are expected to fall within the low micromolar range (≈0.02–0.7 μM). To avoid overestimating physiologically relevant exposure, we selected a comparatively low concentration of astaxanthin corresponding to 40 mg of extract. Given the total astaxanthin content of the extract determined by UPLC-ESI-TQ-MS (139.95 μg/g, comprising the three quantified chromatographic peaks), this amount of extract yields a final concentration of 0.037 μg/mL (0.062 μM) in 150 mL of fermentation medium, representing a conservative lower-bound scenario within the expected *in vivo* range at the colonic level, consistent with colonic bioaccessibility estimates reported by Ma et al. (2026). Faecal fermentations in the presence of astaxanthin and corresponding non-supplemented controls were first monitored by microbial counts. Astaxanthin maintained overall microbiota viability while driving donor-dependent changes in particular bacterial taxa (Table S1).

3.3. Changes in metataxonomic composition during colonic fermentations

For donor V1, α-diversity indices (Observed, Shannon, Simpson) remained quite stable across time. In contrast, in V2 donor, both the Shannon and Simpson indices were significantly lower in the astaxanthin group compared to the control at 24 h, but increased by 48 h, as well as Observed ASVs, suggesting partial recovery of richness and evenness. In V3, both indices increased at 24 h compared to baseline, although no significant differences were observed at 48 h. Overall, these patterns indicate that astaxanthin did not induce major shifts in α-diversity but may transiently modulate richness and evenness in microbial ecosystem (Table S2, Fig. S2).

β-diversity analysis (PCoA based on Bray–Curtis dissimilarity) revealed clear clustering by donor and fermentation time, indicating that inter-individual variation and temporal dynamics were the main drivers of community structure (Fig. S3). This reinforces the strong inter-individual variability in the microbiota response, consistent with previous evidence showing that baseline microbial composition largely determines the outcome of dietary interventions (Johnson et al., 2019). Indeed, changes on structure of microbial communities over time for V1 were similar for both conditions, suggesting poor differences in the communities between the control and astaxanthin conditions. In line, V2 revealed a similar modulation overtime comparing to control. However, for V3, astaxanthin promoted a different microbial profile respect to the control, revealing differences from 24 h (Fig. S3). Collectively, these results underscore that inter-individual variability is a key determinant in the microbiota response to astaxanthin. This aligns with previous evidence showing that baseline gut microbiota composition largely dictates individual outcomes of dietary interventions (Johnson et al., 2019), including astaxanthin bioavailability and systemic effects (Li et al., 2022; Wang et al., 2025). Experimental evidence in animal models further supports the concept of "responder microbiota", where defined microbial consortia enhance the biological effects of astaxanthin (Huan et al., 2025).

Taxonomic analysis revealed that the baseline faecal inocula (V1, V2, and V3) were dominated by members of the phyla Bacillota and Bacteroidota, followed by Actinobacteriota and Pseudomonadota. After fermentation in the presence of astaxanthin, donor-specific shifts were observed (Fig. 2). In donor V1, fermentation resulted in a slight decrease in Bacillota accompanied by a modest increase in Pseudomonadota at 24 h, with profiles at 48 h comparable to the control. In V2, Bacillota levels decreased at 48 h following an initial increase at 24 h, while Actinobacteriota showed a similar temporal pattern. Conversely, Pseudomonadota decreased at 24 h, followed by a clear increase at 48. In V3, a pattern similar to that observed in V2 was found for Actinomycetota and Pseudomonadota. In addition, a significant increase in Bacillota was detected at 24 h, accompanied by a decline in Thermodesulfobacteriota levels (Fig. 2).

