



Optimization of an EPS-enriched gluten-free sorghum bread with improved technological properties and eubiotic potential: evidence from an *in vitro* colonic model

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ARTICLE INFO

Keywords:

Fermented sorghum
Exopolysaccharides
Gluten-free bread
Gut microbiota
MICODE
Prebiotic activity

ABSTRACT

The growing demand for healthy and appealing gluten-free products has prompted research into alternative cereals like sorghum and targeted fermentation strategies. Hence, this study aimed at developing a gluten-free sorghum sourdough bread enriched in exopolysaccharides (EPS) and evaluating its technological, nutritional, and eubiotic potential using the MICODE *in vitro* colon model. Sorghum doughs were fermented with four selected lactic acid bacteria strains and *in situ* EPS production was optimized, with *Leuconostoc pseudosenteroides* DSM20193 showing the highest yield (3.29 g/100 g). The resulting sourdough was incorporated into gluten-free corn-based bread formulations and compared with non-fermented control breads. EPS-enriched bread exhibited improved technological properties, including increased specific volume (up to 78%), reduced hardness (almost 50%), and enhanced sensory acceptability, moreover, fortification with fermented sorghum also determined a reduced starch hydrolysis index compared to corn breads. *In vitro* colonic fermentation demonstrated that EPS-enriched bread upheld microbiota eubiosis while promoting beneficial bacteria groups such as *Bifidobacteriaceae*, *Lactobacillales*, and second-generation probiotic like *Faecalibacterium prausnitzii*, *ibidem* reducing opportunistic groups including *Clostridium* cluster I and *Enterobacteriaceae*. Ecological indicators confirmed enhanced prebiotic activity and eubiotic effects compared to control breads without sorghum or with sorghum flour. Overall, the combination of sorghum and EPS-producing strains represents a promising strategy to develop functional gluten-free products with improved health-related properties.

1. Introduction

The increasing demand for gluten-free products has led to several research into alternative cereals that could replace wheat while maintaining acceptable technological, nutritional, and sensory properties (Dan et al., 2025). Among these, sorghum has become a promising alternative crop given its ability to thrive under harsh conditions and dry climates, thus requiring fewer inputs compared to other cereals. Hence, sorghum could become an important and sustainable food source, particularly in the context of climate change and food security (Fernando et al., 2025).

Besides its agronomic potential, sorghum is also rich in dietary fiber, proteins, and several bioactive compounds, including phenolic acids and flavonoids. These components contribute to its antioxidant, anti-inflammatory, and potential metabolic health benefits (Birhanu, 2021; Fernando et al., 2025). Recent studies have also highlighted sorghum prebiotic potential, demonstrating that its pericarp can selectively stimulate beneficial genera in the gut including *Bifidobacterium* and species belonging to the former *Lactobacillus* genus, thereby promoting gut microbial diversity and health (de Sousa et al., 2019; Fernando et al., 2025; Yang et al., 2022).

Despite these advantages, the large-scale use of sorghum in bakery

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<https://doi.org/10.1016/j.foodres.2026.120554>

Received 21 May 2026; Received in revised form 5 August 2026; Accepted 24 August 2026

Available online 26 August 2026

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products, especially gluten-free bread, remains limited. Gluten-free doughs require the addition of hydrocolloids, starches, or proteins to provide adequate structures. In this context, sourdough-inspired fermentation could lead to improvements in dough rheological and functional features through the *in situ* production of exopolysaccharides (EPS) (Dan et al., 2025). EPS act as hydrocolloids, enhancing dough viscosity and stability, contributing to improved loaf volume and may also exert prebiotic effects, further supporting their inclusion in functional food with potential health-promoting effects (Liu et al., 2025). These modifications could be particularly relevant in gluten-free products which, being lower in fiber and protein, can be nutritionally inferior to their gluten-containing counterparts and have higher glycemic index (Dan et al., 2025). Additionally, sourdough fermentation promotes the degradation of complex proteins, starch matrices and other food constituents. These biochemical transformations can enhance mineral bioaccessibility, promote the release of bioactive peptides, and improve protein digestibility, thereby increasing the overall potential bioavailability of nutrients for human absorption (Saa et al., 2018).

