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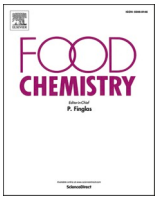

Prebiotic potential of spent brewery grain – *In vitro* study

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ABSTRACT

Spent brewery grain (SBG) is a by-product of the brewery industry. The study aimed to investigate the prebiotic potential of SBG. The chemical composition and fermentation capacity of SBG were checked. The gut microbiota response to SBG was assessed in two *in vitro* models (batch fermentation and dynamic system). Substances with prebiotic properties, including arabinoxylans (16.7 g/100 g) and polyphenols (49.1 mg/100 g), were identified in SBG. Suitable growth and fermentation by probiotic bacteria were observed. The modulatory effect of gut microbiota depends on the *in vitro* system used. In batch fermentation, there was no stimulation of *Bifidobacterium* or lactic acid bacteria (LAB), but short-chain fatty acid (SCFA) and branched short-chain fatty acids (BCFA) synthesis increased. In dynamic, SBG exhibited a moderate bifidogenic effect, promoting *Akkermansia* and LAB growth while reducing *Bacteroides* and *Escherichia-Shigella*. SCFA stabilisation and reduction of BCFA content were noted. Moderate prebiotic effects were observed.

1. Introduction

The problem of food waste and loss is one of the greatest challenges facing modern food systems (Willett et al., 2019). According to the Food and Agriculture Organization, approximately 30 % of food is lost or wasted each year worldwide (FAO, 2019). In the European Union, about 20 % of food production is wasted each year, according to Eurostat. Most waste occurs at the household level (53 %), followed by the processing sector (19 %), production (11 %), catering (12 %) and retail (5 %) (EUROSTAT, 2021). Losses and waste directly contribute to greenhouse gas emissions, agricultural overproduction, energy and economic losses as well as excessive resource consumption (Willett et al., 2019). An additional problem is the by-products of food production and their recycling. Selected by-products are used for further processing in the food industry, some are used as feed for livestock, others are utilised in different sectors of the economy but some are not recycled at all (Van Raamsdonk et al., 2023). However, many by-products have the potential to be recycled as food for human. Several by-products have promising nutritional and processing properties, and the health risk of consumption can be eliminated by appropriate processing (Comunian et al., 2021).

One such by-product is SBG generated during beer production. Approximately 20 kg of SBG is produced for every 100 L of beer (Lynch et al., 2016). The main direction of SBG reuse is feeding livestock. However, the scale of beer production often results in surpluses of SBG that are not utilised and wasted (Nyhan et al., 2023). SBG has a short shelf life in unprocessed form due to its high moisture, protein and fermentable residual sugars (Jackowski et al., 2020). However, the microbiological quality of SBG immediately after production is satisfactory due to the high-temperature treatment during the wort mashing and filtration process (around 85 °C). Therefore, rapid processing of SBG can ensure high microbiological quality and shelf life (Lao et al., 2020;

Abbreviations: Apreb, prebiotic activity; AX, arabinoxylans; AXOS, arabinoxylan-oligosaccharides; BCFA, branched short-chain fatty acids; CFU, colony formation units; GC, gas chromatography; HPLC, high performance liquid chromatography; *Iprob*, prebiotic index; ISAPP, International Scientific Association for Probiotics and Prebiotics; LAB, lactic acid bacteria; S-AX, soluble arabinoxylans; S-NSP, soluble non-starch polysaccharides; I-AX, insoluble arabinoxylans; I-NSP, insoluble non-starch polysaccharides; NSP, non-starch polysaccharides; PCoA, Principal Coordinate Analysis; SBG, spent brewery grains; SCFA, short-chain-fatty acids; SHIME®, Simulator of Human Intestinal Microbial Ecosystem.

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Zeko-Pivač et al., 2022). Moreover, the uses of SBG are broad and include biotechnological, agricultural and nutritional (high-fibre, functional foods and supplement development) applications (Bianco et al., 2020). SBG contains about 20 % protein, 5–8 % fat and a high fibre content of 40–65 % in dry matter. SBG is also a rich source of phenolic compounds (ferulic, p-coumaric, vanillic, caffeic, and sinapic acids), which are increasingly reported to have bifidogenic and LAB stimulation effects (Alves-Santos et al., 2020). Fibre in SBG is formed mainly of non-soluble parts of cellulose, lignin and hemicellulose, the main component of which is AX. AX alone accounts for 20 to 40 % of SGB (Ikram et al., 2017; Lynch et al., 2016). The prebiotic properties of AX and AXOS have been extensively studied recently. It was pointed to the properties that modulate the intestinal microbiota focused on bacterial community, the bifidogenic effect, the synthesis of SCFA, the regulation of the immune response, the increase in the synthesis of intestinal mucus, the regulation of the frequency of bowel movements and other health indicators (Hall et al., 2023; Nguyen et al., 2020; Schupfer et al., 2021). However, studies using a high-fibre diet that included foods rich in AX and AXOS, rather than isolates of these fractions, did not show a direct selective prebiotic effect but a complex influence connected to long-term consumption. The effect of consuming unprocessed high-fibre foods occurs with regular consumption. The scientific community suggested that the efficacy of the intervention was due to a moderate bifidogenic effect and an effect on the SCFA and BCFA profiles (Gill et al., 2021; Vinelli et al., 2022; Yao et al., 2022). Therefore, there is a recent need to investigate the prebiotic potential of unprocessed high-fibre food ingredients, because their effect on the intestinal microbiota is not fully understood (Gill et al., 2021; So et al., 2018; Vinelli et al., 2022). Hence, SBG contains a complex of substances that may have potential prebiotic properties, but there is limited scientific evidence in this area.

The ISAPP defines a prebiotic as a substrate selectively used by host microorganisms to provide a health benefit. In addition, a prebiotic must be resistant to digestion and fermented by the gut microbiota (Gibson et al., 2017). Research focusing on SBG prebiotic activity concerns only on batch fermentation *in vitro* systems of the colon, which is the simplest scheme to study the response of the intestinal microbiota (Bonifácio-Lopes et al., 2022; Calvete-Torre et al., 2023; Lynch et al., 2021; Reis et al., 2014). However, nowadays more advanced *in vitro* digestive systems are accessible. Therefore, further studies of SBG are necessary in other models, such as the SHIME®, which is closer to *in vivo* studies. Moreover, dynamic *in vitro* systems can provide a better understanding of SBG properties compared to batch fermentation (Isenring et al., 2023).

The present study aimed to assess the prebiotic potential of SBG. To achieve this, we investigated the content of NSP, AX, proteins, sugars, and polyphenols. Also, the fermentation capacity, *Ipreb* and *Apreb* of SBG were assessed. Additionally, the gut microbiota response to SBG supplementation was examined in two *in vitro* models to compare their response to potential prebiotics.

2. Materials and methods

2.1. Experiment design

The study included three main steps. The first experiment investigated the chemical composition of SBG, analysing its protein, NSP, polyphenols, polysaccharides, lignin, and sugar content. The second step examined the fermentation dynamics of SBG with probiotic bacteria strains, assessing bacteria number, pH value, and changes in metabolites and sugars before and after fermentation, as well as measuring the *Ipreb* and *Apreb* of SBG. The third part focused on changes in microbiota composition and their metabolites under SBG supplementation in two *in vitro* models: batch fermentation and the SHIME®.

2.2. Spent brewery grains

SBG was obtained from Kampania Piwowarska Dojlidy Brewery (Bialystok, Poland) during the malting process of Żubr lager beer, which uses only barley malt. After brew filtration, SBG was collected, frozen at −20 °C, dried at 95 °C to a stable weight, cooled, and ground to a flour consistency. It was then stored at 4 °C in a vacuum.

2.3. Chemical composition of spent brewery grains

2.3.1. Total protein content

Nitrogen in SBG was determined using the Kjeldahl method (ISO 1871:2009, 2009) with the Digestor 20 AutoLift mineralisation system, scrubber, and Kjeltec 8200 distillation unit (FOSS, Denmark). Results were converted to total protein content by multiplying the nitrogen content by 6.25.

2.3.2. Non-starch polysaccharides, β-glucan and lignin content

NSP content was determined by GC using the standard AACC 32–25, AOAC 994.13 method (AOAC, 1999). The NSP content is the sum of sugars: arabinose, xylose, mannose, galactose and glucose. The S-NSP and I-NSP fractions and the polysaccharide composition of both fractions were determined. The AX content of each fraction was calculated as the sum of arabinose and xylose. After treatment according to AACC 32–25, AOAC 994.13, the samples were separated with 96 % ethyl alcohol (Poch, Polnad) and centrifuged (Eppendorf Centrifuge 5804 R; Hamburg, Germany, 10 min, 5000 rpm) to obtain soluble (supernatant) and insoluble (pellet) fractions. Each fraction was hydrolysed to monosaccharides using 1 M H_2SO_4 (100 °C, 2 h) and then converted to volatile alditol acetates according to Brunton et al. (2007). Samples were separated on a Clarus GC (Perkin Elmer, MA, USA) equipped with an RTX-225 quartz capillary column (0.53/30 m), an autosampler, a split injector and a flame ionisation detector (FID). Carrier gas for analysis: helium. The separation was performed at 225 °C, with an injector and detector temperature of 275 °C. Separation, detection and quantification were performed according to Fraś et al. (2016). The determination was performed in an accredited laboratory, The Plant Breeding and Acclimatization Institute - National Research Institute (PBAI-NRI).

