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In vitro digestibility of bioprocessed brewer's spent yeast: Demonstrating protein quality and gut microbiome modulation potential

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ABSTRACT

With an ever-increasing global population and dwindling natural resources, a shift towards more sustainable food systems is required. Important aspects to aid in this transition are the reduction of food waste, and a movement towards non-animal protein sources. Brewers spent yeast (BSY) is an abundant by-product of the brewing industry, which is generally regarded as waste, despite its high nutritional value. Previous work has shown that fermentation of BSY with *Lactobacillus amylovorus* FST 2.11 resulted in changes in composition, functionality, and improved palatability of the processed raw material (PBSY). In this study, *in vitro* protein digestibility, amino acid bioaccessability, and protein quality of PBSY was explored using the static INFOGEST *in vitro* model. *In vitro* protein digestibility of PBSY (73.0 %) was almost two-fold higher than that of CBSY (40.0 %), while PBSY also displayed significantly higher *in vitro* bioaccessability values for all essential amino acids, except for tryptophan. Investigation of protein quality using the digestible indispensable amino acid score (DIAAS) values and the FAO recommended amino acid scoring pattern for individuals >3 years old showed that the protein quality for CBSY was low (DIAAS of 17.0 %), while PBSY was considered to be of "good" protein quality (DIAAS of 98.2 %). Investigation of the modulation potential of PBSY on the gut microbiome using an *in vitro* colon model system showed an increase in gut microbiome α-diversity indices and an abundance of beneficial Mediterranean diet-responsive taxa after 24 h. Overall, this study highlights the potential of BSY as raw material for the production of a high-quality food ingredient with potential prebiotic effects, aiding in the reduction food waste and supporting global food systems.

1. Introduction

Global food systems are facing mounting pressure, with social, political, and environmental factors such as climate change, pandemics, invasions and wars, loss of biodiversity, dwindling natural resources and increasing global population contributing to significant food insecurity (Van Zanten et al., 2023). With the 2030 expiration of the United Nations Sustainable Development Goals (SDGs) fast-approaching, urgent action is required to facilitate the transformation towards sustainable food systems. Challenging this transition is the high burden of food waste, with approx. 6–10 % of global greenhouse gas emissions arising from the 1.3 billion tonnes of food wasted annually (Rakesh & Mahendran, 2024). Within the framework of a circular bioeconomy, prioritising the valorisation of unavoidable, inedible, but nutritionally valuable biomass from the food industry has the potential to aid in the shift towards more innovative and sustainable food systems (Muscat et al., 2021). Another important aspect in this transformation is the requirement for increased emphasis on non-animal protein source, a movement also known as the protein transition (Duluins & Baret, 2024). To facilitate this, it is essential that the nutritional value of novel alternatives is thoroughly assessed, particularly in terms of protein quality, to ensure the provision of nutritionally adequate protein sources.

Abbreviations: BSY, brewers spent yeast; CBSY, control brewers spent yeast; PBSY, processed brewers spent yeast; AA, amino acids; SAA, sulfur-containing amino acids; DIAAR, digestible indispensable amino acid ratio; DIAAS, digestible indispensable amino acid score.

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Brewers spent yeast (BSY) is a nutritionally valuable brewing by-product, comprising protein (35–60 %), fibre (40–50 %), minerals, vitamins, polyphenols, and antioxidants. Considering the estimated 2.8–3.4 million tonnes of surplus BSY produced annually (Kunze, 1999; Statista, 2023), up to 150,000 tonnes of protein and 160,000 tonnes of fibre are currently wasted, quantities which could potentially fulfil the protein and fibre requirements of millions of people (Consultation, 2013; The Scientific Advisory Committee on Nutrition, 2015; Walpole et al., 2012). To date, the incorporation of BSY into human nutrition has been limited due to its inherent bitterness and ethanol content, with most research focusing on the production of yeast extract, or the extraction of functional components such as β-glucans or mannoproteins, processes whereby other nutritionally relevant fractions of BSY remain unutilised and wasted (Jaeger et al., 2020). Our previous work explored the valorisation of BSY by autolysis and subsequent lactic acid bacteria (LAB) fermentation with *Lactobacillus amylovorus* FST 2.11 to produce a food ingredient (processed BSY; PBSY) which showed fundamental changes in functionality (higher protein solubility, increased foaming stability) and sensory characteristics (reduced bitterness and generation of sour, fruity flavour). In addition, compositional changes were observed (higher levels of free amino acids, degradation of glucans) in comparison to unprocessed BSY (Jaeger et al., 2024a, 2024b). However, the nutritional quality of PBSY remains largely unexplored, particularly with regards to protein quality. While a small number of studies have demonstrated the high digestibility and protein quality of yeast protein concentrate (Ariëns et al., 2021; Wang et al., 2023), PBSY is a more complex matrix, containing yeast cell biomass as well as the solubilised intracellular components. Thus, it is essential to explore the impact of valorisation on the protein quality of BSY if the ingredient is to be considered as a potential protein source in human nutrition.

In recent years, the gut microbiome has gained increasing attention as a key modulator of physical and mental health. It is well known that dietary fibre acts as a modulator of gut microbiota composition, while fermented foods can also have a significantly positive effect on the gut microbiome, in turn inducing beneficial health effects (Balasubramanian et al., 2024; Leeuwendaal et al., 2022). Moreover, studies have shown yeast-derived β-glucans to significantly positively impact the gut microbiota, resulting in immunoregulation and protective effects against genotoxicity and cytotoxicity (Jayachandran, Chen, Sum, Chung, & Xu, 2018a; Mantovani et al., 2008; Steenwijk, 2021). However, little data is available regarding the impact of BSY on the gut microbiome, presenting a significant opportunity for the exploration of the *in vitro* gut microbiome modulation potential of PBSY.

In this study, we demonstrate the potential of PBSY as a sustainable, nutritional, and functional food ingredient by investigating *in vitro* amino acid bioaccessibility and assessing protein quality via *in vitro* digestible indispensable amino acid score (DIAAS) values, using an analytical workflow based on the static INFOGEST 2.0 protocol for the adult population (Brodkorb et al., 2019; Sousa et al., 2023). Moreover, the *in vitro* gut microbiome modulation potential with regard to Mediterranean diet-positive taxa associated with improved cognitive function and reduced frailty in the elderly (Ghosh et al., 2020) was also investigated.

2. Materials and methods

2.1. Production of raw materials

Control BSY (CBSY) and Processed BSY (PBSY) (Suppl. Table S1) were prepared as described previously by Jaeger et al. (2024a).