Moreover, in recent years, there has been growing interest in understanding the interaction between functional foods and the gut microbiota, given its central role in human health. However, while the nutritional and technological benefits of sorghum and sourdough fermentation are increasingly documented (de Sousa et al., 2019; Fernando et al., 2025; Yang et al., 2022), there is still a lack of comprehensive studies evaluating the impact of such products on gut microbiota using advanced *in vitro* models. In this context, gastrointestinal simulation systems such as the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) or the Multi-Unit *In vitro* Colon Model (MICODE), have been widely applied to investigate the modulatory effects on gut microbial composition and metabolic activity of fermented foods (Koirala et al., 2022; Mastrolonardo et al., 2024; Nissen et al., 2021).

Hence, assessing the eubiotic potential of a gluten-free sorghum sourdough bread enriched in EPS could pave the way for new innovative and healthy gluten free food. Moreover, while EPS and sorghum independently exhibit promising prebiotic properties, their combined effect within a complex food matrix remains unexplored. For this reason, the present study aimed at developing a gluten-free sorghum sourdough bread enriched in EPS and evaluating its impact on gut microbiota using an *in vitro* colonic model. New Approach Methodologies (NAMs) are increasingly adopted in food science to assess the functional and health-related properties of novel foods through human-relevant, mechanistic, and ethical approaches. In particular, advanced *in vitro* gut models enable the investigation of food-microbiota interactions without reliance on animal experimentation or invasive human sampling. The application of systems such as the MICODE *in vitro* colon model aligns with the strategic vision of EFSA (European Food Security Authority) and other regulatory bodies, which actively promote the integration of NAMs and gut microbiome data into next-generation food safety and nutritional risk assessment frameworks. Recent EFSA roadmaps explicitly recognize *in vitro* gastrointestinal models as key tools to support evidence-based evaluation of diet-microbiome interactions and their implications for human health (Moreno et al., 2024).

Within this context, while sorghum and EPS independently show promising prebiotic properties, their combined effect within a complex food matrix remains unexplored. To address this gap, this study aimed to develop an innovative, EPS-enriched gluten-free sorghum sourdough bread and using NAMs to evaluate its impact on the gut microbiota.

2. Materials and methods

2.1. Raw materials and microorganisms

For this study, organic white sorghum (*Sorghum bicolor* L.) flour (EcorNaturaSi SpA, Treviso, Italia) having the following proximal composition: carbohydrates, 66 g/100 g; fiber, 7.5 g/100 g; protein, 9.3

g/100 g; fat, 3.2 g/100 g; ashes, 2.1 g/100 g and corn flour (IPAFOOD Srl, Avellino, Italia) having the following composition: carbohydrates, 79.1 g/100 g; fiber, 2.5 g/100 g; protein, 6.7 g/100 g; fat, 1.2 g/100 g; ashes, 1.6 g/100 g, were used.

Commercial fresh baker's yeast (Lesaffre Italia S.p.A., Parma, Italia) was used as bread leavening agent, whereas a xanthan gum powder (Food AR s.r.l., Limbiate, Italia) was used as structuring agent.

Four lactic acid bacteria strains, including *Leuconostoc pseudomesenteroides* DSM20193, previously used for the fermentation of cereals and legumes (Galli et al., 2021; Koirala et al., 2022; Xu et al., 2017), and *Weissella paramesenteroides* A17, *Weissella cibaria* T114 and T118, isolated from sorghum (Verni et al., 2024) and belonging to the Culture Collection of the Food Microbiology Laboratory (Sapienza, University of Rome, Italy), were selected for the ability to synthesize EPS. The strains were cultivated in MRS (De man, Rogosa, Sharp) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) at 30 °C for 24 h. Cells were harvested by centrifugation (MicroCL-21R, Thermo Scientific) at 10,000g for 10 min (4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), resuspended in tap water before the inoculum.