The determination of (1–3)(1–4)]-β-D-glucan content was performed according to 995.16 AOAC, 32–23 AACC with β-Glucan Assay Kit K-BGLU (Megazyme, Neogen, MI, USA).

Lignin was determined according to Dence (1992). Samples (50 mg) were treated with 72 % (*w/v*) sulfuric acid (0.75 mL) for 3 h at ambient temperature. Then, 9 mL of water was added, mixed and incubation continued for 2.5 h at 100 °C. The residues were recovered by filtration through sintered glass funnels under vacuum. The solid fraction was washed three times with water to remove the acid. The glass filters were dried at 50 °C in an oven until constant weight was obtained.

2.3.3. Organic acids and sugars

Samples were diluted in Milli-Q water (200 mg/5 mL) and extracted twice by sonication for 30 min at 30 °C (IS-6; 35 kHz; Inter Sonic; Olsztyn, Poland). After the first sonication, the sample was centrifuged (Eppendorf Centrifuge 5804 R; Hamburg, Germany, 5000 rpm, 15 °C, 10 min). The extraction was repeated twice. The supernatant was filtered through a 0.45 μm syringe filter into the vials. Organic acids and sugars were analysed using Shimadzu HPLC with LC-20 CE pump, CBM-20 A controller, CTD-20 AC oven, SIL-20 AC autosampler, RID-10 A detector and UV/Vis SPD-20AV detector (Kyoto, Japan). An Aminex HPX-87H column 300 × 7.8 mm (Bio-Rad, Warsaw, Poland) was used with an isocratic flow of 0.5 mL/min of 10 mM H_2SO_4 at 40 °C. Quantification was based on detection at 210 nm wavelength UV/Vis for organic acids, RI for sugars and external standard curves of 0.10–60 μg per injection of each analyte. All external standards were purchased from Sigma Aldrich (Poznan, Poland) with a purity of 99 %, except for maltotriose with a purity of 95 %.

2.3.4. Polyphenols content

SBG (200 mg) was extracted with 5 mL of 80 % (*v*/v) methanol using an ultrasonic bath (15 min, 30 °C; IS-6; 35 kHz; Inter Sonic; Olsztyn, Poland). The samples were centrifuged (Eppendorf Centrifuge 5804 R; Hamburg, Germany, 10 min, 5000 rpm, 0 °C). Extraction was repeated twice. The supernatant was transferred to a vial and filtered through a 0.22 um synergy filter. The polyphenol content was determined according to Kazimierczak et al. (2020). An HPLC system described in 2.3.3 equipped with a Fusion-RP 80 A column (250 mm × 4.60 mm, 4 μm, Phenomenex, CA, USA) was used. Acetonitrile (Poch; Poland) with MiliQ standard water was used as the mobile phase (phase A was 10 % C_2H_3N (v/v) in H_2O and phase B was 55 % (v/v) C_2H_3N in H_2O). The analysis time was 42 min. The gradient flow of 1 mL/min was applied as follows 1.00–22.99 min, 95 % phase A and 5 % phase B; 23.00–27.99 min, 50 % phase A and 50 % phase B; 28.00–32.99 min, 80 % phase A and 20 % phase B; and 33.00–42.00 min, 95 % phase A and 5 % phase B. The single run lasted 42 min. The wavelength range used was 270–360 nm. For the identification of phenolic compounds, external standards from Fluka and Sigma-Aldrich (Poznań, Poland) with purities of 95.0–99.9 % were used. The concentrations of phenolic compounds were calculated from the standard curves.

2.4. Fermentation dynamic of SBG and prebiotic scores

2.4.1. SBG medium preparation

To prepare growth media, 1 g of SBG was mixed with 100 mL of distilled water, autoclaved at 121 °C for 15 min and cooled. MRS broth (NeoGen, MI, USA) was used as a control medium for fermentation.

2.4.2. Fermentation conditions and number of bacteria

Lacticaseibacillus rhamnosus ATCC 53103 (GG) and *Bifidobacterium animalis* subsp. *lactis* BB-12 were activated from frozen cultures (−80 °C) on MRS agar (NeoGen, MI, USA) and incubated anaerobically at 37 °C for 48 h. A selected colony was then transferred to MRS broth (NeoGen, MI, USA) and incubated anaerobically for 24 h at 37 °C. The culture was then centrifuged for 10 min at 5000 rpm, and washed with PBS (Sigma Aldrich, Poznań, Poland). Next, centrifuged again under the same conditions and suspended in 10 mL of fresh PBS. The SBG medium and the MRS broth were inoculated by adding 1 % (100 uL/10 mL) of the bacterial suspension. Incubation was 24 h at 37 °C under anaerobic conditions.

2.4.3. pH value

The pH value was measured with an Orion Star A211 (Thermo Fisher, Massachusetts, USA) at 20 °C before and after fermentation.

2.4.4. Organic acids and sugars

Samples were diluted in Milli-Q water, centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge 5804 R; Hamburg, Germany), and the supernatant was filtered through a 0.22 μm syringe filter into vials. Analysis conditions are detailed in section 2.3.3.

2.4.5. Prebiotic index and prebiotic activity

Ipreb and *Apreb* were analysed according to Huebner et al. (2007) and Palframan et al. (2003). *Escherichia coli* ATCC 10536, *E. coli* ATCC 11775, *E. coli* ATCC 25922, *E. faecalis* ATCC 51299, *E. faecalis* ATCC 29212, *E. faecalis* 29,433, *L. rhamnosus* GG, *Lactoplantibacillus plantarum* 299v, *Lactobacillus acidophilus* ATCC 4356, *B. animalis* subsp. lactis BB-12 and *B. infantis* 35,624 were used. All bacteria were activated from frozen (−80 °C) cultures. *E. coli* was cultured on nutrient agar (Oxoid, UK), while other bacteria were cultured on MRS agar (NeoGen, MI, USA). After 48 h incubation at 37 °C, a selected colony was transferred to culture broths. Liquid cultures were performed at 37 °C for 24 h. *E. coli* was cultured in nutrient broth (Oxoid, UK) and the remaining bacteria in MRS broth (NeoGen, MI, USA).

To investigate the prebiotic scores, cultures of strains from the same species of bacteria were mixed in equal proportions. The culture medium was prepared with 5 g/L casein peptone, 3 g/L yeast extract, and glucose as the control at 10 g/L or 10 g/L of SBG or inulin (positive prebiotic control). The medium was inoculated with 1 % (10uL/1 mL) bacterial suspension. Plate cultures for numbering bacteria were performed immediately after inoculation and after 24 h of anaerobic incubation at 37 °C. Culturing was performed according to Naghili et al. (2013). Briefly, bacterial suspensions were serially diluted in buffered peptone water (Oxoid, UK) and immediately inoculated onto the appropriate selective media. *E. coli* was cultivated on TBX agar (Biokar Diagnostics, France), *Lactobacillus* at MRS agar, *Bifidobacterium* at BSM agar (Millipore, MA, USA), *E. faecalis* at COMPASS® *Enterococcus* Agar (Biokar Diagnostics, France). Bacterial cultures were incubated under anaerobic conditions at 37 °C for 24 h for *E. coli* and 48 h for the rest of the bacteria. After this time the colonies were counted.

The *Ipreb* is a ratio of bacterial growth in the medium with the addition of the tested substance (potentially prebiotic) compared to their growth in the control sample with glucose. The *Ipreb* was calculated using the Eq. (1).

$$Ipreb = \frac{CFU\ of\ bacteria\ in\ samples\ with\ SBG\ or\ inulin}{CFU\ of\ bacteria\ in\ samples\ with\ glucose} \quad (1)$$

Equation 1. *Ipreb* calculating formula.

Apreb reflects a given substance's ability to support a probiotic or beneficial bacteria's growth relative to enteric bacteria and relative to growth on a non-prebiotic substrate, such as glucose. The *Apreb* was calculated using the Eq. (2).

$$Apreb = \frac{(LogP24 - LogP0)\ SBG\ or\ inulin}{(LogP24 - LogP0)\ glucose} - \frac{(LogE24 - LogE0)\ SBG\ or\ inulin}{(LogE24 - LogE0)\ glucose} \quad (2)$$

Equation 2. *Apreb* calculating formula; *LogP24*- decimal logarithm of the tested bacteria number of CFU in the sample after 24 h incubation; *LogP0*- decimal logarithm of the tested bacteria number of CFU in the sample initially; *LogE24*- decimal logarithm of *E. coli* CFU in the sample after 24 h incubation; *LogE0*- decimal logarithm of *E. coli* CFU in the sample initially.

2.5. In vitro intestinal systems

2.5.1. Faecal human microbiota inoculum

The human microbiota was obtained from a healthy 39-year-old female volunteer who had not taken antibiotics for 12 months prior. The participant gave written consent to take part in the study. Consent for the use of this material in the study was obtained from the Research Ethics Committee (decision KE-U/12/2022).