Delving deeper at the phylogenetic level, astaxanthin supplementation induced time-donor-dependent and time-resolved changes in genus-level composition (Fig. 3A, Table S4). At 24 h, microbial shifts suggested potential health-associated modulation in donors V2 and V3, whereas responses in V1 were more heterogenous. In V1, astaxanthin led to decreases in *Streptococcus*, *Phascolarctobacterium*, *Coprococcus* and *Alistipes*, alongside increasesing *Escherichia-Shigella*, *Parasutterella* and Erysipelotrichaceae UCG-003. In contrast, donors V2 and V3 showed a decrease in *Escherichia-Shigella,* and an increase in genera such as *Blautia* and *Flavonifractor*, which are associated with benefitial metabolic functions including SCFA production and flavonoid metabolism (Liu et al., 2021; Rodríguez-Castaño et al., 2020). Notably, *Bifidobacterium* abundance also increased in both donors. This observation is consistent with previous *in vitro* fermentation studies reporting increases in *Bifidobacterium* following astaxanthin supplementation (Ren et al., 2025a, 2025b). Further donor-specific responses were observed. In V2, astaxanthin supplementation was associated with increased abundance of *Mediterraneibacter* and *Monoglobus.* genera involved in the degradation of complex carbohydrates such as pectins (Crost et al., 2023; Meiners et al., 2025). In V3, enrichment of butyrate-producing genera such as *Roseburia* and *Coprococcus* (Nie et al., 2021; Yang et al., 2023) was observed together with increases in *Rumminococcus*, *Phascolarctobacterium* and *Dorea*. Concomitantly, potentially detrimental taxa, including *Lachnoclostridium*, *Alistipes*, and *Escherichia–Shigella* decreased, supporting a shift toward a more favorable microbial profile (Cai et al., 2022; Zhao et al., 2023) (Fig. 3A–Table S4). At 48 h, a convergent response across donors was observed, characterized by increased abundance of *Faecalibacterium*, a key butyrate-producing genus associated with anti-inflammatory effects and gut homeostasis (Danne et al., 2026; Sabater et al., 2026). However, donor-specific patterns remained evident. In V2 and V3, microbial profiles evolved and reorganized, retaining some original members while incorporating taxa recognized for their homeostatic functions, with increases in SCFA-producing genera such as *Coprococcus* and commensal taxa including *Parabacteroides* and *Gemmiger*. In V2, despite the increase in *Escherichia-Shigella*, additional increases were observed in *Intestinimonas*, *Lacticaseibacillus*, and *Bacteroides*, alongside reductions in opportunistic genera such as *Enterococcus* and *Pseudomonas*. In V3, increases in *Dorea* and *Ruminococcus* were accompanied by decreases in genera often