2.2. Sorghum fermentation

2.2.1. Set-up and optimization of EPS *in situ* synthesis

Aiming at selecting the optimal fermentation conditions for *in situ* EPS synthesis, several sorghum sourdoughs were prepared modifying time and temperature of fermentation as well as percentage of sucrose added. More specifically, sorghum flour and water were mixed at a final dough yield (DY) [dough weight × 100/flour weight] of 300, corresponding to a flour:water ratio of 33:67. *Leuc. pseudomesenteroides* DSM20193, *W. paramesenteroides* A17, *W. cibaria* T114 and T118 were singly inoculated in sorghum doughs at the final cell density of 6.5 log cfu/g. Doughs were fermented at 25 or 30 °C for 16 or 24 h, whereas sucrose was added to the dough, prior to fermentation, at 5 or 10% w/w, to promote EPS synthesis.

Doughs pH was monitored with a FiveEasy Plus pH meter (Mettler-Toledo, Columbus, OH, USA), whereas the viscosity was measured by using a rotational viscometer Lch. NDJ-8S (Lachoi Scientific Instrument Co., Shaoxing, China). The viscosity was determined on 200 g samples, placed in cylindrical containers having 7 cm diameter and 12.5 cm height, at 25 °C, by using the speed/rotor combinations suggested by the viscometer provider, to fall within the correct calibration ranges of the instrument.

2.2.2. Characterization of sorghum doughs

The conditions that better promoted EPS synthesis, measured indirectly through the viscosity change, were further characterized by determining LAB microbial growth, sugars and organic acids concentration, as well as EPS content and confronted with controls prepared without sucrose.

In detail, for the microbiological analysis, 10 g of each sample were diluted with 90 mL of physiological solution (0.9%), homogenized with a stomacher and subjected to serial decimal dilutions. LAB were determined on MRS agar (Oxoid) at 30 °C for 48 h, under anaerobiosis.

The analysis of organic acids and sugar was carried out on water/salt-soluble extracts obtained as described by (Weiss et al., 1993). Lactic acid and acetic acids were quantified using the Megazyme K-DLATE and KACETRM kits (Megazyme International, Wicklow, Ireland), respectively. The analysis of glucose, fructose and sucrose was carried out by using the Megazyme K-FRUGL and K-MASUG kits (Megazyme International) following the manufacturer's instructions.

EPS were isolated and quantified on 5 g of samples using the gravimetric method reported by Ketabi et al. (2008), which consisted in three phases: i) protein precipitation with trichloroacetic acid (20%) ii) EPS precipitation with ethanol and recovery by centrifugation, and iii) EPS weighing after freeze-drying in a Lyovapor™ L-210 (Buchi Ltd., Newmarket, Suffolk, UK).

2.3. Bread production and characterization

2.3.1. Optimization of bread formulation and bread making

Before bread making, the dough formulation was optimized modifying sorghum and xanthan percentage. Nine corn-based doughs containing 1.5, 3 or 4.5% w/w xanthan and 5, 15 or 25% w/w sorghum were produced. Doughs had DY 230 corresponding to a flour/water ratio of 43.5/56.5% (w/w). Baker yeast (1.5% on dough weight) was added to all doughs. Aiming at selecting the best formulation, doughs leavening performance of the different samples, was evaluated determining the volume increase (ΔV , mL) and was expressed as the percentage of volume increase. The formulation with 4.5% xanthan and 25% sorghum was then selected for the baking trials.

Five experimental breads manufactured from doughs with DY 230 (Table S1), as described above, were produced as follows: control bread made using only corn flour; bread made using corn (75%) and sorghum (25%) flours; three more breads obtained with the addition sorghum sourdoughs fermented (at 25 °C for 16 h with 10% sucrose) with *Leuc. pseudomesenteroides* DSM20193, *W. cibaria* T114 and T118. Doughs were mixed in a mixer bowl (Electrolux assistant, EKM4000) for 5 min, divided into 300 g pieces, shaped, and rested in baking trays for 2 h at 30 °C. Loaves were baked in a ventilated oven (VEVOR EB-4D, Vevor, Taicang, China) at 220 °C for 25 min. After baking, breads were cooled for 2 h at room temperature. Baking was done on two different days (two independent baking trials) and three loaves were prepared for each type of experimental bread. Each sample was analyzed twice.