2.2. In vitro simulated gastrointestinal digestion

Static *in vitro* digestion of the BSY ingredients was performed according to the INFOGEST 2.0 protocol (Brodkorb et al., 2019), with the pancreatin suspension prepared as described by Sousa et al. (2023). All reagents and enzymes were purchased from Merck, except for pooled human saliva which was sourced from Lee BioSolutions. Enzyme activities were measured using previously described protocols (Brodkorb et al., 2019). Bile salt concentrations were measured according to manufacturer kit instructions. All digestions were carried out in triplicate.

Briefly, to prepare digested samples for faecal fermentations, 50 g of dry BSY ingredient was incubated with an equal volume of pooled human saliva at 37 °C for 2 min (16 rpm) on a rotating mixer. The oral bolus was then diluted 1:1 (v/v) with simulated gastric fluid, 0.3 M $CaCl_2(H_2O)_2$, pepsin (2000 U/mL), and ultrapure water, and the pH was adjusted to 3 using 1 M HCl. The mix was incubated at 37 °C for 2 h, after which gastric digestion was stopped by raising the pH to 7 with 1 M NaOH. The mixture was diluted 1:1 (v/v) with simulated intestinal fluid, 0.3 M $CaCl_2(H_2O)_2$, bile salts (10 mM), pancreatin (100 U trypsin/mL), and ultrapure water, and incubated at 37 °C for 2 h at 16 rpm to simulate the intestinal phase. The resulting digesta were dialysed (1 kDa molecular weight cut off) at 4 °C for 24 h to account for absorption in the small intestine (Lynch et al., 2021). The retentate was freeze-dried and stored at − 20 °C until use in batch faecal fermentations.

The *in vitro* protein digestibility of the BSY ingredients was determined according to the workflow described by Sousa et al. (2023). Briefly, the ingredients were diluted with the required amount of ultrapure water to give a protein content of 40 mg/mL (based on total nitrogen × 6.25). 3 mL aliquots of the normalised BSY solutions were subject to static *in vitro* digestion as described above. However, in this case, dialysis was not performed after the intestinal phase; instead, enzymatic reactions were stopped by the addition of protease inhibitor (Pefabloc, 500 mmol/L). Digesta were then immediately snap-frozen in liquid nitrogen and frozen at −80 °C until use. A protein-free blank (ultrapure water) was also digested in parallel for the estimation of the contribution of the digestive enzymes.

2.3. Sample separation into bioaccessible and non-bioaccessible fractions

The separation of digests into bioaccessible (supernatant) and non-bioaccessible (pellet) fractions was performed as described by Sousa et al. (2023), with some modifications. Briefly, digesta were defrosted and precipitated with 80 % methanol (v/v) at −20 °C for 1 h, followed by centrifugation at 2000×*g* for 15 min at 4 °C. The supernatants were collected, and 5 mL aliquots were dried in a vacuum centrifuge (VacSafe 15, Labogene, Denmark) and resuspended in 200 μl of ultrapure water. The pellets were washed twice with 100 % methanol (2000×*g* for 5 min at 4 °C), dried, and divided into two portions for total amino acid analysis, and tryptophan analysis.

2.4. Amino acid analysis of raw materials and in vitro digesta

For total amino acid analysis, samples were hydrolysed in the presence of 3 mL of 6 M HCl (Sigma-Aldrich, Steinheim, Germany) at 110 °C for 23 h in a heating block. After cooling, 800 μl of 50 mM Norvaline (internal standard) in 0.1 M HCl was added and mixed. The solution was filtered under vacuum and the filtrate was transferred to a volumetric flask and diluted to 50 mL with Ultra-Pure Water (MilliQ). Then, 1 mL aliquots were filtered using a 0.45 μm filter (Sarstedt, Nümbrecht, Germany)) and the filtrate retained for analysis. Samples were derivatized using AccQ-Tag Ultra Derivatization Kit (Waters Corp, Milford, MA, USA). Analysis was then performed on a Thermo Scientific Dionex UltiMate 3000 RS Series (Thermo Fisher Scientific, Waltham, MA, USA) with a Waters AccQ-Tag Ultra C18 (150 mm × 4.6 mm, 2.5 μm) (Waters Corp, Milford, MA, USA) using eluent A (AccQ-Tag Ultra Eluent A Concentrate) (Waters Corp, Milford, MA, USA)/ultrapure water (MilliQ Water) 5/95 v/v and eluent B (acetonitrile, HPLC-grade) (Sigma-Aldrich, Steinheim, Germany. After separation, the amino acids were detected photometrically (λ = 260 nm). External calibration was

performed with Amino Acid Standard (AccQ-Tag, Pico-Tag, AccQ-Tag Ultra), using Norvaline as internal standard (Waters Corp, Milford, MA, USA). Tryptophan was quantified by basic hydrolysis; sample aliquots were mixed with 3 mL of 4 M NaOH and incubateds at 110 °C for 20 h. After cooling, samples were acidified to pH 6.5 using HCl. The solutions were diluted to 25 mL with sodium borate buffer (pH 9), the samples were centrifuged and filtered using a 0.45 μm filter (Sarstedt, Nümbrecht, Germany)), and the filtrate used for analysis. Tryptophan was measured using fluorescence detection (excitation: 280 nm; emission: 350 nm) with the previously described measuring system. Sulphur-containing AA (methionine and cysteine) were not included in analysis due to methodological limitations.

2.5. Determination of in vitro digestibility, amino acid bioaccessibility, DIAAR, and DIAAS

In vitro protein digestibility was determined as described by Sousa et al. (2023) using the following equation:

$$\textit{In vitro}\ \text{protein digestibility}\ (\%) = \frac{AA\ in\ food\ supernatant\ (mg) - AA\ in\ blank\ supernatant (mg)}{(AA\ in\ food\ supernatant\ (mg) - AA\ in\ blank\ supernatant\ (mg)) + \max(0; AA\ in\ food\ pellet\ (mg) - AA\ in blank\ pellet\ (mg))} \times 100 \quad (1)$$

The same equation was used for the determination of individual AA bioaccessibility by considering the total amounts of the individual AA (mg) in the supernatants and pellets of the food and blank samples.

The *in vitro* digestible indispensable amino acid ratio (DIAAR) of the essential amino acids was calculated using Eq. (2), considering the FAO reference values for individuals >3 years (FAO, 2013) and the amino acid content of the test protein (Table S2).

$$\textit{In vitro DIAA ratio}\ (DIAAR) = \frac{digestible\ indispensable\ amino\ acid\ (mg)\ in\ 1\ g\ test\ protein}{digestible\ indispensable\ amino\ acid\ (mg)\ in\ 1\ g\ reference\ protein} \quad (2)$$

The DIAAS was calculated using the following equation:

$$DIAAS\ (\%) = lowest\ DIAAR \times 100 \quad (3)$$

2.6. Participant recruitment and faecal sample processing for batch fermentations

Faecal samples were collected from healthy, community-dwelling, older donors (aged 65+) and older donors residing in long-term residential care, following a screening process and food frequency questionnaire. This procedure was approved by the local Clinical Research Ethics Committee (review reference numbers: ECM 4 (w) 11/1/2022 & ECM 3 (y) 20/06/2023) and all subjects gave their informed consent before they participated in the study.