2.5.2. The simulator of human microbial intestinal ecosystem (SHIME®) dynamic in vitro model

An instrumental model SHIME® (ProDigest, Gent, Belgium) was applied in the study. The experiment was run in a MULTISHIME® setup focused on the distal colon where according to the literature the major part of AX is fermented (Z. Chen et al., 2019; Salden et al., 2018). All conditions and all reagents used were according to the SHIME® manual from ProDigest (Gent, Belgium). Briefly, the study was performed regarding the intestinal lumen. The standard nutritional medium (PD-NM001B, ProDigest, Gent, Belgium) in this design was sequentially flowing through three separated digestive compartments stomach/duodenum joint vessels, proximal colon bioreactors, distal colon bioreactors (three bioreactors connected to a single proximal colon - three repetitions of this colon section). The instrument was inoculated with faecal microbiota. The fresh faecal sample was suspended in a phosphate buffer (1:5 ratio), homogenising and centrifuging for 2 min at 500 rpm. The supernatant was injected into SHIME® filled with standard nutrient medium (proximal and distal colon) and left overnight for initial

stabilisation. Afterwards, a two-week stabilisation period was conducted, in which the standard nutritional medium (210 mL) and pancreatic liquid (90 mL) were dozed automatically three times a day to the system. During the experiment, the standard media were supplemented with 5 g/L SBG. The SBG was added before autoclaving of media (121 °C, 15 min). The feeding with SBG lasted 8 days (Fig. 1). After that, the 8-day washing was applied with medium without SBG supplementation. Sampling was after a stabilisation period (point 0 0d), after 8 days (8d) of SBY supplementation and after 8 days of washing (16d). The SHIME® feeding program is in the repository dataset (RepOD, University of Warsaw, Poland) connected to this paper.

2.5.3. Batch fermentation in vitro model

The static system was performed according to Pérez-Burillo et al. (2021) with modification. The intestinal microbiota inoculum was collected from the SHIME® system from the descending colon after the stabilisation period at 0 point time (L-ST 0 h). 25 mL of intestinal fluid was poured into 50 mL falcons with previously weighted sterile SBG (addition was the same as in the SHIME® system). Fermentation specific to the colon lasts 24 (L-ST 24 h) and 48 h (L-ST 48 h) under anaerobic conditions at 37 °C (Fig. 1).

2.5.4. SCFA and BCFA analyse

Samples from SHIME® and batch fermentation were diluted in Milli-Q water, centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge 5804 R; Hamburg, Germany), and the supernatant was filtered through a 0.22 μm syringe filter into vials. Analysis conditions are detailed in section 2.3.3.

2.5.5. Bacterial DNA extraction and metabarcoding

Total genomic DNA was extracted using Genomic Mini AX Bacteria+ (A & A Biotechnology, Gdansk, Poland) according to the manual. After isolation DNA quality was checked by running the sample on 1 % agarose gel and template quantity was measured by fluorimetry using Qubit 2.0 and High Sensitivity Picogreen reagents (Thermo Fisher Scientific, MA, USA). Amplification and sequencing of conserved bacterial 16S rRNA gene fragments covering V3 and V4 regions were performed externally by DNA Sequencing and Oligonuleotide Synthesis Laboratory IBB PAS (Warsaw, Poland). For amplification of 450 bp long fragments following primers were used: 16S_V3-F 341-357F: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG 3′ and 16S_V4-R 785-805R: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC 3′ (Klindworth et al., 2013).

Obtained amplicons were checked on 1 % agarose gel, purified by Ampure XP magnetic beads (Beckman Coulter, CA, USA). Amplicon libraries were pooled in equimolar ratios and indexed according to Nextera indexing strategy by PCR and sequenced on MiSeq instrument paired-end mode using 600 cycle v3 chemistry kit (Illumina, CA, USA. Further analyses were performed locally. Obtained sequencing reads were quality-controlled using FastQC (Andrews, 2010) and accepted 16S rDNA amplicon sequences were classified using Qiime2 (Bolyen et al., 2019) with dada2 pipeline and taxonomic assignment based on Naïve Bayes classifier trained on Silva database v. 138 as downloaded in April 2024, presenting the bacterial community composition (OTUs - operational taxonomic units). The alpha-diversity (Shannon, Chao1, and Simpson indexes) were calculated using the phyloseq R version 1.22.3 package (McMurdie & Holmes, 2013). For plotting, ggplot2 R version 3.3.5 package was used.

2.6. Statistical analysis

Statistical analyses were performed in Statistica 13.3 (StatSoft, Poland) and Prism 10 (GraphPad Software, MA, USA). Basic descriptive analyses were performed. Percentage and proportional data were subjected to compositional data transformation. The Shapiro-Wilk test, Brown-Forsythe test and Levene test were performed to assess the parametricity of the data. ANOVA and *t*-tests were performed. The correlation matrix was prepared using Pearson's correlation. Bray–Curtis distance matrix was used for PCoA. Statistical significance was set at $\alpha = 0.05$ for all analyses.

3. Results

3.1. Chemical composition of spent brewery grains

The protein content in SBG was at 20.1 g/100 g (Table 1). Fibre in SBG was mainly rich in I-NSP fractions, including I-AX and lignin. S-AX and β-glucans were at a low level (Table 1). A relatively high content of maltose and maltotriose was found in SBG. The sum of polyphenols identified in SBG was 49.1 mg/100 g using the HPLC method. The highest amounts were kaempferol-3-glucoside, quercetin, kaempferol, myricetin and chlorogenic acid.

3.2. Fermentation dynamic of SBG and prebiotic scores

The results of the SBG fermentation, *Ipreb* and *Apreb* are presented in Fig. 2. The intensity of bacterial growth (Fig. 2A) was better in the MRS medium. However, in the SBG a significantly increased number of *Lacticaseibacillus rhamnosus* GG was observed compared to the initial number ($p < 0.05$). *Bifidobacterium animalis* subsp. *lactis* BB-12 in SBG showed a slight increase in live cells after fermentation ($p > 0.05$). The initial pH of the growth media was similar between SBG and MRS (Fig. 2B). A decrease in pH was observed in all samples analysed after fermentation. The decrease in pH caused by L. *rhamnosus* GG was greater in SBG than in MRS. In both media, *B. animalis* BB-12 had a lower ability

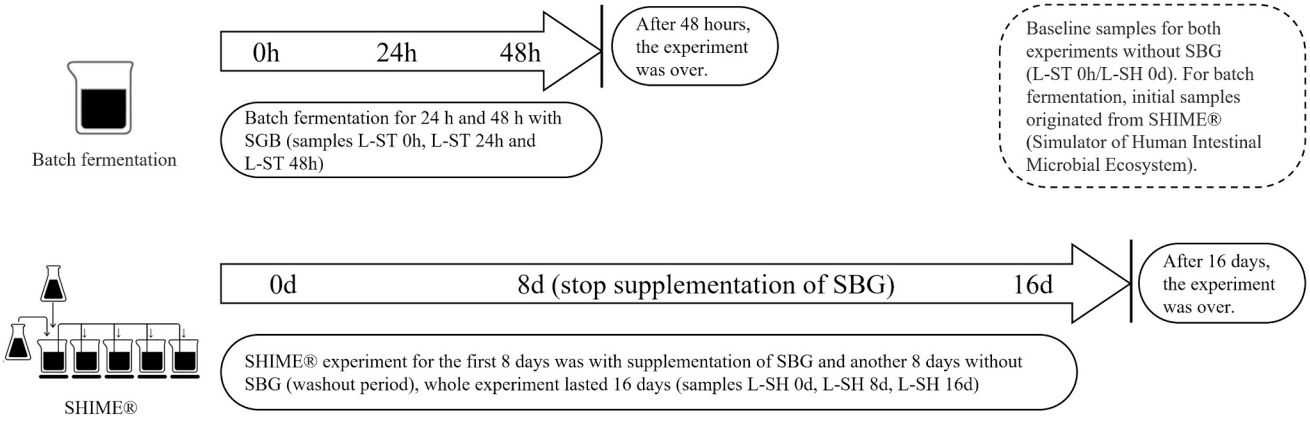

Fig. 1. Batch fermentation and SHIME® experiment scheme.

Table 1
Content of selected chemical compound in the analysed spent brewery grains; $n = 3$.

Proteins	20.1 ± 0.3	
Fiber fractions		
Total NSP	29.7 ± 0.2	
I-NSP	28.2 ± 0.3	
S-NSP	1.5 ± 0.1	
Total AX	16.7 ± 0.2	
I-AX	16.2 ± 0.2	g/100g
S-AX	0.5 ± 0.1	
Lignin	9.0 ± 0.2	
β-glucans	0.6 ± 0.0	
Water-soluble carbohydrates		
Maltotriose	3.4 ± 0.3	
Maltose	7.3 ± 0.4	
Glucose	0.6 ± 0.1	
Polyphenols		
Chlorogenic acid	4.0 ± 0.1	
Caffeic acid	2.6 ± 0.0	
p-coumaric acid	1.8 ± 0.0	
Vanillic acid	3.1 ± 0.4	
Salicylic acid	2.7 ± 0.4	
Sinapic acid	1.3 ± 0.1	mg/100g
Quercetin-3-o-rutinoside	3.5 ± 0.6	
Kaempferol 3-glucoside	12.2 ± 1.3	
Myricetin	5.3 ± 0.8	
trans-Cinnamic acid	0.4 ± 0.0	
Quercetin	6.8 ± 2.0	
Kaempferol	5.4 ± 0.5	

to acidify the environment than L. *rhamnosus* GG.