2.3.2. Biochemical and nutritional characterization

Acidification and proteolysis in bread doughs were determined by measuring the pH as described above whereas total titratable acidity (TTA) was determined by titration and defined as the amount of 0.1 M NaOH required to adjust the end pH of 10 g dough in sterile water to 8.3. Residual sugars (determine glucose, fructose, and sucrose) content in breads was estimated by using the Megazyme K-SUFRG kit (Megazyme International) following the manufacturer's instructions. Total free amino acids (TFAA) were measured using the cadmium-ninhydrin assay as reported by Verni et al., (2024).

Breads starch hydrolysis index (HI) was determined by mimicking the *in vivo* digestion of starch (De Angelis et al., 2009). In detail, bread portions, containing 1 g of starch, were treated with amyloglucosidase, and the glucose assay kit (GOPOD-format, Megazyme) was used to quantify the released glucose, following the manufacturer's instructions. The degree of starch digestion was expressed as the percentage of hydrolyzed starch after 180 min. Control wheat bread was used as a reference to estimate the hydrolysis index (HI = 100). To calculate the predicted glycemic index (pGI), the equation proposed by Capriles and Arêas (2013), was used:

$$pGI = 0.459 \times HI + 39.7$$

2.3.3. Technological characterization and sensory analysis

The specific volume of the breads was measured using the rapeseed displacement method (AACC, 2000), whereas the chromaticity coordinates of the breads were obtained by a CS-10 colorimeter (CHN Spec Technology, Hangzhou, China) and reported as color difference, ΔE^*ab , calculated by the following equation:

$$\Delta E^*ab = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

where ΔL , Δa and Δb are the differences for L , a and b values between sample and reference (a white ceramic plate having $L = 92.2$, $a = 0.15$, and $b = 0.85$).

Texture profile analysis was performed by using a Texture Analyzer FRTS- 100 N (Imada, Toyohashi, Japan) equipped with a 3 cm cylinder probe FR-HA-30 J on 200 g loaves. The instrument settings were test speed 1 mm/s, 30% deformation of the sample, and two compression

cycles.

Sensory analysis of the breads was carried out by 15 trained panelists (6 men and 9 women; average age: 34 years; range: 23–50 years) with demonstrated abilities and prior expertise in the sensory analysis of leavened baked goods. A two-hour training session was performed, and the assessors evaluated the descriptors to be included in the sessions. Sensory attributes were scored on a scale from 0 to 10, with 10 being the highest score. Sensory evaluations were carried out following the independent method of the “Sensory analysis – Methodology - Flavour Profile” methods (ISO 6564-1985) with some modifications. In detail, the library of the Environmental Biology Department of the Sapienza University of Rome (Italy) was used instead of cabinets as previously proposed by Elia (2011). Enrolled panelists, who did not suffer from any food intolerances or allergies, received information on the objectives of this study and provided written informed consent. Three separate sessions were conducted, and beverages were served in a randomized order and encoded with three-digit random numbers. A glass of water was drunk by the panelists among samples.