Faecal samples from donors were transferred to an anaerobic cabinet less than 1hr after passing. Approx. 200 mg of the sample was immediately frozen and stored at −80 °C, to be used for microbiome profiling. The remainder of the sample was homogenised in a previously prepared reduced sterile solution of phosphate-buffered saline containing 20 % glycerol. This inoculum was divided into aliquots of approx. 20 mL and stored at −80 °C.

2.7. Faecal fermentations

Batch fermentation cycles simulating the colonic fermentation of the chosen ingredients were conducted over a 24 hr period, following protocols described previously (Ntemiri et al., 2020, 2017). Samples from two healthy participants (H2 & H3) and two frail participants (R6 & R8) were used for fermentation inoculation. For each fermentation cycle, four parallel single vessels with a 150 mL working volume in each vessel were used. Each fermentation cycle comprised of a 150 mL working volume of basal media, 1 % (w/v) faecal inoculum and 5 g of pre-digested food ingredient (CBSY or PBSY).

Briefly, a basal media was prepared in a volume of 150 mL, according to the following recipe; casein (3 g/L), peptone water (2 g/L), yeast extract (2 g/L), bile salts (0.5 g/L), tween 80 (2 mL/L), vitamin K (10 μL/L), haemin solution (10 mL/L), $NaHCO_3$ (2 g/L), KH_2PO_4 (0.04 g/L), NaCl (0.1 g/L), $CaCl_2{\cdot}6H_2O$ (0.01 g/L), $MgSO_4{\cdot}7H2O$ (0.01 g/L), resazurin (1 mg/L), and antifoam solution (1 mL/L). As per our previous studies, the basal fermentation media was supplemented with a mix of prebiotic fibres to minimise the loss of microbiota diversity caused by adaptation to *in vitro* conditions (Ntemiri et al., 2020). This mixture (named MIX) was prepared according to the following recipe: arabinogalactan (2 g/L), pectin (2 g/L), soluble starch (5 g/L), xylan (2 g/L), inulin (1 g/L), amylopectin (1 g/L), beta-glucan (0.5 g/L), glucose (2 g/L), mucin (4 g/L). This basal media was autoclaved at 121 °C for 15 min and added to sterilized, small scale bioreactor vessels (MiniBio 500, Asistec). Following a brief cooling period, the pre-digested food prototype was added aseptically to the bioreactor.

During the fermentation cycle, a continuous flow of NO_2 was used to maintain anaerobic conditions. Temperature (37 °C), dissolved oxygen (0 %) and pH (6.8) were automatically monitored and controlled. Samples were withdrawn from the bioreactor at T0, T16 and T24 and centrifuged at 5000 rpm for 15 min. Following this, the supernatant and pellet were separated, and stored at −80 °C.

2.8. DNA extractions from stool samples and faecal fermentation homogenates

DNA was extracted from faecal samples for microbiome profiling as previously described by our laboratory (Ntemiri et al., 2020). 200 mg of the stored pellet was homogenised mechanically using a Mini-Beadbeater (Biospec Products, Inc., Bartlesville, OK, USA) in a sterile bead-beating tube containing 1 mL lysis buffer and zirconia glass beads of three sizes- 0.1, 0.5 and 1.0 mm (Thistle Scientific Ltd., Glasgow, UK). Following this, samples were incubated at 70 °C for 10 min and extraction was carried out according to the QIamp Fast DNA Stool (Qiagen) Kit protocol. The NovaSeq 6000 system (Illumina Inc., CA, USA) was used for amplicon sequencing of the 16S rRNA variable region V3 & V4. Library preparation and sequencing were performed by

Novogene Co., Ltd, Beijing, China.

2.9. Statistical analysis

Digestion experiments and amino acid analyses were performed in triplicate unless otherwise stated. All analyses were subjected to testing of normality, variance and statistical difference of means using independent *t*-test testing with the IBM SPSS software (IBM SPSS Statistics Ver. 28.0.0.0 (190) using a significance level of 5 %.

2.10. Bioinformatic analysis

The raw Illumina reads obtained from amplicon sequencing of the 16S rRNA variable region V3 & V4 were quality-filtered using TrimGalore v0.6.7 ("Trim galore-A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files", 2015). The quality filtered reads were then taxonomically classified using both SPINGO (for classification at the species and genus levels) and Lotus2 (Özkurt et al., 2022) with DADA2 clustering (for identification of amplicon sequence variants, ASVs). Alpha diversity indices (Observed ASVs, Chao1, ACE, Shannon, InvSimpson, Simpson, Fisher) were computed from ASVs using vegan and phyloseq R packages. Rarefied reads, i.e., 0.9 * minimum reads across all samples (30 K reads/sample), were used to estimate alpha diversities. The nonmetric multidimensional scaling (NMDS) ordination plots based on Bray-Curtis dissimilarity were generated to visualize the microbiota. Multivariable permutational analysis of variance (PERMANOVA) with Bray-Curtis dissimilarity was performed at the ASV, species, and genus levels (permutations = 999). Pairwise comparisons were computed using Wilcoxon rank-sum test. The effect size (i.e., Hedges' G) between T24/ T16 and T0 for each species within each microcosm was used to evaluate the shift of species. The Healthy Food Diversity (HFD) index of donors was evaluated from the semi-quantitative food frequency questionnaire (Drescher et al., 2007).

To test if the sequences annotated as *Lactobacillus helveticus*, which were consistently enriched in the PBSY microcosms across donors, were the strain *L. amylovorus* FST 2.11 that was initially used to ferment BSY, the sequences were BLASTed against the constructed database. The database includes 26 complete genomes of *L. helveticus*, 9 complete genomes of *L. amylovorus*, and 3 complete chromosomes of *L. amylovorus* downloaded from NCBI on October 31st, 2023. 99.3 % of the sequences (10,526 out of 10,605) has the same highest BLAST alignment similarity to both *L. helveticus* and *L. amylovorus*.