The content of organic acids and sugars in the samples subjected to incubation with each probiotic depended on the type of medium and the bacteria (Fig. 2 C, D). The total sugar content was significantly reduced after incubation ($p < 0.05$). In the SBG medium, maltotriose was partially degraded by both bacterial strains, while L. *rhamnosus* GG showed a greater ability to catabolize this trisaccharide ($p < 0.05$). Maltose was completely reduced from the medium by L. *rhamnosus* GG, and partially by *B. animalis* BB-12 ($p < 0.05$). Glucose was catabolised best from all the sugars by both strains regardless of media ($p < 0.05$). Organic acid and sugar content was lower in SBG before fermentation than in the MRS medium. Also, after fermentation, the concentrations of organic acids were lower in SBG ($p < 0.05$). The observed trends in metabolite changes were similar for both the tested media and the bacteria. The main metabolites formed in both media and by both probiotics were lactic and acetic acid. In addition, *B. animalis* BB-12, increased significantly the content of succinic acid content in SBG ($p < 0.05$) but not in MRS ($p > 0.05$). On the other hand a slight reduction of malonic acid content was observed in media incubated with L. *rhamnosus* GG, but not *B. animalis* BB-12. The propionic acid content did not change significantly across media and probiotics ($p > 0.05$).

Ipreb values (Fig. 2E) were higher for SBG samples than for inulin for all tested bacterial groups, but for *Bifidobacterium*, the difference was not significant ($p > 0.05$). The *Apreb* (Fig. 2F) of SBG was higher than 0.9 and comparable to inulin ($p > 0.05$). Only in the case of *Bifidobacterium*, the value of *Apreb* of SBG was significantly lower than that of inulin ($p < 0.05$). The high values for the *Ipreb* indicate good growth of the tested bacteria with SBG. Moreover, *Apreb* values demonstrated that LAB, *Bifidobacterium* and *E. faecalis* growth was better with the addition of SBG than *E. coli* (Fig. 2F).

3.3. Gut microbiota, SCFA and BCFA modulation

The levels of lactic acid, SCFA (acetic acid, propionic acid, butyric acid) and BCFA (isobutyric acid, isovaleric acid, valeric acid) in the batch fermentation (Fig. 3A) were changing over time more dynamically than in SHIME® (Fig. 3D). In the batch fermentation, the levels of all compounds tested increased significantly after 24 and 48 h ($p < 0.05$), except for valeric acid, which remained at a similar level throughout the experiment. The lactic acid content decreased significantly between 24 and 48 h in the batch fermentation system. The percentage of SCFAs in batch fermentation (Fig. 3B) showed a decreasing trend due to the increase in BCFAs.

No lactic acid was detected in the SHIME® (Fig. 3D). By supplementing SBG to SHIME® nutrient medium (from 0d to 8d), a significant decrease in the content of isobutyric and isovaleric acids was observed ($p < 0.05$), while valeric acid remained at a constant level. Simultaneously, SCFA levels were stable during SBG supplementation (Fig. 3D). After 16 days (L-SH 16d) of the experiment, and thus after an 8-day washout period without SBG, a significantly higher concentration of propionic acid was observed. However, isobutyric acid and isovaleric acid levels returned to near initial concentrations. In addition, valeric acid was significantly lower than at the beginning of the experiment. In SHIME®, the percentage of all SCFAs increased significantly ($p < 0.05$) within 8 days of SBG supplementation. After 16 days (8 days without SBG supplementation), the percentage of acetic acid decreased significantly due to the increase of propionic acid ($p < 0.05$). The remaining acids returned to initial levels, except for valeric acid, the percentage of which decreased significantly ($p < 0.05$).

Sequencing data were deposited in the RepOD repository. All data regarding sequencing statistics and biodiversity indices are available in Supplementary files 1 and 2. 640,902 paired-end raw sequencing reads for five samples were obtained with the mean number of reads per sample 128,180. After filtration and denoising, the average number of reads for the sample was 55,163 (Supplementary 1). The alpha-diversity indices (Chao1, Shannon, and Simpson) differ between the samples, with the highest value for the time 0 sample (0 was the same for both batch fermentation and SHIME®), indicating that bacterial diversity at the start of the experiment was the most diverse (sample deriving from faeces after the stabilisation period). In the L-ST 24 h sample (batch fermentation), an extensive reduction compared to the baseline sample in alpha-biodiversity occurred. The rest of the samples present similar levels of alpha-diversity indicators. Nevertheless, alpha-biodiversity in samples from both models was reduced compared to the levels at the beginning of the experiment (time 0).

In the batch fermentation after 24 h (L-ST 24 h) a decrease in the relative abundance of many genera of bacteria was observed (Fig. 3C), including: *Agathobacter, Akkermansia, Anaeroglobus, Bacteroides, Bifidobacterium, Cloacibacillus, Collinsella, Escherichia-Shigella, Eubacteriaceae, Faecalibacterium, Fusobacterium, Holdemania, Hungatella, Lactobacillus, Megasphaera, Pseudomonas, Sanguibacteroides, Subdoligranulum, Sutterella* and *Victivallis* Those observations are in line with the decrease of alpha-diversity indices. After 48 h of batch fermentation (L-ST 48 h), a partial return of alpha-diversity and the increase of relative abundance of bacteria to the initial state was observed. However, there were noticeable changes in the microbiota composition between the baseline and L-ST 48 h sample. The relative abundance of *Blautia, Butyricicoccus, Cloacibacillus, Coprococcus, Eubacterium, Faecalibacterium, Flavonifractor, Lachnospiraceae* UCG-004, *Phascolarctobacterium, Subdoligranulum* increased notably. A decrease in the relative abundance of *Agathobacter*, *Bacteroides*, *Bifidobacterium*, *Eisenbergiella*, *Escherichia-Shigella*, *Fusobacterium*, *Lactobacillus*, *Parabacteroides*, and *Pseudomonas* was also observed after 48 h (L-ST 24 h).

In SHIME®, the dynamics of changes in microbiota composition (Fig. 3F) were different than in batch fermentation. With SBG supplementation (L-SH 8d), the relative abundance of *Akkermansia, Anaeroglobus, Bifidobacterium, Butyricicoccus, Collinsella, Eubacterium, Fusobacterium, Lachnoclostridium, Lactobacillus, Lactococcus, Odoribacter, Veillonella, Victivallis* increased. The relative abundance of *Agathobacter, Bacteroides, Clostridium, Eisenbergiella, Escherichia-Shigella, Faecalibacterium, Flavonifractor, Intestinimonas, Ruminococcus, Subdoligranulum* and UBA1819 decreased. After cessation of SBG supplementation (L-SH 16d), the composition of the microbiota changed compared to previous periods. The relative abundance of some of the bacterial taxa returned to the initial state (*Agathobacter, Bacteroides, Cloacibacillus, Eisenbergiella,*