2.4. Study of the eubiotic features of bread

2.4.1. Human colon microbiota and *in vitro* gut model

Aiming at studying the eubiotic potential of the sorghum sourdough bread, CtB, SB and fSB-DSM were selected for an *in vitro* gut model. Human gut microbiota (GM) was sourced from fecal samples of healthy adult individuals. The sample collection followed established protocols from previous studies (Connolly et al., 2012; Diotallevi et al., 2021; Nissen et al., 2021; Marangelo et al., 2025; Gianotti et al., 2025). Specifically, samples were collected on two occasions from three different participants, aged between 30 and 45 years, who had not received antibiotic treatment for at least 3 months and had not deliberately consumed prebiotics or probiotics prior to the experiment. The participants were fully informed about the purpose and procedures of the study and verbally consented to the use of their fecal samples, in line with the Bioethical Committee of the University of Bologna. Fecal samples (Bristol stool types 2–5) were provided during the experiment. For sample collection, donors were supplied with a collection kit, which included a stool collector (Sarstedt AG & Co. KG, Nümbrecht, Germany) and an anaerobic jar containing an O₂-consuming catalyst (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). The samples were promptly transported to the laboratory and processed within 2 h. A 10% (w/v) GM suspension was prepared immediately before the experiment by homogenizing 2 g of each donation in 54 mL of pre-reduced phosphate-buffered saline (PBS) (Addazii et al., 2026; Nissen et al., 2023), followed by two washes of the pellet in PBS (16,000g for 5 min) (Gianotti et al., 2025).

Prior to the *in vitro* colonic fermentation, bread samples were subjected to sequential enzymatic treatment carried out according to the consensus method developed within a large European framework (COST Action InfoGest). The digestion procedure consisted of three consecutive phases, and all the gastrointestinal fluids were prepared according to the INFOGEST protocol (Brodtkorb et al., 2019). Briefly, 2 g of homogenized bread were added to 2 mL of simulated salivary fluid (containing 150 U/mL of porcine α -amylase) to create the oral step of the digestion. The bolus was incubated for 5 min at 37 °C and then we added 4 mL of simulated gastric fluid (containing 2000 U/mL of pepsin), reproducing the gastric phase. After 120 min at 37 °C, 8 mL of pancreatic (containing pancreatin, 200 U/mL based on trypsin activity) and bile fluids were added. The chyme was incubated for a further 120 min at 37 °C. The digestates were gently centrifuged (4 min at 6000 $\times g$) to separate the denser portion, which represents the insoluble portion of emulsion that is used for *in vitro* fermentation (Nissen et al., 2023). Short-term batch proximal colon fermentations were conducted for 48 h in independent vessels using an *in vitro* colon model, MICODE (Nissen et al., 2024; Gianotti et al., 2025). The preparation of the experiments was made according to published procedures (Connolly et al., 2012; Wang et al.,

2020) and described in detail in Nissen et al. (2023). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (BM) (Connolly et al., 2012; Diotallevi et al., 2021) and left running to reach and maintain the proximal colon ecological conditions (0.0% of DO₂ and pH 5.75). In each bioreactor anaerobic conditions (0.0–0.1% w/v of DO₂), temperature at 37 °C, agitation at 300 rpm, and pH at 5.75 were constantly kept and monitored over the experiment with a dedicated software Lucullus 3.1 (Getinge AB, Goteburg, Sweden). Once the exact environmental settings were reached, the four vessels were aseptically injected with 9 mL of GM suspension and 1 mL of the denser part of digestates of sorghum breads samples (to a final concentration of 1%, w/v), while an additional vessel was set as blank control (BC, basal medium and 1% GM only). Batch cultures were run under these controlled conditions for a period of 49.22 h during which samples were collected at 3 time points (Baseline, 24 and 48 h). The baseline (BL) was defined on the first pH changes detected by Lucullus (1 read/10 s) and was set after 1.22 ± 0.12 h. Sampling was performed with a dedicated double syringe filtered system (Getinge AB, Sweden) connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a close control, monitoring and recording of fermentation parameters the software Lucullus 3.1 (Getinge AB, Sweden) was used. Fermentations were conducted in duplicate independent experiments, using for each a new pool of feces from the same three healthy donors.