3. Results

3.1. In vitro protein digestibility and individual amino acid bioaccessibility

Assessment of overall *in vitro* protein digestibility (Suppl. Table S3) resulted in a significantly higher value for PBSY (73.0 % ± 4.9 %) than CBSY (40.0 % ± 3.4 %). Regarding the *in vitro* bioaccessibility of individual amino acids (Fig. 1; Suppl. Table S4), significantly higher values were obtained for PBSY compared to CBSY ($p < 0.05$), for all amino acids except tryptophan. Lysine was the least bioaccessible essential AA in CBSY (12.7 %) increasing by almost 60 % in PBSY (72.8 %). CBSY also showed low bioaccessibility levels for some non-essential AAs such as glycine (16.2 %) and aspartic acid (14.6 %), with the bioaccessibility of these AAs increasing approximately 3-fold in PBSY (61.2–70.2 %). The least increases in AA bioaccessibility between CBSY and PBSY were observed for tryptophan, proline, and glutamic, with increases of 13–17 % observed. Proline was found to be the most bioaccessible AA in CBSY (61.8 %), while the same was true for tyrosine in PBSY (90.5 %).

3.2. In vitro DIAAR and DIAAS

Digestible indispensable amino acid ratio (DIAAR) values were calculated for all essential amino acids (Fig. 2) using the amino acid

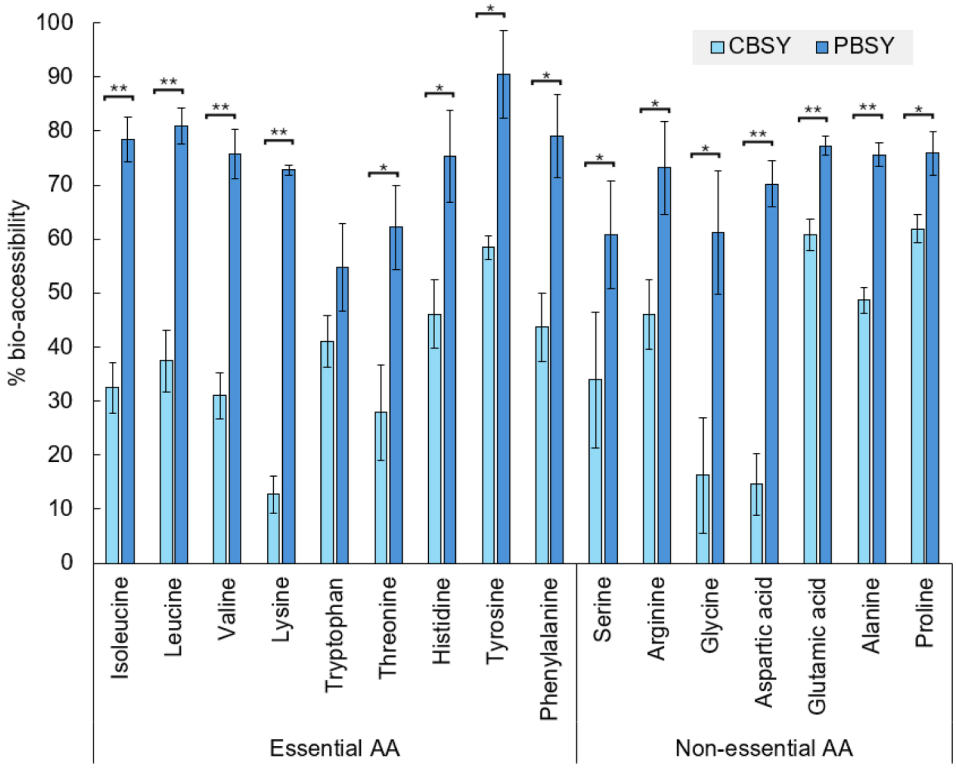

Fig. 1. Bioaccessibility (%) of essential and non-essential AAs after *in vitro* digestion, presented as mean ± standard deviation of triplicate experiments. Significance $p < 0.05$ indicated by * and $p < 0.001$ indicated by **.

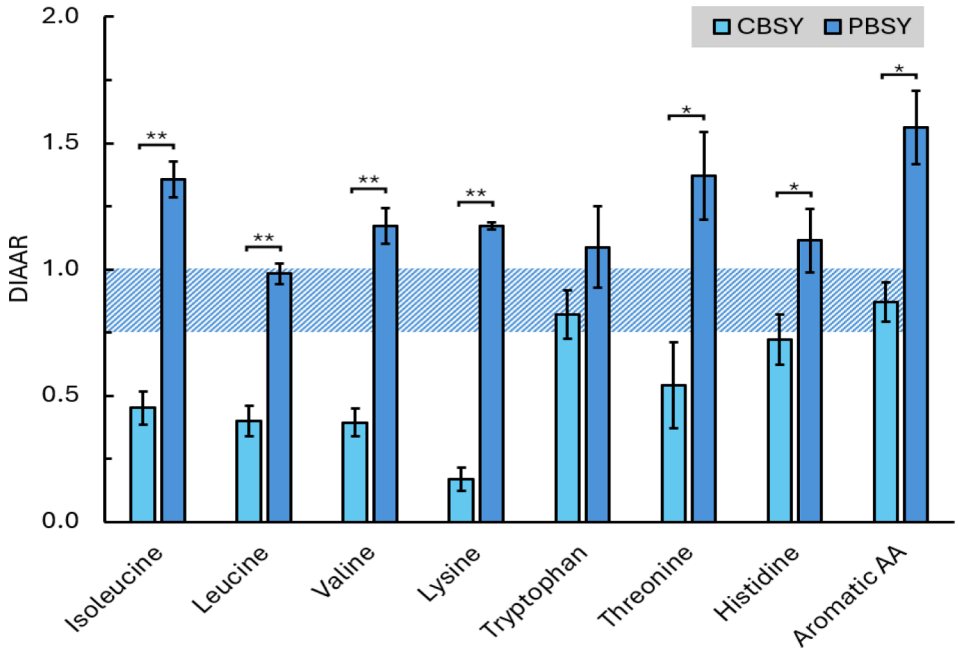

Fig. 2. DIAA ratio (DIAAR) for essential amino acids, represented as mean ± standard deviation of triplicate experiments. The shaded band 0.75–1.0 indicates "good" and "excellent" source of each essential AA as described by FAO (2013), respectively. "Aromatic AA" consists of the sum of phenylalanine and tyrosine. Significance $p < 0.05$ indicated by * and $p < 0.001$ indicated by **.

composition of the CBSY and PBSY ingredients (Suppl. Table S2) and the FAO 2013 reference protein values (Suppl. Table S5). In CBSY, the DIAAR values of several essential AA fell below the 0.75 threshold for 'good quality' protein. The lowest DIAAR was observed for lysine in CBSY, with a value of 0.2. Higher DIAAR values were observed for some of the other essential AA (isoleucine: 0.5; leucine: 0.4; valine: 0.4), with tryptophan and aromatic AA being the only essential AAs to exceed a DIAAR of 0.75. DIAAR values were significantly higher in PBSY ($p < 0.05$) in all cases except for tryptophan, with values of > 0.75 observed for all AA, and values >1 observed for all AA except leucine. Determination of *in vitro* DIAAS resulted in a significantly higher value for PBSY (98.3 %) than CBSY (17.0 %), an increase of >80 %. Lysine and leucine were found to be the first limiting amino acids for CBSY and PBSY, respectively.