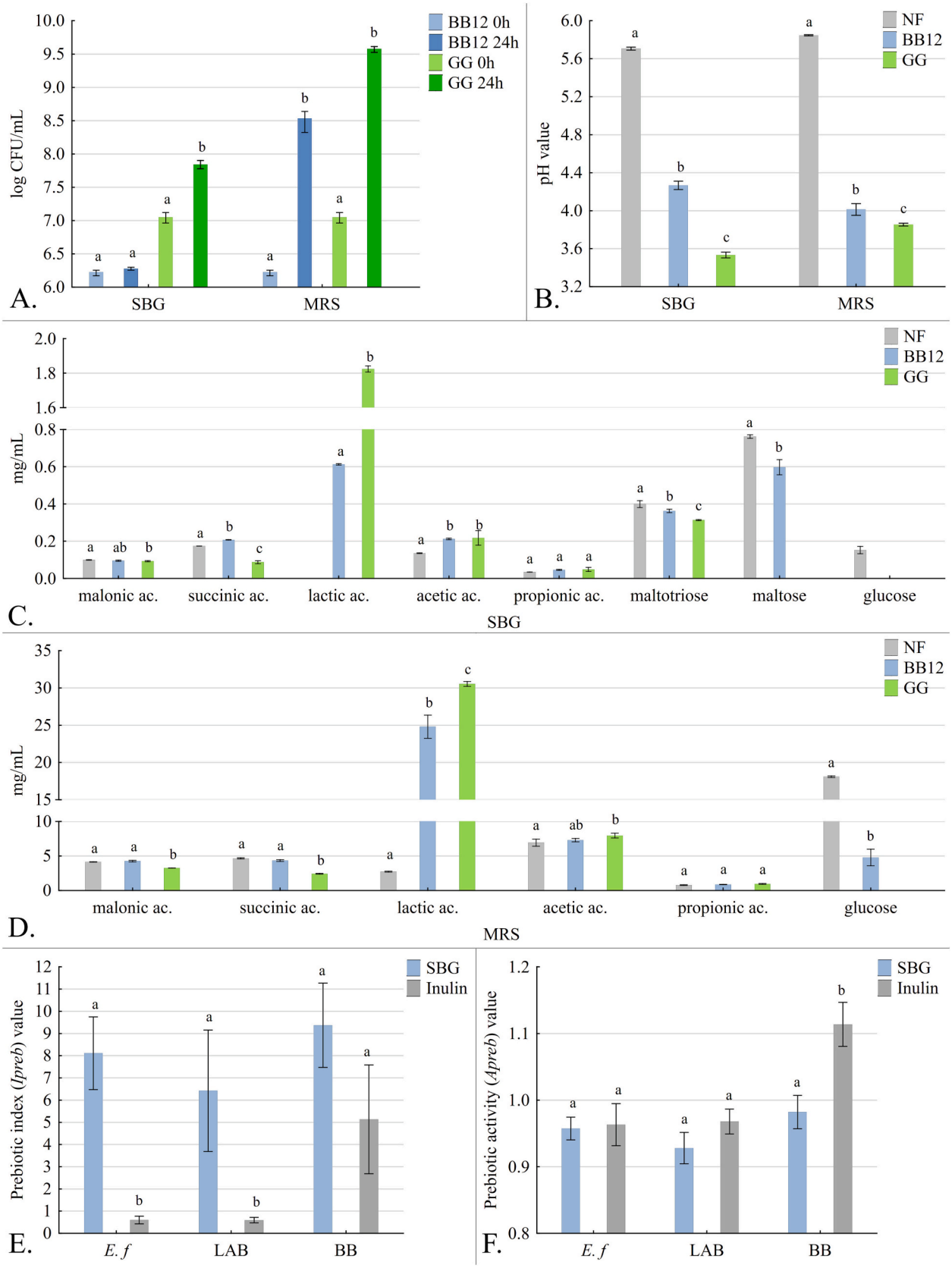

Fig. 2. Fermentation dynamic based on the bacterial count (A), pH values changes (B), organic acid and sugars variation in spent brewery grains (SBG; C) and MRS medium (D); prebiotic index (E) and prebiotic activity (F) of the SBG and inulin; "h" means hours, "ac." means acid, "NF" means not fermented medium, "BB12" means *Bifidobacterium animalis* subsp. *lactis* BB-12, "GG" means *Lacticaseibacillus rhamnosus* GG (GG), "*E.f* " means *Enterococcus faecalis* strains mix, "LAB" means lactic acid bacteria strains mix, "BB" means *Bifidobacterium* strains mix; lowercase letters indicate statistical differences between samples in Tukey's test after ANOVA analysis or *t*-test ($p < 0.05$); error bars in chart A indicate the minimum-maximum ranges, in the rest they indicate standard deviations; n = 3.

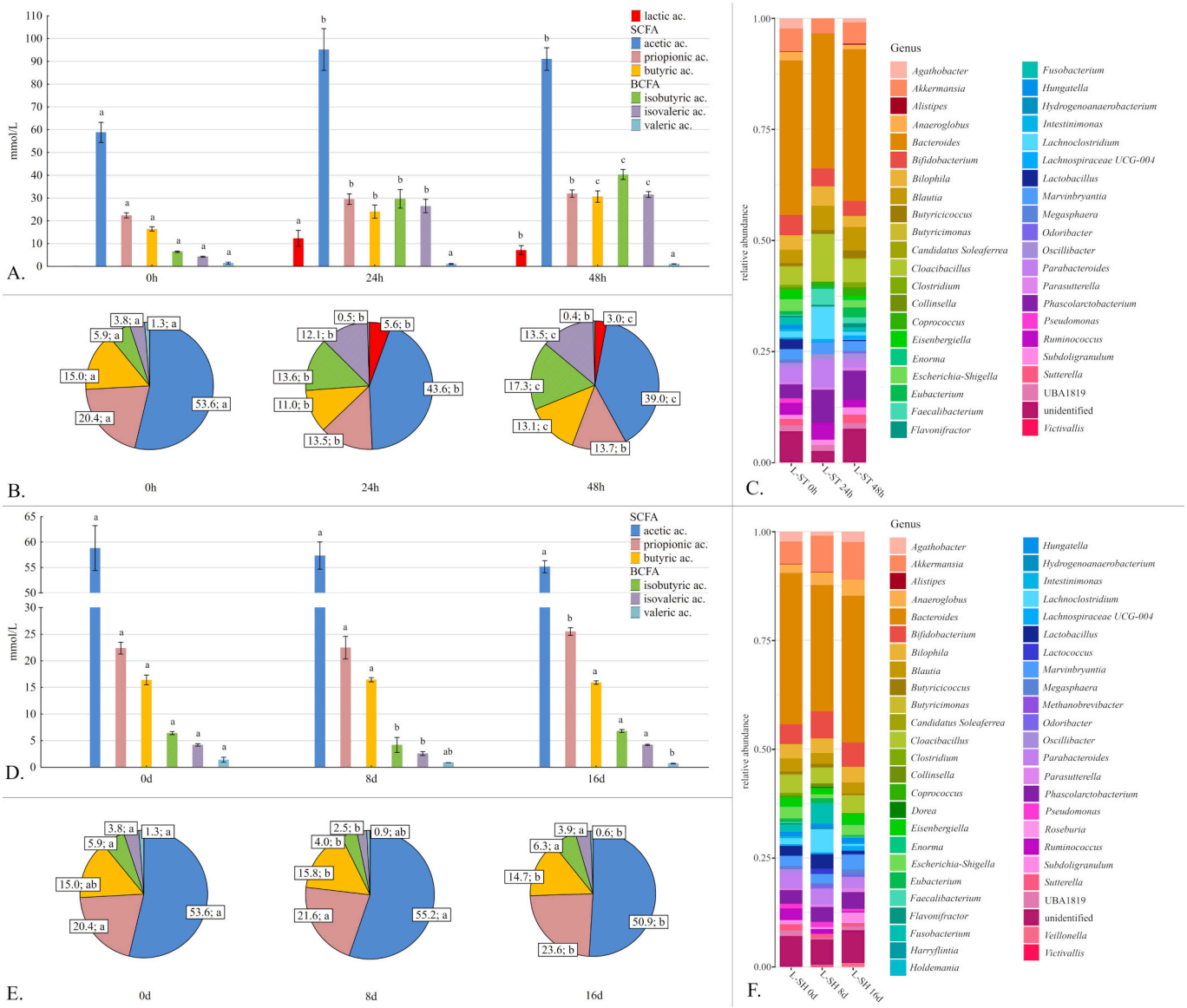

Fig. 3. Lactate, short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) in the batch fermentation (L-ST): content in mmol/L (**A**) and in percentage share (**B**) and in the SHMIE (L-SH): content in mmol/L (**D**) and percentage share (**E**) in different times of the experiment; relative abundance of microbiota composition at the genus level for L-ST (**C**) and for L-SH (**F**); "ac." means acid; "h" means hours, "d" means days; lowercase letters indicate statistical differences between samples in Tukey's test after ANOVA analysis ($p < 0.05$), error bars indicate standard deviations; for SCFA and BCFA, n = 3; for sequencing samples from 3 replications were merged into one ($n = 1$).

Escherichia-Shigella and *Faecalibacterium*). On the other hand, *Akkermansia, Anaeroglobus, Bifidobacterium, Hungatella, Marvinbryantia, Megasphaera, Phascolarctobacterium, Subdoligranulum, Veillonella* and unidentified bacteria were more abundant than at initial state. *Bilophila, Blautia* and *Eubacteriaceae* remained at similar levels of abundance compared to that after the SBG supplementation period. When comparing the batch fermentation system and SHIME®, differences in SCFA and BCFA and its microbiota taxonomic composition were noticed. The changes observed in batch fermentation were more varied, while the changes in the SHIME® system were balanced.

The correlation between the relative abundance of bacterial genera and metabolites was determined by Pearson's correlation (Fig. 4A, B and Supplementary 3), where significant ($p < 0.05$) pars were marked. Batch fermentation (Fig. 4A) and SHIME® exhibit (Fig. 4B) different correlations between metabolites and microbial groups. The correlation in batch fermentation was directly related to the loss of biodiversity in the system and the reduction in the relative abundance of certain taxa. Noteworthy, the strong positive correlation between *Blautia, Butyricicoccus, Cloacibacillus, Coprococcus, Eubacterium, Faecalibacterium, Lachnoclostridium, Lachnospiraceae* UCG-004, *Oscillibacter, Parasutterella, Subdoligranulum* and SCFAs and BCFAs except valeric acid. These bacterial genera during incubation were probably mainly responsible for the synthesis and transformation of fatty acids.