2.4.2. Extraction and analysis of colonic metabolites from colonic effluates of GM

The analysis of volatile organic compounds (VOCs) from effluates of colonic fermentation was conducted using an Intuvo 9000 GC System (Agilent Technologies, Santa Clara, CA, USA) coupled with an 5977B GC/MSD System mass spectrometer operating in electron impact mode (ionization voltage of 70 eV) (Agilent Technologies, USA). The system was equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, NL). The preconditioning, absorption, and desorption steps for SPME-GC-MS analysis, the Solid Phase Micro-Extraction (SPME) GC-MS procedure along with data processing and the identification of compounds were conducted as previously reported with minor adjustments (Nissen et al., 2021; Nissen et al., 2023; Gianotti et al., 2025). In short, 2 mL of MICODE effluates were placed in 10-mL glass vials and supplemented with 4 µL of an internal standard mixture (acetic acid, 4-methyl-2-pentanol, and phenol at 10000 ppm) to achieve a final concentration of 5 mg/L. Samples were equilibrated for 10 min at 45 °C. A carboxen-polydimethylsiloxane SPME fiber (85 µm) was then exposed to the sample for 40 min. Studies (Nissen et al., 2020, 2021). The identification of compounds was carried out by matching mass spectra against available databases (NIST 11 MSMS library and NIST MS Search program 2.0, NIST, Gaithersburg, MD, USA). Each VOC was quantified relatively in percentage terms (LOD = 0.001 mg/kg). Additionally, the primary microbial metabolites linked to prebiotic activity in BL and EP samples were quantitatively measured in mg/kg (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2024), and results were presented as changes. All data were expressed as normalized mean values derived from duplicates in two independent experiments.

2.4.3. Extraction and enumeration of GM populations by real time qPCR

GM DNA was extracted from the MICODE effluates at each time points (BL, T1, and EP) using the Nucleo Spin DNA stool Kit (Macherey Nagel, Darmstadt, Germany). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK) and samples of high purity were chosen. DNA samples from the BL and other time points were used for quantitative PCR (qPCR) analyses. To highlight changes in the microbiota after fermentation, enumeration of bacterial groups was made by qPCR and SYBR Green I chemistry, following previous protocols (Nissen et al., 2021; Nissen et al., 2024). Specifically, some bacterial groups were selected as generally accepted indicators of eubiosis

or dysbiosis of colon microbiota, and their perturbations were considered closely correlated (directly or inversely) to the prebiotic potential of foods. Fourteen different bacterial taxa (Table S2) were assessed by qPCR on a QuantStudio5® System with SYBR Green I chemistry (Applied Biosystem, Thermo Fisher, USA). Gene standards were prepared from the relevant PCR run amplicons of the target bacterial species using the GeneJet amplicon purification kit (Thermo Fischer Scientific, USA) as previously described (Nissen et al., 2021; Nissen et al., 2024). For each of the target microorganisms, qPCR reactions were set up as follows: a holding step at 95 °C or 96 °C for 30–60 s and a cycling step at 95 °C for 6 s and 60 °C for 25–50 s, repeated 40 times, followed by melting curve analysis to define the reaction specificity. Quantifications were performed separately with a five-point standard of the given amplicon. Reactions were prepared with 1 ng DNA, 2× Power up SYBR Green (Thermo Fisher Scientific, USA) and 250 nM of each primer (Eurofins Genomics, Ebersberg, Germany). All results were expressed as mean values of sextuplicate.

2.5. Statistical analysis

Data from sorghum sourdoughs and bread biochemical and technological characterization were subjected to one-way ANOVA; pair-comparison of treatment means was obtained by Tukey's procedure at $P < 0.05$, using the statistical software Statistica 12.5 (StatSoft Inc., Tulsa, USA).