3.3. Alpha diversity index

The alpha diversity indices, derived at the amplicon sequence variant (ASV) level, generally reduced over time, however the average alpha diversity indices in the PBSY supplemented vessels were higher than those in the CBSY supplemented fermentations, irrespective of the time point (Fig. 3). Examining the PBSY/CBSY ratio for key microbiome statistics, the average fold difference of the alpha diversity indices at T24 was higher than that at T0 and/or T16 (Fig. 4A), indicating higher alpha diversity indices at T24. Over time, donor-associated differences in microbiome composition at the genus level decreased from 78.1 to 18.1 %, while the variation associated with medium supplementation type increased from 9.1 % to 16.3 % (Fig. 5, Bray-Curtis dissimilarity). Similarly, the variation associated with the Rockwood Clinical Frailty Scale and the Healthy Food Diversity Index of the donors decreased to a smaller extent than the variation associated with media supplementation at T24 (Table 1).

3.4. Identification of diet-responsive taxa

In a previous study on Mediterranean diet intervention in the NU-AGE project, we identified a set of diet responsive taxa that correlated with retention of health in pre-frail subjects (Ghosh et al., 2020). In the current study, we identified 22 diet responsive taxa (12 NU-AGE Diet-Positive and 10 Diet-Negative) across all four donors at baseline and tested how they responded to the supplementation type. Most of the NU-AGE Diet-Negative species decreased over time in response to nutrient supplementation, but many of these species increased in Basal medium. NU-AGE Diet-Positive species also primarily decreased over time (Fig. 4B) concordant with the decline in alpha diversity that is a feature of batch fermentation. In terms of the total relative abundance of 12 Diet-Positive species, the fold difference (PBSY/CBSY) increased with time, and at T24, the sum of the diet-positive species abundance in the PBSY-supplemented medium was greater than the CBSY except for one donor, H2 (Fig. 4C).

Compared to the CBSY and Basal supplemented media, a sequence identified as *Lactobacillus helveticus* was significantly enriched in the PBSY-supplemented medium across at least three donors ($p < 0.05$), although this is likely *L. amylovorus* FST 2.11 that was initially used to ferment BSY (Suppl. Fig. S1). Regarding differentially abundant species between CBSY and PBSY at 24, among the species detected in at least three donors and being significant in at least two donors ($p < 0.05$, Wilcox test), nine species (*Lactobacillus helveticus, Butyricimonas virosa, Ilumatobacter fluminis, Steroidobacter denitrificans, Eubacterium eligens, Gaiella occulta, Melghirimyces thermohalophilus, Nitrospira japonica, Solirubrobacter ginsenosidimutans*) were consistently more abundant in the PBSY across donors, and one species (*Cronobacter sakazakii*) were more abundant in the CBSY. Of the nine PBSY-enriched species, *L. helveticus, B. virosa, I. fluminis*, and *S. denitrificans* were significantly more abundant in at least three donors ($p < 0.05$, Wilcox test). Differentially abundant species were also observed between PBSY and Basal (see supplementary text).

4. Discussion

The protein transition is an emerging concept which encompasses a shift from a diet high in animal-derived protein sources towards one which is richer in alternative proteins, with the aim of mitigating environmental impact of protein production, improving animal welfare, and providing healthier diets to the growing global population (Duluins & Baret, 2024). An important narrative within this transition is the