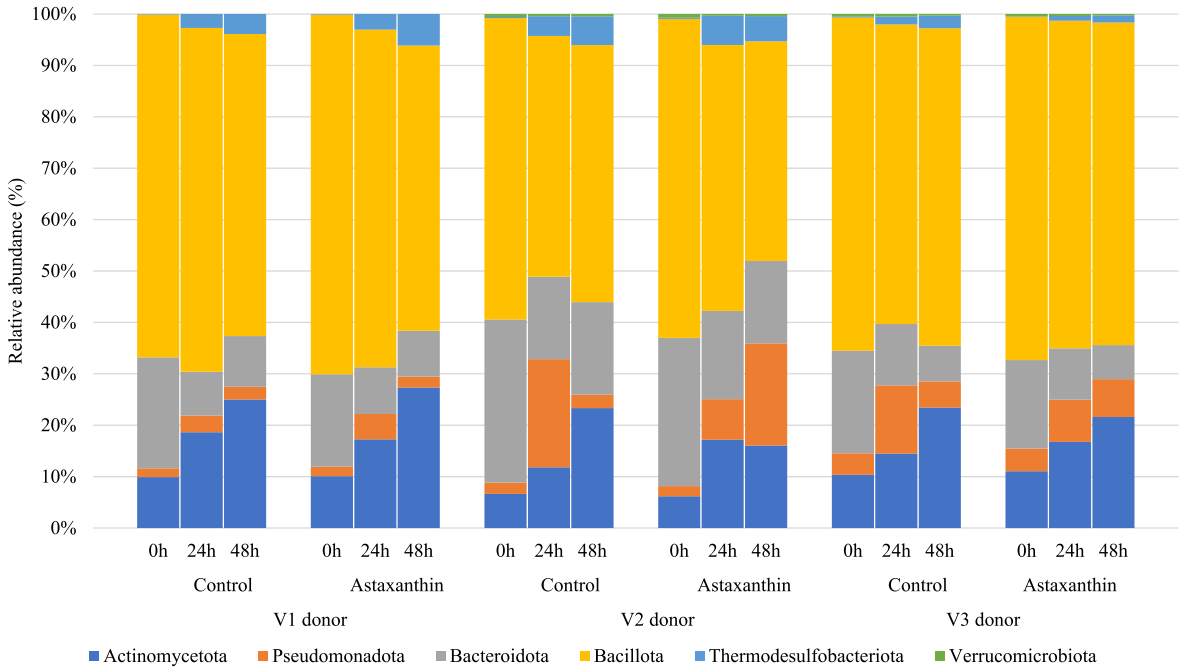

Fig. 2. Changes on relative abundance at phylum level during colonic fermentation with astaxanthin at 0, 24, and 48 h.

associated with gut dysfunction, including *Collinsella*, *Bilophila*, *Desulfovibrio*, and *Sutterella* (Sayavedra et al., 2025; Singh et al., 2023; Zheng et al., 2020). In contrast, V1 exhibited a relatively stable microbial profile over time, with limited differences between control and astaxanthin conditions. Apart from the increase in *Faecalibacterium*, only modest changes were observed, including slight increases in *Parabacteroides* and *Bilophila*, and a reduction in opportunistic taxa such as *Pseudomonas* and *Alistipes* (Fu et al., 2024).

To further summarize genus-level changes, a PCA biplot was constructed using the main fermentation timepoints (24 and 48 h) (Fig. S4). The analysis revealed clear separation between control and astaxanthin-treated samples, supporting both donor-dependent responses and temporal modulation. Despite inter-individual variability, a shared microbial signature emerged under astaxanthin exposure. In V1, the 48 h profile showed increased contribution of genera linked to colonization resistance and microbial homeosthasis such as *Faecalibacterium*, *Bifidobacterium*, and Ruminococcaceae UCG-002, whereas control samples were more associated with genera including *Streptococcus* and *Bacteroides*. In V2, early increases in *Bifidobacterium* and *Flavonifractor* as health promoting-taxa, along with other pathobionts (*Enterococcus* and *Bilophila*) at 24 h were not sustained at 48 h, where taxa such as *Coprococcus* and *Sellimonas,* linked to recovery of gut homeostasis (Muñoz et al., 2020), became more prominent. In V3, the 24 h profile was enriched in commensal and health-associated genera (*Bifidobacterium*, *Coprococcus*, *Blautia*, *Dorea*), together with key contributors such as *Faecalibacterium*, *Ruminococcus*, and *Mediterraneibacter*. Astaxanthin-associated profiles remained characterized by the contribution of genera such as *Coprococcus*, *Blautia*, *Enterocloster*, and *Faecalibacterium* at the fermentation endpoint, highlighting the continued presence of taxa typically associated with robust microbial activity (Fig. S4).

Overall, astaxanthin promoted biologically structured modulation of the gut microbiota, despite marked interindividual variability. In particular, donors V2 and V3 showed a progressive enrichment of butyrate-producing taxa, among others; whereas V1 exhibited a more limited but still directionally consistent response; suggesting that the effects of astaxanthin are strongly shaped by the baseline microbiota configuration, in line with previous studies on carotenoid–microbiota interactions (Dai et al., 2022; Li et al., 2022; Pratap et al., 2022; Ren, Qi, et al., 2025). The enrichment of metabolically active taxa, including butyrate producers and *Bifidobacterium*, further supports a selective modulatory effect, while transient increases in opportunistic genera likely reflect short-term ecological adaptation.