The microbiomic results were obtained from qPCR quantifications (Table S2) and were expressed as shifts, calculated as $\log_2(F/C)$ and processed with MANOVA, using “time” and “food matrix” as categorical predictors, followed by a Duncan *post hoc* test ($P < 0.05$) (Table S3) (Casciano et al., 2026; Nissen et al., 2021). Graphics were produced with BoxplotR (Spitzer et al., 2014). Targeted metabolomic results were obtained from SPME-GC/MS values that were calculated by comparison with a known quantity of chemical standard and processed with MANOVA, using “time” and “food matrix” as categorical predictors, followed by a *post hoc* Tukey HSD test ($P < 0.05$). Baseline values of this quantification is reported in Table S4. Untargeted metabolomic results were obtained from SPME-GC/MS values normalized with the mean centering method and statistically computed with MANOVA using “time” and “food matrix” as categorical predictors and processed with Principal Component Analysis (PCA) ($P < 0.05$) (Table S5) (Nissen et al., 2020). Additionally, Ven Diagrams and Pairwise Intersection map, supported by Table S6, were produced with multiple comparator tool (<https://mobiertools.com/listcompare.php> last accessed 09/04/2026). Heatmap of the volatilome was produced with Heatmapper (Babicki et al., 2016).

Datasets mining and multivariate statistic for omics were conducted as previously presented (Granato et al., 2014; Nissen et al., 2021), also using the statistical software Statistica 8 (StatSoft Inc., USA).

3. Results and discussion

3.1. Optimization of sorghum fermentation conditions

Aiming at *in situ* synthesizing EPS, four lactic acid bacteria strains were selected as starters for sorghum fermentation. Sucrose was added to the dough since it is one of the main sugars used as substrate for EPS synthesis (Guérin et al., 2020). In a preliminary phase, sorghum doughs were fermented with *Leuc. pseudomesenteroides* DSM20193, *W. cibaria* T114 and T118, and *W. paramesenteroides* AI7 at 25 and 30 °C for 24 h and previously used as EPS-producing starters during cereals fermentation (Galli et al., 2021; Koirala et al., 2022; Verni et al., 2024; Xu et al., 2017). During incubation, the pH decreased significantly, from 6.28 to approximately 3.5 for doughs fermented with *W. cibaria* and *W. paramesenteroides* strains, whereas higher pH values (4.15 ± 0.02) were observed for doughs fermented with *Leuc. pseudomesenteroides* DSM20193.

However, the viscosity increase was found to be minimal or nearly

absent ($P > 0.05$), with the highest value obtained for *Leuc. pseudomesenteroides* DSM20193 and when fermentations were carried out at 25 °C rather than 30 °C (Table 1).

Since the pH reached was extremely acidic, and acidity is in fact one of the main process parameters to consider when the goal is to synthesize EPS, as it adversely affects its production (Wongsuphachat & Maneerat, 2010), the optimization process included reducing fermentation time from 24 to 16 h. This modification led to higher pH values at the end of fermentation, reaching 3.8 for doughs fermented with *W. paramesenteroides* AI7, and slightly above 4 for those fermented with the rest of the doughs. This modification significantly affected dough viscosity, with *Leuc. pseudomesenteroides* DSM20193, showing the greatest increases, exceeding 10 Pa·s (Table 1).

Aimed at increasing EPS synthesis, sucrose concentration was also raised from 5% to 10% resulting in a considerable increase in viscosity ($P < 0.05$), especially for doughs fermented with *W. cibaria* T114 and T118, compared to the respective doughs containing 5% sucrose.

3.2. Biochemical characterization of fermented sorghum

Based on the results obtained during the optimization process, sorghum doughs fermented at 25 °C for 16 h, with 10% sucrose, were selected for further characterization. Specifically, starters growth and acidification were monitored, comparing them with doughs fermented with the same strains but without sucrose addition (EPS-). Despite the relatively short fermentation times, cell density increased more than 2 log cycles, reaching 9 log cfu/g, without showing significant differences ($P > 0.05$) between EPS+ (sucrose containing) and EPS- doughs, which did not contain sucrose.