In SHMIE® (Fig. 4B), other pairwise correlation values were observed between taxonomic groups and SCFAs and BCFAs than in batch fermentation. Bacterial genera strongly positively correlated with acetic acid where *Alistipes*, *Clostridium*, *Coprococcus*, *Enorma*, *Eubacterium*, *Parabacteroides*, *Ruminococcus* occurred. For propionate, a strong positive correlation was observed with *Anaeroglobus*, *Bilophila*, *Eisenbergiella*, *Enterococcus*, *Holdemania*, *Hungatella*, *Lachnospiraceae* UCG-004, *Marvinbryantia*, *Megasphaera*, *Methanobrevibacter*, *Parasutterella*, *Phascolarctobacterium*, *Subdoligranulum*, UCG-005 and *Veillonella* occurence. The highest positive correlation for butyric acid was with *Alistipes*, *Butyricicoccus*, *Eubacterium*, *Lactobacillus*, *Parabacteroides*, *Pseudomonas*

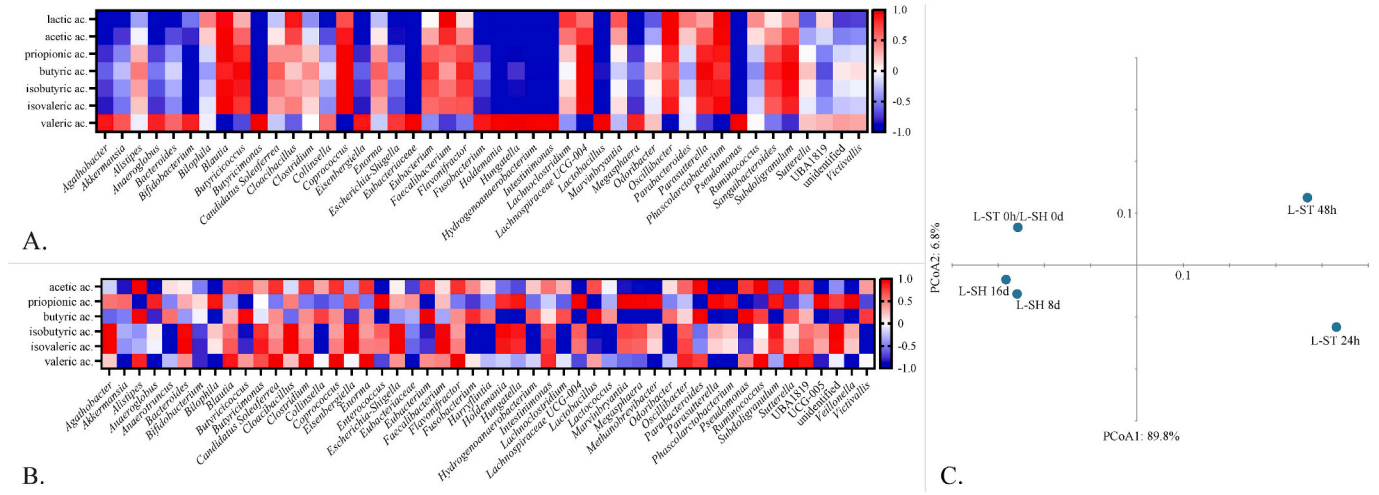

Fig. 4. Correlation between microbial taxa relative abundance and metabolites in the batch fermentation model (A) and dynamic fermentation model (B); Principal Coordinates Analysis (PCoA) of the tested colon fermentation models based on the Bray–Curtis distance (C); "L-ST" means batch fermentation model; "L-SH" means SHIME®; "h" mean hours, "d" mean days.

and *Victivallis*. Within isobutyric acid and isovaleric acid, correlation pairs for individual microorganisms were similar and these were *Agathobacter*, *Bacteroides*, *Cloacibacillus*, *Eisenbergiella*, *Escherichia-Shigella*, *Faecalibacterium*, *Holdemania* and *Hungatella*. In the case of valeric acid, the strongest positive correlations were shown by *Blautia*, *Candidatus Soleaferrea*, *Clostridium*, *Coprococcus*, *Enorma*, *Flavonifractor*, *Ruminococcus* and *Sutterella*. In the case of SHIME®, a greater variation in correlations was observed than in the batch fermentation system. This was related to the bacterial diversity, the relative abundance of taxa in the samples and the dynamics of changes in both *in vitro* systems.

The extensive distance between the batch fermentation (L-ST) and SHIME® (L-SH) indicates the low similarity of these *in vitro* models (Fig. 4C). These results show the overall variability under the influence of SBG supplementation in both models. In batch fermentation, differences were more significant. In SHIME® the changes between the start and other measurement points were smaller. The transformations in SHIME® reflected a greater influence of SBG on the gut microbiota and its metabolites.

4. Discussion

4.1. Chemical composition of spent brewery grains

SBG is a complex by-product that could be processed into a valuable food ingredient due to its high nutritional value, particularly the fibre, protein and phenolic compounds. The literature provides similar data on SBG protein, NSP, β-glucan, lignin and AX content (Ikram et al., 2017; Jackowski et al., 2020; Lynch et al., 2016). Relatively new data in this study pertain to maltotriose, maltose, and glucose. These sugars remain in the SBG after the lautering process. Only the study by Jin et al. (2022) has described the content of fermentable sugars so far (total content 8.2 ± 5.8 g/100 g), and the results are comparable across both studies. However, most of the studies have focused on the effects of acidic or enzymatic hydrolysis treatment of polysaccharides rather than sugars naturally present in SBG (Rojas-Pérez et al., 2022; Sganzerla et al., 2022). The greatest variation in published data relates to polyphenol content (Birsan et al., 2019; Macias-Garbett et al., 2021). This deviation is due to the differences in analytical methodologies and especially sample preparation. Like in the present study, SBG dried at high temperatures has a lower concentration of phenolic compounds than freeze-dried (Guido & Moreira, 2017). On the other hand, high-temperature treatment is less expensive, inactivates bacteria and moulds, and can be used without special equipment. In addition, a more complex polyphenol extraction method gives better quantification results (Guido & Moreira, 2017). The most commonly reported polyphenols in SBG are ferulic acid, p-coumaric acid, vanillic acid, caffeic acid, sinapic acid, salicylic acid, chlorogenic acid, quercetin, and kaempferol (Guido & Moreira, 2017; Macias-Garbett et al., 2021). In this study, high-temperature drying most probably caused the degradation of some phenolic compounds (Table 1) (Antony & Farid, 2022; Y. Zhang & Liu, 2022). Moreover, the level of ferulic acid, the most abundant polyphenol in SBG, was below the detection limit in our study. This was probably due to the temperature and time of dehydration. This phenolic acid could also not be extracted well due to the stable chemical bond with AX (Bento-Silva et al., 2018; Macias-Garbett et al., 2021). We did not use hydrolysis during extraction to release ferulic acid from chemical bonds with AX. Nonetheless, the content of the rest of the detected polyphenols aligns with the literature evidence (Birsan et al., 2019; Macias-Garbett et al., 2021). On the other hand, polyphenols exert their prebiotic effects through several molecular mechanisms involving interactions with gut microbes, mostly *Bifidobacterium,* LAB and the host (Alves-Santos et al., 2020). In summary, the content of the above ingredients in SBG, in terms of the definition of a prebiotic, indicates that it is a raw material rich in indigestible fractions of dietary fibre (AX and lignin) and polyphenolic compounds. However, SBG also contains digestible ingredients such as proteins and sugars.

4.2. Fermentation dynamic of SBG and prebiotic scores

Regarding the characteristics of prebiotic substances, the study was initially focused on the fermentation potential of SBG by defined probiotic bacteria. The observed bacterial growth (Fig. 2C) in SBG indicates its suitability for fermentation by *B. animalis* BB-12 and L. *rhamnosus* GG. The literature described SBG as a useful fermentation material with many potential applications. Many microorganisms, such as LAB, *Propionibacterium, Bacillus subtilis*, yeasts and moulds grew rapidly and produced typical metabolites in SBG (Neylon et al., 2021; Shetty et al., 2023). The effects of SBG fermentation include valorisation of this raw material into organic acids, alcohols, and bioactive peptides (*e.g.* bacteriocins), obtaining proteins with higher nutritional value, improving digestibility or increasing technological utility. The valorisation of SBG has been studied for various purposes, in the synthesis of active ingredients, in animal feed and in the production of supplements and food (Fernandes et al., 2021; Jackowski et al., 2020; Neylon et al., 2021; Shetty et al., 2023).

Regarding fermentation of SBG by L. *rhamnosus* GG and *B. animalis*

BB-12, typical changes in the SBG chemical composition included mainly a reduction in the levels of sugars (glucose, maltose, maltotriose). The catabolism of these sugars was associated with the presence of α-1,4-glycosidase and α-glucosidase bacterial enzymes, which can degrade disaccharides and maltotriose to simple sugars (Gänzle & Follador, 2012; Pokusaeva et al., 2011). The organic acid profile indicates the correct formation of typical metabolites such as lactic acid and acetic acid (Pokusaeva et al., 2011; Suissa et al., 2023). Malonic and succinic acids according to the metabolic pathways were not utilised by *B. animalis* BB-12. A different phenomenon was observed in L. *rhamnosus* GG, where succinic acid was metabolised by succinate dehydrogenase in the incomplete citric acid cycle (Suissa et al., 2023). Furthermore, the unchanged propionic acid content confirms the metabolic changes, as it is not a typical metabolite for L. *rhamnosus* GG and *B. animalis* BB-12 (Pokusaeva et al., 2011; Suissa et al., 2023).

Apreb and *Ipreb* are indicators of prebiotic activity typically used in *in vitro* studies of polysaccharides and oligosaccharides. The higher the values of these indicators, the greater the prebiotic properties of the tested substance. If the *Ipreb* value is greater than one, the substance stimulates the growth of microorganisms compared to the control carbohydrate, here glucose (Figueroa-González et al., 2019; Huebner et al., 2007). To our knowledge, no data have been published on these indicators in SBG. Nevertheless, Paesani et al. (2019) showed that the *Aperb* for wheat AXOS was 0.36 for *Lactobacillus* and *Bifidobacterium*, while the *Ipreb* was 4.09. On the other hand, the values obtained for inulin by Paesani et al. (2019) were also measurably lower than in our studies (Fig. 2E, F). In another study, unprocessed cereal drinks such as barnyard, foxtail and kodo millet were tested and the authors obtained an *Apreb* value of 0.45 (Arya & Shakya, 2021). In addition, the authors studying AX and AXOS found a strong correlation between the chemical structure of AX and the rate of fermentation by probiotic bacteria. *Ipreb* was observed to increase when AX was treated with the enzyme xylanase, resulting in the formation of more xylose compared to untreated AX. This reaction increased the number of saccharides available to the bacteria and stimulated their growth. It was also shown that a high degree of polymerisation of AX reduced the dynamics of fermentation compared to AX with a low degree of polymerisation (Pollet et al., 2012; Wang et al., 2020). On the other hand, when testing unprocessed food raw materials such as SBG in this method, the prebiotic activity of polysaccharides may be masked by nutrients such as sugars, proteins or lipids used in metabolic pathways by bacteria. Nevertheless, these methods provide a general comparison of the growth of *Enterobacteriaceae* and probiotics in the presence of a specific substance.