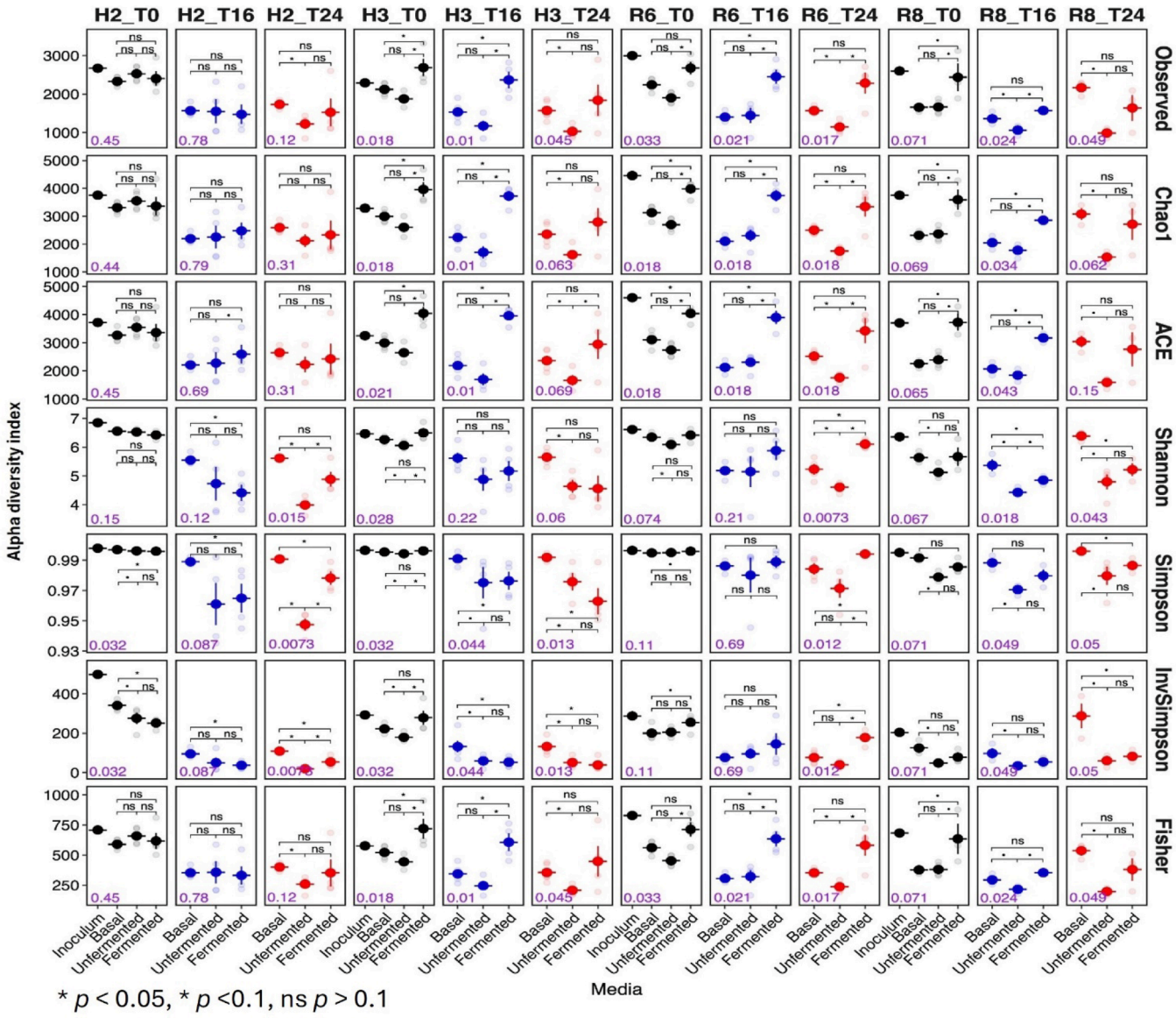

Fig. 3. ASV-level alpha-diversity indices over time. $p < 0.05$, Wilcox test between two media (in black); $p < 0.05$, Kruskal test across four media (in purple); Error bar indicates 95 % confidence interval; Observed, observed ASVs number; Chao1, a nonparametric estimator of species richness; ACE, abundance-based coverage estimator; Shannon, combining richness and diversity. It measures both the number of species and the inequality between species abundances; Simpson, a measure of diversity which considers the number of species present, as well as the relative abundance of each species; InvSimpson, the inverse of the classical Simpson diversity estimator; Fisher, a parametric index of diversity that assumes that the abundance of species follows the log series distribution.

provision of nutritionally adequate alternative protein sources, as non-animal protein is often associated with an incomplete essential AA profile and lower digestibility (Leroy et al., 2021). Moreover, macro-nutrient and micronutrient profiles of alternative protein sources can be negatively impacted as a consequence of obtaining a higher protein content, for example, in the production of protein isolates. In fact, it may be more beneficial to choose processing methods which maintain un-altered the nutritional value of the protein source, but enhance acces-sibility and availability of the nutrients (Van Zanten et al., 2023). In the current study, the *in vitro* bioaccessibility of all measured essential AAs were significantly higher in PBSY than in CBSY (except for tryptophan), despite the comparable protein content of the ingredients. In terms of protein quality, calculation of *in vitro* Digestible Indispensable Amino Acid ratio (DIAAR) values using the FAO 2013 reference protein values for individuals >3 yrs showed an increase in *in vitro* DIAAR for all essential AA in PBSY, with this difference being significant ($p < 0.05$) for all measured essential AA except tryptophan. While the DIAAR values for several essential AAs in CBSY fell within the 'low quality' range (<0.75), DIAAR values for PBSY were >0.75 ("good" quality) for all AA, and >1 ("high" quality) for all AA except leucine, indicating higher protein quality. The *in vitro* DIAAS value determined for PBSY is in line with what was reported by Ariëns et al. (2021) for yeast protein concentrate, also using the static INFOGEST protocol. However, it should be noted here that determination of the sulfur-containing amino acid (SAA) contents of the digesta supernatants was not possible due to high variability in the data measurements (data not shown), and thus these amino acids were not considered in the evaluation of the protein quality of CBSY and PBSY. While several studies have shown SAA to be the limiting amino acid in yeast protein extracts (Ariëns et al., 2021; Cao et al., 2025; Zhu et al., 2022), this appears to be highly dependent on the specific yeast ingredient and protein extraction method utilised, with others reporting threonine, leucine or histidine as the limiting amino acid (Cao et al., 2025; Jacob et al., 2019; Wang et al., 2023). Never-theless, it should be considered that SAA could potentially be the