3.4. Changes in microbiota functionality during colonic fermentations

3.4.1. Short-chain fatty acids profile

In contrast to control fermentations, astaxanthin supplementation did not consistently enhance total SCFA production and, in several cases, resulted in lower metabolite levels, suggesting that shrimp-derived astaxanthin does not function primarily as a direct fermentable substrate for the gut microbiota. Instead, its effects appeared to be mediated through selective modulation of microbial metabolic activity, generating donor-dependent shifts in fermentative pathways (Fig. 4, Table S5). In donor V1, astaxanthin supplementation resulted in significantly lower concentrations of most SCFAs at 24 h compared to the control, including propionate, butyrate, and total SCFAs. By 48 h, these were attenuated, although certain branched fatty-acids, particularly 2-methyl-butyrate and iso-valeric acid, remained reduced. In V2, astaxanthin induced a distinct metabolic response characterized by transient increases at 24 h in acetic acid, branched-chain fatty acids (iso-butyric and iso-valeric acids), and notably iso-caproic acid, followed by significantly lower acetic acid production at 48 h. Similarly, donor V3 displayed transient elevations in branched-chain fatty acids at 24 h, while acetic and propionic acids decreased by 48 h among with significantly increased concentration of butyric acid, despite previous lower levels. This delayed response, together with shifts in minor fatty acids, also suggests a time-dependent functional adaptation potentially linked to the enrichment of butyrate-producing taxa observed in the meta-taxonomic analysis.

The temporal and donor-dependent increases in branched-chain fatty acids (e.g., iso-butyric, iso-valeric, 2-methyl-butyrate) may reflect transient enhancement of protein fermentation pathways. However, the reduction of these metabolites by the fermentation endpoint under astaxanthin supplementation suggests a progressive shift away from proteolytic metabolism toward a more saccharolytic and metabolically favorable microbial profile. This interpretation is consistent with previous evidence indicating that astaxanthin-mediated microbiota