4.3. Gut microbiota, SCFA and BCFA modulation

The classification of prebiotic substances, as defined by ISAPP, includes non-digestible substrates utilised by host microorganisms and confer a health benefit (Gibson et al., 2017). The most important feature of prebiotic substances is their ability to be fermented by intestinal microbiota, expressed, among others, by desired changes in SCFA and BCFA content.

Currently, there is limited evidence regarding the response of gut microbiota, SCFA, and BCFA to SBG. Researchers so far have focused mainly on the prebiotic properties of isolated AX or AXOS fractions from SBG or other cereal materials. In a study by Lynch et al. (2021), SBG was tested as one of the samples in a batch fermentation system under controlled bioreactor conditions with continuous pH monitoring (pH 6.8). The composition of the fermented sample included 40 % rice along with barley malt. A significant decrease in the relative abundance of *Bacteroides*, *Blautia* and *Faecalibacterium* was observed, along with an increase in *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Agathobacter*, *Subdoligranulum*, *Phascolarctobacterium*, *Eubacterium* and *Escherichia-Shigella*. In SCFA synthesis a non-significant increase in the acetate, propionate, and butyrate content was observed. For BCFA, a significant decrease in isovaleric acid content and an increase in valeric acid content were noted. Calvete-Torre et al. (2023) examined four different SBGs with varying proportions of barley malt with other grains such as rice, unmalted barley, and rice flour. This experiment was conducted in batch fermentation without pH stabilisation. An increased number of readings for *Bilophila*, *Parabacteroides*, *Phascolarctobacterium*, *Senegalemassilia*, *Collinsella*, *Coprococcus*, *Lachnoclostridium*, *Clostridium* and *Escherichia-Shigella* were observed. The content of SCFA and BCFA significantly increased for all analysed acids. In the study by Bonifácio-Lopes et al. (2022), the impact of SBG flour on gut microbiota was investigated. The study was conducted under *in vitro* batch fermentation without acidity stabilisation. The authors observed increased copy numbers in RT-PCR assays for *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Escherichia-Shigella*. Significant increases were noted in the contents of succinate, lactate, acetate, propionate and butyrate. The authors did not analyse BCFA. No further studies testing SBG were found. Two other studies examined the modulation of gut microbiota by AX and AXOS fractions isolated from SBG (Gómez et al., 2015; Reis et al., 2014). Both research used batch fermentation without acidity control. The researchers observed a significant increase in *Bifidobacterium*, *Enterococcus*, *Bacteroides*, *Prevotella*, *Clostridium*, *Eubacterium* and an overall increase in the total bacteria copy number. Both studies observed significantly increased SCFA concentration, but BCFA was not analysed.

Comparing literature data on SBG, AX, and AXOS with our batch fermentation results reveals several similarities in microbiota changes and SCFA and BCFA concentrations. In the presented study, no stimulation of the development of *Bifidobacterium* or *Lactobacillus* genera was observed (Fig. 3C). However, the increase in the relative abundance of *Subdoligranulum, Faecalibacterium, Eubacterium*, family and *Phascolarctobacterium*, but the decrease of the *Bacteroides* and *Escherichia-Shigella* genera were observed, what remains consistent with the findings of Harris et al. (2019) and Lynch et al. (2021). Other research utilizing batch fermentation observed a similar trend to this study in changes in SCFA and BCFA when the fermentation acidity was not regulated. The lack of pH monitoring led to significant intense fatty acid synthesis. Such results were reported in all the mentioned research concerning SCFA and BCFA in batch fermentation (Bonifácio-Lopes et al., 2022; Calvete-Torre et al., 2023; Gómez et al., 2015; Reis et al., 2014).

There is a notable gap in the current knowledge on the prebiotic properties of SBG in advanced, dynamic *in vitro* systems such as SHIME®, animal models or human trials. The presented analysis on the SHIME® model is similar to the investigation by Lynch et al. (2021), which used controlled pH conditions in batch fermentation. When comparing SHIME® and Lynch et al. (2021) results, a similar increase in the relative abundance of *Bifidobacterium*, *Lactobacillus* and *Enterococcus* and a decrease in *Bacteroides* was observed (Fig. 3F). However, the similarity between batch fermentation and SHIME® remains low due to variations in experiment duration, system parameters and differences in nutrient and prebiotic feeding (Isenring et al., 2023; Roupar et al., 2021). On the other hand, extensive data has been documented on the prebiotic properties of AX and AXOS in dynamic systems, animal models or human studies. These studies highlight the ability of AX and AXOS to promote the increase in relative abundance of *Bacteroides*, *Bifidobacterium*, *Blautia*, *Dorea*, *Eubacterium*, *Lactobacillus*, *Faecalibacterium*, *Prevotella*, *Roseburia* and *Enterococcus* (François et al., 2012; Kjølbæk et al., 2020; Schupfer et al., 2021, 2023; Walton et al., 2012; Zambrana et al., 2019). Other research has reported significant reductions in *Campylobacter, Clostridium* and *Escherichia-Shigella* (Schupfer et al., 2021). In addition, an increase in butyrate-producing bacteria was observed, whose relative abundance also increased in our study (*Butyricicoccus*, *Bilophila*, *Marvinbryantia*, *Odoribacter*, *Lachnoclostridium*) (Damen et al., 2011; Schupfer et al., 2023; Van den Abbeele et al., 2011). Conversely, another research group observed a reduction in alpha biodiversity due to AX's promotion of the growth-selected species of bacteria like *Bifidobacterium* (Müller et al., 2020). In summary, AX and AXOS studies often observed a moderate bifidogenic effect, supported *Bacteroides* growth, and simultaneously retained *Firmicutes* and *Bacteroides* proper ratio.

However, a clear and specific influence on certain microbiota taxa cannot be consistently attributed to all AX and AXOS. This effect depends on the structure and degree of branching of AX and AXOS, which is determined by the origin of the raw material (Z. Chen et al., 2019; Neyrinck et al., 2018). The molecular weight of AX and the number of side chains, which vary between raw materials, further influence these properties. For example, AX from barley has a higher molecular weight than AX from rice, resulting in smaller and less branched molecules that are more easily fermented by the gut microbiota (Z. Chen et al., 2019; Wang et al., 2020). Notably, the hydrolysis products of AX, namely AXOS, showed more substantial bifidogenic effects than AX due to its less branched structure, shorter chemical forms and lower molecular weight (Broekaert et al., 2011).

The presented research indicates that SBG supports the growth of *Bifidobacterium*, LAB, *Akkermansia*, *Lachnospiraceae*, and cellulose-degrading bacteria in SHIME® (Fig. 3F). The increase in the relative abundance of cellulose-degrading bacteria is often observed when analysing the impact of minimally processed high-fibre raw materials with complex chemical characteristics, such as bran and whole grain foods (So et al., 2018). SBG also contains other dietary fibre fractions besides AX, primarily cellulose and lignin. Although these are not considered substances with direct prebiotic effects. Noteworthy, according to recent reports, cellulose stimulates the growth of bacteria from the *Ruminococcaceae* family (*Ruminococcus*, *Faecalibacterium*, *Subdoligranulum*, *Oscillibacter*, formerly *Blautia*), which have a positive impact on host health (Moraïs et al., 2024). Cellulose-degrading bacteria are also frequently associated with the mucosal layer of the intestine, contributing to its integrity (Di Vincenzo et al., 2024). In addition, a high-fibre diet increases the relative abundance of *Akkermansia* (Ramos Meyers et al., 2022; Vinelli et al., 2022; Zhang, Hu, et al., 2022). *A. muciniphila* degrades mucin and produces propionate and acetate. It has also been observed that although *A. muciniphila* degrades mucus, it increases the expression of the Muc2 gene. This enhances mucus production, potentially improving the mucin layer, its bacterial community, and the intestinal wall, thereby positively influencing gut-associated lymphoid tissue and supporting the maintenance of homeostasis (Ramos Meyers et al., 2022). Furthermore, in the absence of dietary fibre, *A. muciniphila* significantly reduces mucus levels in the gut, decreasing its relative abundance and increasing intestinal inflammation (Zhang, Hu, et al., 2022). Researchers focusing on the consumption of unprocessed fibre sources noted an increase in *Lachnospiraceae* count (Yao et al., 2022), which was documented in our study as well. Bacteria from this family are commensal microorganisms involved in the synthesis of SCFAs, mainly propionate and acetate. However, in pathological conditions they are associated with obesity, diabetes, inflammatory bowel disease and other disorders (Vacca et al., 2020). Various dietary fibre fractions such as AX, AXOS, β-glucans, xylooligosaccharides, cellulose, and lignin in unprocessed (high-fibre) foods have broad effects on the microorganisms that establish the gut microbiota (So et al., 2018; Vacca et al., 2020). However, the literature often highlights the deep incorporation of the prebiotic fraction of dietary fibre into chemical structures with other molecules that limits the bioavailability of these molecules for microbes (Makki et al., 2018). Consequently, fibre-rich foods stimulate a slower increase in *Bifidobacterium* and LAB than isolated prebiotic fractions. On the other hand, these foods stimulate cellulose-degrading bacteria such as, *Lachnospiraceae* and others that affect the mucosal layer, and produce metabolites that have beneficial effects on the organism (Makki et al., 2018). However, the time required to observe these effects is typically seen with long-term habitual consumption of fibre-rich foods (Makki et al., 2018; Zhang, Fan, et al., 2022).