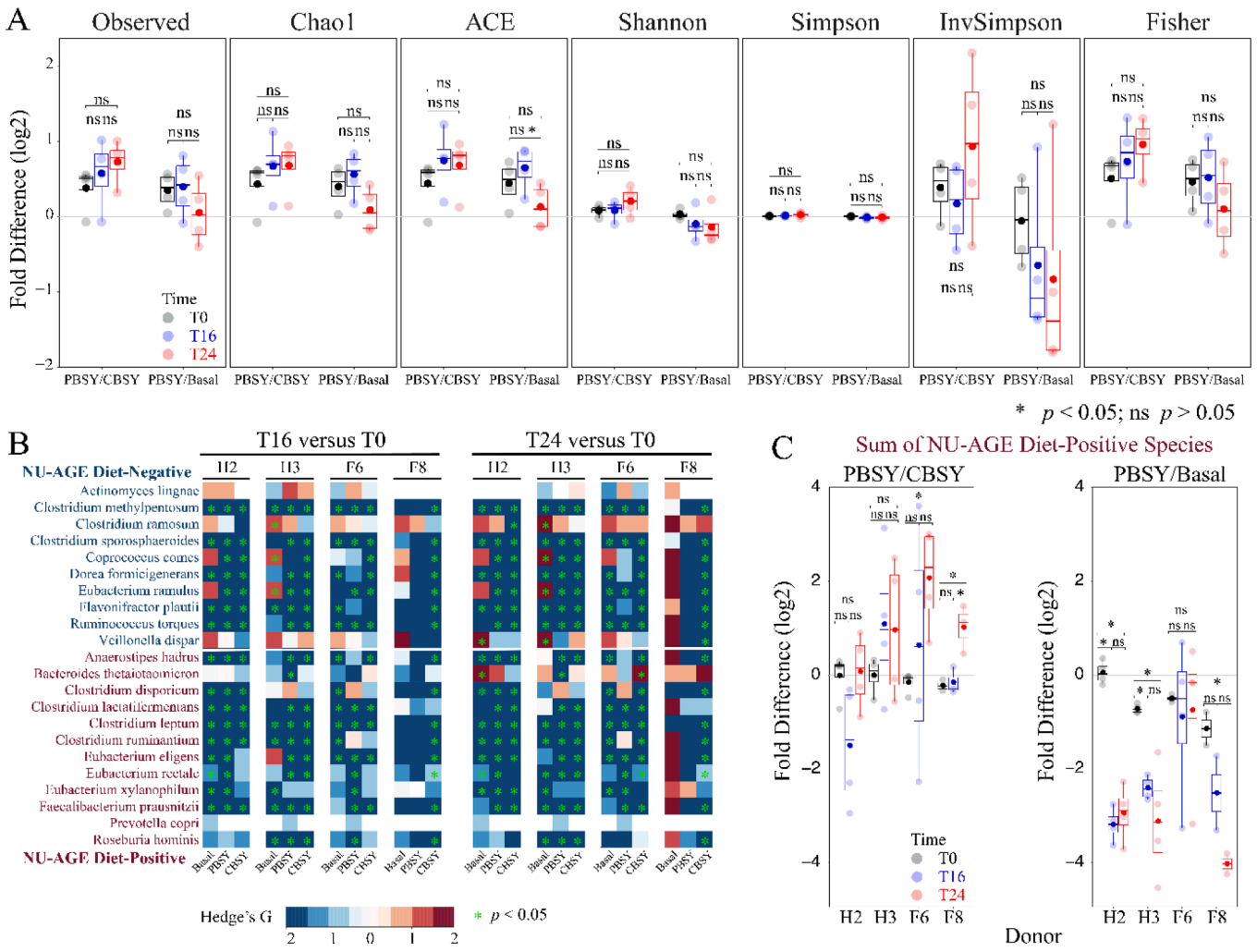

Fig. 4. The fold difference (log2 ratio) of alpha diversity indices (Observed ASV, Chao1, ACE, Shannon, Simpson, InvSimpson, Fisher) in PBSY to CBSY, and Basal (A); Changes (Hedge's G of relative abundance at T16 or T24 to T0) of NU-AGE Diet-related species over time (B); The fold difference (log2 ratio) of the sum of the NU-AGE Diet-Positive species (C). Smaller, darker circles indicate average value (A,C). *, $p < 0.05$ (Wilcoxon test).

limiting amino acid for the ingredients in the current study, and that the inclusion of SAA data would result in a truer and more accurate assessment of the protein digestibility and quality of CBSY and PBSY. Moreover, Sousa et al. (2024) recommend the use of a protein-free cookie as a blank control to decrease the level of auto-digestion of the digestive enzymes, which can impact the estimation of protein hydrolysis. In the current study, high variability was observed in the AA measurements of the cookie blank digesta due to co-elution of unknown compounds (data not shown), and so ultra-pure water was used as the blank control instead. Because of this, it is possible that a higher degree of digestive enzyme autolysis occurred in the blank control, and that the digestibility and CBSY and PBSY may be under-estimated. While these are limitations of the current study, the available results still allow for a comparison of the changes in protein and amino acid bioaccessibility between the two ingredients. The improvement in the protein quality of PBSY compared to CBSY can be directly linked to the protein degradation and release of free AA and peptides which occurred as part of the BSY valorisation process (Jaeger et al., 2024a, 2024b), with the positive impact of autolysis on yeast digestibility previously documented in animal agriculture studies (Agboola et al., 2022; Kaewtapee et al., 2022; Liu et al., 2021). In addition, the *L. amylovorus* FST 2.11 strain used in the fermentation has been characterised as possessing proteolytic activity (Axel et al., 2016). Overall, the enhancement of protein quality with little impact on the overall nutritional profile is noteworthy, highlighting the feasibility of the applied process for potentially valorising BSY into a nutritionally adequate alternative protein source to aid in the protein transition.

There is an emerging consensus that the protein transition should not simply reduce food to a single macronutrient, as such an approach can lead to a false assumption that all proteins are nutritionally interchangeable based solely on their protein content or quality, essentially disregarding any other macronutrients and micronutrients they may provide and their associated health benefits (Duluins & Baret, 2024; Leroy et al., 2021). This is particularly applicable in the current study, as PBSY contains fermentation metabolites and a significant proportion of dietary fibre which can modulate gut microbiota composition. Moreover, research investigating the link between valorised foods and the human gut microbiome is limited, and provides an opportunity to identify sustainable foods which promote health, thereby addressing 'SDG 3: good health and well-being' (O'Toole & Paoli, 2023). We therefore used an artificial colon model, seeded with faecal material of two healthy or two frail older subjects, to assess the impact of PBSY on its interaction properties with the human gut microbiota. It should be noted here that although faecal samples from the elderly population were used in the artificial colon model study, the BSY ingredients were digested using the adult INFOGEST protocol, and so the digestion conditions were not adapted to those recommended for older individuals.

A global statistic for ecosystem complexity is the alpha diversity

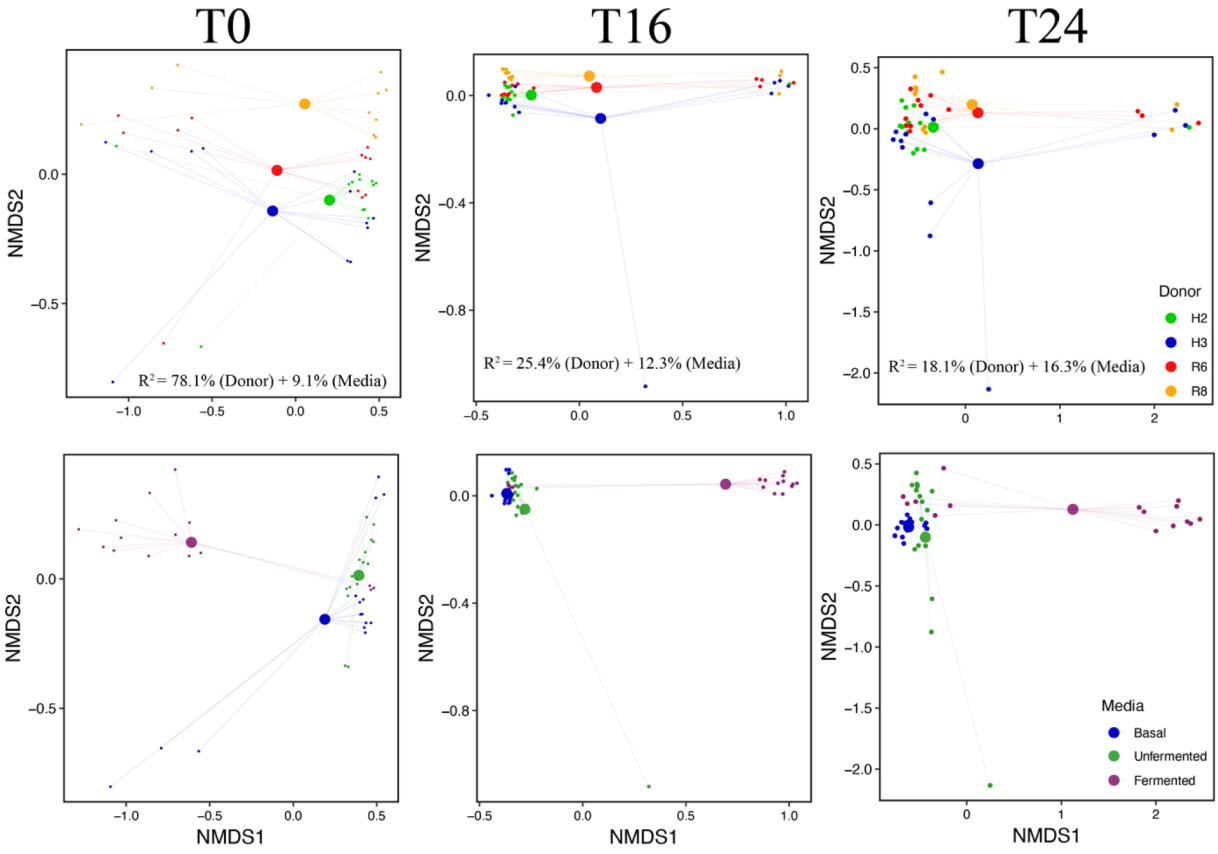

Fig. 5. Donor-associated and media-associated differences in microbiome composition (genus) over time. R^2 indicates the variation explained by Donor and Media in the permutational analysis using the adonis2 model below. The statistical significance level was less than 0.001 for both the donor and media group at each time point. adonis2(genus ~ Donor + Media, method = "bray", permutations = 999, by = "margin").