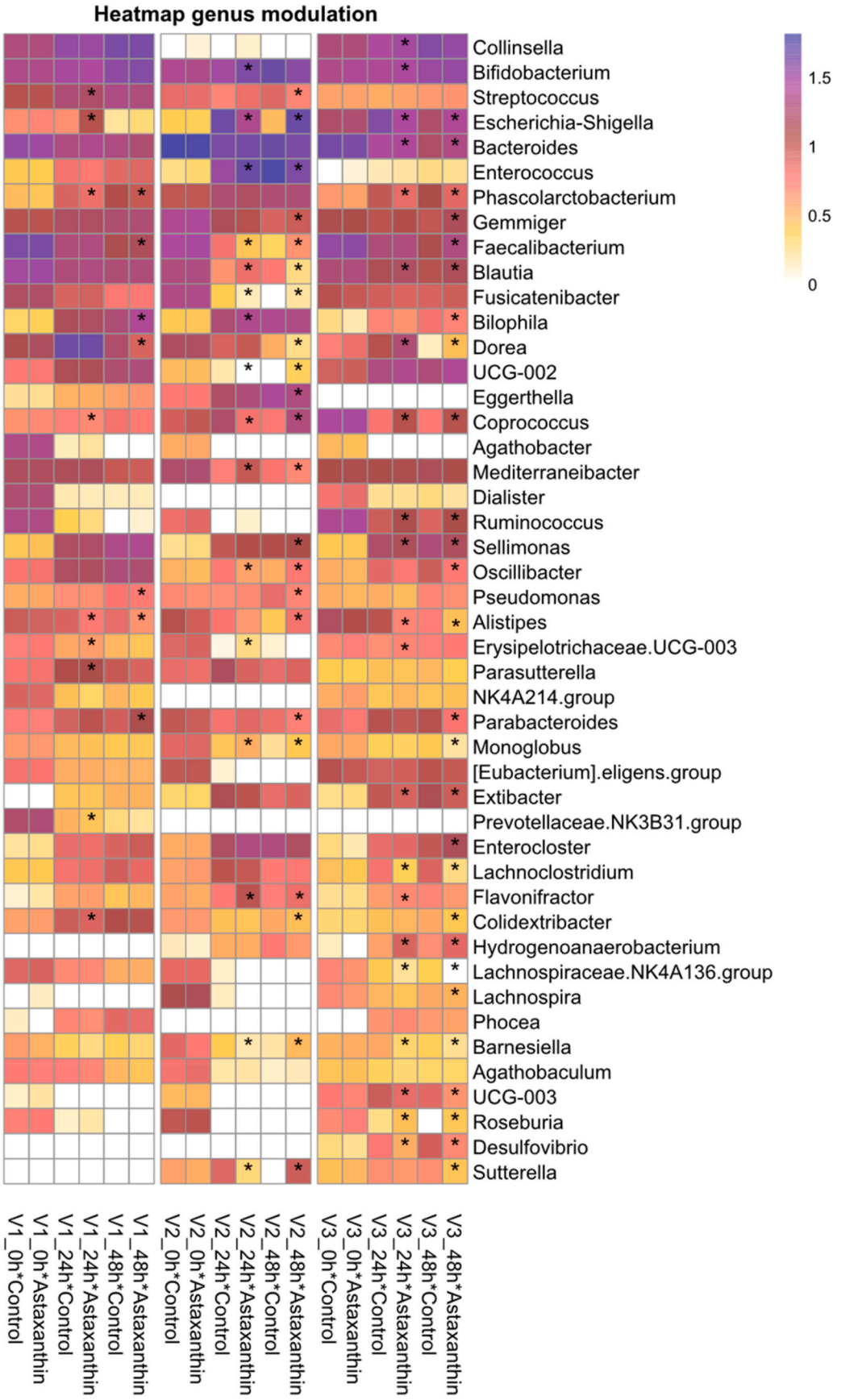

Fig. 3. Heatmap showing modulation of microbial taxa at genus level during colonic fermentation in presence of astaxanthin. Only taxa with a mean relative abundance of 1% in at least one timepoint are showed. Statistical analysis was performed by two-way ANOVA test and Games-Howell posthoc correction, considering significant those changes respect to control for 24h and 48h with an adjusted p-value < 0.05 (*).