Literature data on SCFA and BCFA concerning SBG use suggest an increasing content of both fatty acids groups. However, these results refer to batch fermentation systems without pH regulation (Bonifácio-Lopes et al., 2022; Calvete-Torre et al., 2023). In contrast, where pH regulation was applied to batch fermentation, the results were similar to those presented in our SHIME® experiment. They showed no effect on SCFA and reduction in isovalerate concentration (Lynch et al., 2021). On the other hand, studies investigating isolated AX or AXOS fractions observed an increase in SCFA concentration and a decrease in specific BCFA concentrations in dynamic models, animal studies or human studies (Nguyen et al., 2020; So et al., 2018). However, the differences in chemical composition between the pure AX and AXOS and the SBG are too extensive to extrapolate these results to the SBG. A systematic review by Vinelli et al. (2022) found no significant effect of dietary fibre from different unprocessed food sources on changes in SCFA *in vivo*. Numerous studies suggest that the gut microbiota community composition is modulated without affecting SCFA levels (O. Chen et al., 2021; Müller et al., 2020; Vinelli et al., 2022; Wilms et al., 2021). This phenomenon is primarily attributed to the interdependencies between gut microbiota microorganisms and their metabolites, including SCFA and BCFA. It means that healthy gut microbiota's metabolites are constantly used and produced by microbes in this community, ensuring homeostasis (Peterson et al., 2022; Vinelli et al., 2022). In contrast, a greater response in fatty acid synthesis was observed in studies using selective dietary fibre fractions that allowed high levels of SCFA production by targeted groups of microorganisms (Vinelli et al., 2022). The key factors that modulate the level of SCFA production seem to be fibre dose, type, degree of processing and chemical structure (Yao et al., 2022). The effect of dietary fibre on BCFA production varies, but most studies suggest that high-fibre diets reduce BCFA levels (Vinelli et al., 2022). Thus, our study results are consistent with the others, given the reduction in BCFA content during the SBG intervention period and the stable SCFA levels in SHIME®. An important finding in this study is the return of the BCFA content to the baseline level after the washout period in SHIME®. This suggests a direct modulatory effect of SBG. Only the synthesis of propionic acid increased after the washout period (L-SH 16d) probably due to the increase in abundance of the *Lachnospiraceae* family and synthesis through the acrylate pathway by *Akkermansia* (El Hage et al., 2019; Kirmiz et al., 2020; Zaplana et al., 2024). However, metabolic pathways for propionate production involve many taxa of microorganisms and pathways of this fatty acid need to be elucidated.

Regarding the correlation of microbial taxa and fatty acids (Fig. 4A, B). In most studies, *Akkermansia*, *Bacteroides*, *Bifidobacterium*, *Prevotella*, *Ruminococcus*, *Blautia*, *Clostridium*, *Streptococcus*, *Phascolarctobacterium*, *Dialister* and *Veillonella* are closely correlated with acetate synthesis. Bacteria associated with propionate synthesis include *Bacteroides*, *Coprococcus*, *Megasphaera*, *Roseburia*, *Ruminococcus*, *Akkermansia*, *Veillonella* and *Propionibacterium*. For butyrate, the key bacteria are *Bacteroides*, *Anaerostipes*, *Coprococcus*, *Eubacterium*, *Faecalibacterium*, *Roseburia*, *Ruminococcus* and *Lachnospiraceae*. The bacterial groups associated with BCFA synthesis mainly include *Bacteroides*, *Prevotella*, *Megasphaera*, *Escherichia-Shigella* and *Clostridium* (Lange et al., 2023; Morrison & Preston, 2016; Ramos Meyers et al., 2022; Rios-Covian et al., 2020; Salazar et al., 2022). In the case of batch fermentation, the correlations did not broadly align with the literature data. This was due to the high variability in the batch fermentation, which resulted in a loss of alpha-biodiversity in this model. However, the SHIME® results were more consistent with the *in vivo* studies. Essentially, data linked with SCFA and BCFA to specific taxonomic units in the human gut microbiota most often come from animals and human trials that are not free from variability and heterogeneity in microbiota due to differences among populations influenced by lifestyle, diet, diseases, geographic region, development level, and other factors (Hou et al., 2022).

4.4. Difference between in vitro systems

Many differences have been observed between used *in vitro* systems. Other authors have highlighted differences between batch fermentation and colon models such as SHIME®, TIM-2 or less commonly used models. The most common differences indicate lower stability, inability to stabilise microbiota, short-term responses, and the influence of environmental conditions that degrade microbiota biodiversity in batch

fermentation (Isenring et al., 2023; Roupar et al., 2021). In the research presented, a decrease in alpha biodiversity was also observed in batch fermentation. This was most likely due to changes resulting from the characteristics of batch fermentation and exposure of the gut microbiota to altered environmental conditions during the laboratory procedures. This occurred despite the highest possible timed and environmentally rigorous procedures used. These procedures included sampling, transfer to anaerobic conditions and temperature changes (Isenring et al., 2023). In addition, the movement and periodic refreshing of the bacterial feed media that occurs in SHMIE® is not practised in batch fermentation. This leads not only to pH changes in the latter but also to changes in substrate availability for fermentation and metabolic products of the microbiota between these two systems fermentation (Isenring et al., 2023; Roupar et al., 2021). Hence, the lack of similarity was shown in the PCoA analysis (Fig. 4C). The differences between the *in vitro* models have practical implications for the prebiotic properties analysis. Thus, a higher degree of stability was achieved in SHIME®, and these results can be to a higher degree discussed and extrapolated to animal and human studies.

5. Conclusion

The study demonstrated the prebiotic potential of SBG. Substances with prebiotic properties were identified in SBG, including dietary fibre fractions and polyphenols. SBG was also shown to contain digestible nutrients - sugars and protein. The results indicated that SBG is a suitable growth medium for probiotic bacteria. In addition, SBG has a high *Ipreb* and *Apreb* and modulated intestinal microbiota. However, changes in the microbiological community were dependent on the *in vitro* system used. In the SHIME®, SBG showed a moderate bifidogenic effect, stimulating the growth of *Akkermansia*, LAB and reducing the relative abundance of *Bacteroides*, *Clostridium* and *Escherichia-Shigella*. Stabilisation of the SCFA content and reducing the BCFA content were demonstrated as well in SHIME®. However, the effect of BCFA decrease was not maintained after the end of SBG supplementation. A different gut microbiota response was observed in batch fermentation with no *Bifidobacterium* or LAB stimulation effect. Batch fermentation resulted in increased SCFA and BCFA synthesis. The observed differences between the *in vitro* systems indicate the greater utility of SHIME® for research on the prebiotic properties of fibre-rich food ingredients due to similarities with fibre-microbiota interactions reported *in vivo*.

This study provided new insights into the properties of SBG and its effects on the gut microbiota, SCFA and BCFA. It is also the first study that comprehensively describes the aspects of the chemical composition, fermentation capabilities and gut microbiota response under the influence of SBG in different *in vitro* systems. In addition, the same initial composition of the gut microbiota in both *in vitro* models allowed a direct comparison of the systems used and the identification of specific differences between them. This study had several limitations that should be addressed in the future. Only one SBG from one brewery was tested. The short duration of SBG supplementation in the SHMIE can be extended to observe changes in microbiota, SCFA and BCFA over time. Nevertheless, the results indicate that SBG is a promising by-product that could be used to create valuable dietary supplements and functional foods with prebiotic properties. Future standardisation of SBG will be required to achieve greater analysis repeatability and a consistent chemical composition.

The research provides a substantial basis for further analysis into the prebiotic potential of SBG in more complex *in vitro* models, *e.g.* cell lines, and further *in vivo* in animals and humans. This study demonstrated moderate prebiotic effects of SBG in *in vitro* models. Moreover, future studies on the prebiotic activity of SBG should prioritize evaluating its impact on the microbiome through long-term supplementation and *in vivo* models. Additionally, conducting cohort population studies could provide insights into the microbiota response within intervention groups and reveal potential health benefits associated with SBG consumption.

Ethical statement

Before the study, consent was obtained from the Research Ethics Committee (decision KE-U/12/2022).

Declaration of generative AI in scientific writing

The authors declare no use of AI technology.

CRediT authorship contribution statement

Marcin Kruk: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Piotr Lalowski:** Writing – review & editing, Formal analysis. **Magdalena Płecha:** Writing – review & editing, Methodology, Data curation. **Alicja Ponder:** Writing – review & editing, Methodology. **Agnieszka Rudzka:** Writing – review & editing, Resources. **Dorota Zielińska:** Writing – review & editing, Supervision. **Monika Trząskowska:** 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.

Data availability

The data are available in an open repository at the following link: https://doi.org/10.18150/EI2LQX

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141254.

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