Table 1
Frailty and HFD-index associated variation over time at ASV, species, or genus level.

	ASV			Species			Genus		
	Frailty	HFD	Media	Frailty	HFD	Media	Frailty	HFD	Media
T0	0.027	0.019	0.069**	0.141***	0.107***	0.110***	0.144***	0.059**	0.091**
T16	0.022	0.018	0.077***	0.066**	0.067**	0.116***	0.029	0.030	0.120**
T24	0.023	0.021	0.076***	0.060***	0.084***	0.141***	0.019	0.042*	0.161***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Media: Basal, Fermented BSY, Unfermented BSY; adonis2(abundance ~ Frailty + HFD + Media, permutations = 999, method = "bray", by = "margin").

index which reflects the diversity of species within a sample (Thukral, 2017). A complex diet typically encourages higher levels of gut microbiome alpha diversity and is associated with host health, exemplified by the effect of the Mediterranean diet on gut microbiome and health (Ghosh et al., 2020). As expected in the artificial colon model, the alpha diversity indices generally reduced over time, although the average alpha diversity indices in the PBSY supplemented vessels were higher than those in the CBSY supplemented fermentations, irrespective of the time point. Moreover, the variation associated with the Healthy Food Diversity (HFD) Index and the Rockwood Clinical Frailty Scale of donors decreased to a lesser extent than the variation associated with media supplementation at T24. These results indicate that the effect of the PBSY on the microbiome becomes greater than donor, frailty or habitual dietary influences, which are established as major microbiome modifiers (Claesson et al., 2012; Li et al., 2022; Wu et al., 2011).

Several species were significantly enriched in PBSY compared to CBSY at T24, including *Butyricimonas virosa,* a species that has been linked with the induction of beneficial effects on host energy metabolism (Lee et al., 2022), and *Ilumatobacter fluminis* which was previously found to be significantly more abundant in the gut microbiome of laying hens fed with a mannan-rich fraction derived from the cell walls of *Saccharomyces cerevisiae* (Leigh et al., 2024). β-glucan is an another yeast cell wall fraction which has been shown to stimulate growth of beneficial microbes in the gut and in turn induce positive health effects particularly regarding immunoregulation and protective effects against genotoxicity and cytotoxicity (Jayachandran, Chen, Sum, Chung, & Xu, 2018b; Mantovani et al., 2008; Steenwijk et al., 2021). The potential of hydrolysed yeast β-glucan (HG) as an microbiome modulator *in vitro* was previously demonstrated by Pi et al. (2022), with increased abundances of beneficial bacteria observed, including *Bifidobacterium*, *Faecalibacterium,* and *Prevotella.* Although in the current study PBSY contained less β-glucan than CBSY (14.99 ± 0.38 g/100 g DM and 24.97 ± 0.75 g/100 g DM, respectively), it was potentially more accessible in PBSY due to the valorisation process degrading the structure of the yeast cell wall, possibly contributing to the enhanced microbiota modulation potential by PBSY (Jaeger et al., 2024b).

Non-absorbed protein and bio-active peptides may also be involved in gut microbiota modulation, with colonic AA/protein fermentation

metabolites including branched chain fatty acids, amines, organic acids, ammonia, sulfuric compounds, phenolic/indole compounds and gaseous compounds (Wu et al., 2022). In general, an excess of colonic protein fermentation is associated with negative health outcomes, as some of these metabolites are toxic to the host. (e.g. ammonia, amides, phenols, indoles and hydrogen sulfides) (Peled & Livney, 2021). While this could be a potential problem in the case of CBSY due to the higher proportion of non-bioaccessible AA present after *in vitro* digestion, the issue is potentially of less significance for PBSY, with valorisation reducing the amount of higher MW peptides and proteins reaching the colon. However, small amounts of AA from PBSY will be available for colonic fermentation by commensal bacteria, and their metabolites could potentially act as signalling molecules in the microbiota-gut-brain axis. For example, the neurotransmitter precursors tryptophan and tyrosine can be converted by gut microbiota to serotonin and dopamine, respectively, signalling molecules which are involved in regulation of mood and mental health (Murray et al., 2023).

5. Conclusion

Valorised BSY has significant potential to reduce global food waste, improve food security and support the global shift towards sustainable food systems. The increased digestibility and essential AA bioaccessability of PBSY highlights its potential as a high-quality alternative protein source which can play an important role in the protein transition. Moreover, the demonstrated microbiome modulation potential of PBSY presents an opportunity for the application of PBSY as a prebiotic supplement. However, it is important to note that this study represents preliminary findings, and further studies in this area are essential to fully understand the potential for the use of BSY in human nutrition and health applications. Of interest would be an in-depth screening of the microbiome modulation potential of CBSY and PBSY using a dynamic *in vitro* colon model and monitoring of metabolite production e.g. short chain fatty acids. Moreover, future work should focus on the application of BSY ingredients in various food matrices and subsequent investigation of the resulting nutritional, functional, and microbiome modulation characteristics.

Ethical statement

The collection of human faecal samples was approved by the local Clinical Research Ethics Committee (review reference numbers: ECM 4 (w) 11/1/2022 & ECM 3 (y) 20/06/2023) in compliance with the Declaration of Helsinki. All subjects gave their informed consent before they participated in the study, in full compliance with the approved guidelines and regulations.

CRediT authorship contribution statement

Alice Jaeger: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Laura Nyhan:** Writing – review & editing, Project administration, Investigation. **Aylin W. Sahin:** Writing – review & editing, Supervision, Conceptualization. **Emanuele Zannini:** Writing – review & editing, Project administration, Funding acquisition. **Dara Meehan:** Writing – original draft, Investigation, Formal analysis. **Junhui Li:** Writing – review & editing, Supervision, Conceptualization. **Paul W. O'Toole:** Writing – review & editing, Supervision, Conceptualization. **Elke K. Arendt:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.115732.

Data availability

Data will be made available on request.

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