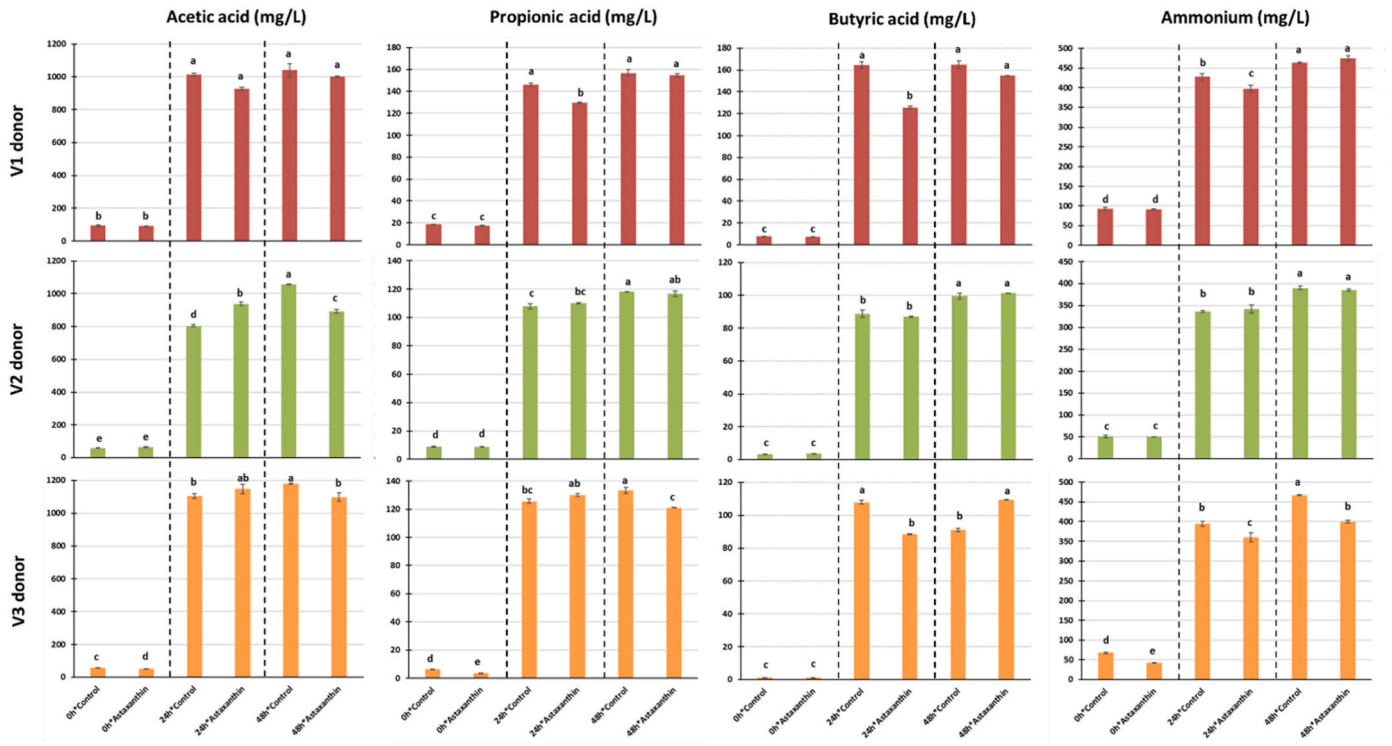

Fig. 4. Concentration of major short-chain fatty-acids and ammonium ion (mg/L) during colonic fermentation (0 h, 24 h, and 48 h) of astaxanthin-supplemented samples and their respective controls for each donor. Data are expressed as mean ± standard deviation. Statistical analysis was performed by two-way ANOVA test and Tukey posthoc correction and letters were used to denote groups that did not differ significantly from each other.

modulation may influence metabolic pathways associated with diverse biological processes and disease-related conditions, including hypermotility (Yasuda et al., 2023), ulcerative colitis (Huan et al., 2025), obesity (Wang et al., 2019), or cecal injury (Chen et al., 2021). Conversely, previous *in vitro* studies have generally reported increased SCFA production following astaxanthin supplementation, in contrats to the overall lower SCFA levels observed in the present work (except for acetate at 24 h in V2 and butyrate at 48 h in V3). For instance, Ren, Qi, et al. (2025) and Dai et al. (2022) reported increases in major SCFAs (acetate, propionate, and butyrate), although butyrate remained unchanged in the latter study. These discrepancies may largely reflect differences in experimental design, particularly the substantially higher astaxanthin concentrations and pooled fecal inocula used in previous models. Similarly, *in vivo* studies assessing astaxanthin-microbiota interactions are typically conducted under pathological conditions, where increases in SCFAs are expected and associated with improved metabolic balance (Huan et al., 2025; Zhang et al., 2024). In contrast, observations from healthy-state controls in these studies align more closely with our findings, showing either reductions or minimal changes in major SCFAs such as acetate and butyrate (Chen et al., 2021;Martin et al., 2023; Yasuda et al., 2023), as well as decreases in minor SCFAs like valerate (Wu et al., 2020).

Overall, these results suggest that astaxanthin does not primarily enhance microbial fermentation capacity in terms of total SCFA output, but rather modulates the qualitative metabolic profile of the microbiota in a donor-dependent manner, reflecting a selective modulation of microbial metabolic pathways.

3.4.2. Microbial ammonium production

Overall, ammonium levels increased over time, reflecting active nitrogen metabolism during fermentation. However, both the magnitude of this increase and the differences between control and astaxanthin-supplemented samples varied among donors (Fig. 4; Table S6). Although both conditions showed significant temporal changes, ammonium concentrations were slightly but significantly lower under astaxanthin at 24 h, indicating a mild attenuation of proteolytic activity. In V2, ammonium accumulated steadily throughout fermentation, with no significant differences between treatments at any time point. By contrast, donor V3 exhibited consistently lower ammonium concentrations under astaxanthin supplementation compared with control conditions across all fermentation time points. This sustained reduction suggests a more pronounced suppression of microbial proteolysis and/or amino acid deamination in response to astaxanthin.

Despite the clear interindividual variability observed, the overall trend was consistent with the reduced production of branched-chain fatty acids (isobutyrate, isovalerate, and 2-methyl-butyrate) detected under astaxanthin supplementation, which are commonly co-generated with ammonium through amino acid fermentation pathways (Louis & Flint, 2017).

3.5. Correlation networks linking microbial taxa with SCFAs and ammonium

Spearman correlation analysis revealed significant associations between several genera and the production of SCFAs and ammonium during colonic fermentation (Fig. 5), providing further insight into the metabolic pathways modulated by astaxanthin supplementation. Notably, iso-caproate production was positively associated with members of the genera *Flavonifractor*, *Extibacter*, and *Enterocloster*, among others. These taxa have previously been linked to the metabolism of flavonoids and ellagitannins (Pidgeon et al., 2025; Rodríguez-Castaño et al., 2020). In the present context, their association with iso-caproate may suggest a role in astaxanthin biotransformation or in downstream metabolic pathways that favor the production of branched- and medium-chain fatty acids, although direct mechanistic evidence remains limited. In contrast, members of the genus *Bifidobacterium* were positively associated with increased levels of iso-butyrate and 2-methyl-butyrate, consistent with previous reports indicating that certain

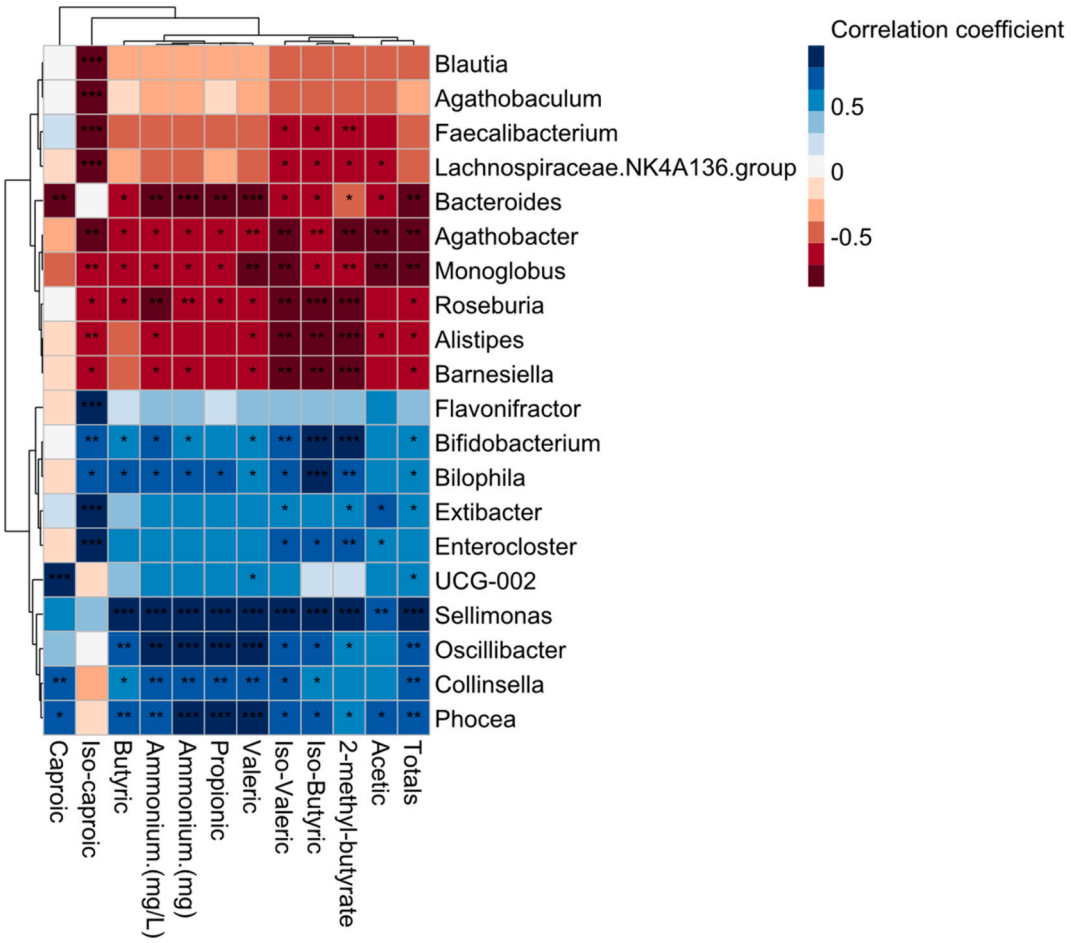

Fig. 5. Spearman analysis revealing significant associations between taxa al genus level, and SCFAs and ammonium production during astaxanthin intervention. Only taxa with a mean relative abundance of 0.5% in at least one timepoint were used for analysis. Heatmap shows only those associations with an adjusted p-value $<$ 0.05 and a correlation coefficient $>$0.75.

Bifidobacterium species can contribute to branched-chain fatty acid production either directly or through cross-feeding interactions (Bernabeu et al., 2024; De Vuyst & Leroy, 2011). Thus, the increase in the abundance of this genus at 24 h for V2 and V3 donors would explain, at least partially, the temporal modulation of these branched-chain short-chain fatty acids. Furthermore, ammonium production was positively correlated with the relative abundance of *Sellimonas*, *Collinsella*, *Oscillibacter*, and *Phocea* (Fig. 5). Some of these taxa have been associated with low-fiber dietary patterns (Gomez-Arango et al., 2018) or high-protein dietary patterns (Meslier et al., 2020; Muñoz-Yánez et al., 2025; Wu et al., 2024), and with proteolytic metabolic activity (Amaretti et al., 2019; Muñoz-Yánez et al., 2025). Interestingly, these taxa were generally detected at lower relative abundances under astaxanthin treatment, suggesting that their reduction may contribute to the decreased ammonium production observed. Conversely, ammonium levels were negatively associated with the relative abundance of *Roseburia* and *Bacteroides*, commensal genera typically linked to saccharolytic metabolism and carbohydrate fermentation pathways (e.g., butyrate and propionate production) rather than proteolysis (Louis & Flint, 2017; Mukhopadhya et al., 2025). Their relative enrichment following astaxanthin supplementation further supports a shift toward more saccharolytic microbial activity.

Overall, these correlation patterns support a metabolic shift between proteolytic and saccharolytic fermentation processes within the microbial ecosystem in response to astaxanthin. Considering that proteolytic metabolism has been associated with pro-inflammatory effects (Diether & Willing, 2019), our findings suggest that astaxanthin may promote a microbial metabolic profile more compatible with intestinal homeostasis and gut health.

This study has some limitations that should be acknowledged. The use of a static *in vitro* fermentation models does not fully reproduce the complexity of the human gastrointestinal tract, including host-microbiota interactions and absorption processes. In addition, the physiologically relevant astaxanthin concentration used, while representative of colonic exposure conditions, is susceptible to rapid microbial transformation, making targeted analytical approaches insufficient to capture the full spectrum of biotransformation products. Future studies employing non-targeted metabolomics strategies and *in vivo* validation will be necessary to fully elucidate the fate and functional impact of shrimp-derived astaxanthin in the gut ecosystem.

4. Conclusions

The results reveal that shrimp-derived astaxanthin supplementation maintained microbial viability while modulating both community composition and metabolic activity in a donor-dependent manner. Across colonic fermentations, the carotenoid tended to attenuate proteolytic metabolism, evidenced by lower ammonium and branched-chain fatty acid levels, and to moderate saccharolytic fermentation, preserving a balanced SCFA profile. These functional effects coincided with donor-dependent compositional shifts, with increases in potentially health-associated taxa (*Bifidobacterium* and *Faecalibacterium*) emerging primarily in donors showing higher responsiveness. Collectively, these results indicate a possible tendency for astaxanthin to act less as a direct

fermentable substrate and more as a selective modulator of microbial metabolism, with effects observed at physiologically relevant colonic concentrations and dependent on the baseline microbiota composition of each donor. The decrease in astaxanthin during colonic fermentation may involve microbial processes, although transformation products could not be identified and further investigation is warranted. Finally, this work supports the valorization of crustacean by-products as a sustainable and biologically active source of astaxanthin, with promising applications in the development of functional food and nutraceutical ingredients oriented toward gut health. Future studies including larger donor cohorts will be required to confirm whether these donor-dependent patterns are generalizable to a broader population.

CRediT authorship contribution statement

Mikel Roldán: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Martina Čagalj:** Methodology, Data curation. **Toni Jurić Šolto:** Methodology, Data curation. **Edgard Relaño de la Guía:** Methodology, Data curation. **Natalia Molinero:** Writing – original draft, Data curation. **Carolina Cueva:** Writing – review & editing, Methodology. **Vida Šimat:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **M. Victoria Moreno-Arribas:** Writing – review & editing, Writing – original draft, Resources, Project administration, Conceptualization.

Ethical statement

The authors declare that they have no known competing financial interests, ethical issues or personal relationships that could have appeared to influence the work of this review.

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Declaration of competing interests

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2026.109788.

Data availability

We've shared the link related to data.

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