The increase in cell density of the starters corresponded to an increase in the concentration of organic acids, main metabolites of carbohydrate metabolism (Fig. 1). Lactic acid, present only in trace amounts before fermentation, reached on average 16.6 mmol/kg in fermented doughs, whereas acetic acid, which was not detected before fermentation, ranged from 1.64 to 6.96 mmol/kg in fermented sorghum. The highest values for both lactic and acetic acid were observed in EPS-doughs (Fig. 1). In EPS+ doughs fermented with *Leuc. pseudomesenteroides* DSM20193, the sucrose added was almost completely consumed and used for exopolysaccharides synthesis, which reached 3.29 ± 0.39 g/100 g in sorghum dough fermented with *Leuc. pseudomesenteroides* DSM20193. When *Weissella* strains were used for fermentation, sucrose consumption ranged from 73 to 92% with doughs fermented with *W. cibaria* T118 and *W. paramesenteroides* AI7 showing the highest and lowest consumption, respectively (Fig. 1). These results are in line with EPS synthesis that reached 2.89 ± 0.12 , 2.54 ± 0.27 , and 1.19 ± 0.37 g/100 g for *W. cibaria* T118, T114, *W. paramesenteroides* AI7 fermented dough, respectively. The release of fructose in EPS+ doughs (Fig. 1) is also consistent with EPS synthesis since dextransucrase cleaves sucrose into fructose and polymerizes glucose into dextran (Kothari et al., 2015). Nevertheless, since *W. paramesenteroides* AI7 was the strain that

synthesized the lowest amount of EPS, thus determining the lowest viscosity increase (Table 1), it was excluded from the baking trials.

3.3. Optimization of bread formulation

In order to formulate gluten-free breads with an adequate nutritional composition and desirable technological properties, the bread recipe was optimized by modifying sorghum content, added at 5%, 15%, and 25% to corn flour. A corn-based gluten-free bread was selected as the reference formulation because it represents a conventional gluten-free bread matrix, allowing the independent evaluation of i) sorghum flour incorporation and ii) the additional effects of sourdough fermentation and *in situ* EPS production. Nevertheless, although the use of EPS has positive effects on the volume of wheat-based breads, these effects are not always as evident or significant in gluten-free products (Schwab et al., 2008; Wolter et al., 2014). Therefore, xanthan powder was also included in the formulation as a structuring agent, at different concentrations (1.5, 3, 4.5%). The selection of the optimal formulation was based on the increase in dough volume during proofing with baker's yeast (Table S7). Increasing xanthan concentration led to a clear rise in dough volume, with the highest value observed for the formulation containing 5% sorghum and 4.5% xanthan. However, since the goal was to also develop a nutritionally balanced bread, the formulation containing 25% sorghum and 4.5% xanthan was selected. This choice was made because using 25% sorghum allows for a substantial increase in fiber content, enabling the bread to be classified as a "source of fiber" according to current regulations (EC Reg. 1924/2006). Indeed, gluten-free products, unless enriched with protein isolates or fiber, are usually characterized by high carbohydrate content, resulting in a high glycemic index (Romao et al., 2021).

3.4. Bread characterization

3.4.1. Biochemical and nutritional characterization

Based on these results, five types of experimental breads were produced and characterized: a bread made exclusively with corn flour (CtB), one made with corn flour and 25% sorghum flour (SB), and three made with corn flour and fermented sorghum (25%) using *Leuc. pseudomesenteroides* DSM20193, *Weissella cibaria* T114, and T1-S8 (fSB-DSM, fSB-T118, and fSB-T114, respectively). The control doughs containing only corn flour showed pH and TTA values of 5.7 and 3.2 mL, respectively (Table 2). The addition of sorghum flour did not affect the pH but significantly increased titratable acidity, exceeding 6 mL. As expected, the addition of fermented sorghum further increased acidity, reaching pH and TTA values of 4.6 and 10 mL, respectively. The highest TTA values were observed in fSB-DSM, followed by fSB-T118 and fSB-T114. Since carbohydrate malabsorption, particularly that of fructose, may occur in patients with celiac disease due to mucosal inflammation, villous atrophy, and impaired nutrient absorption (Schnedl et al., 2021), the soluble sugar content of the breads was also determined. The concentrations of glucose, fructose, and sucrose differed significantly among bread formulations. CtB contained significantly higher ($P < 0.05$) glucose levels than the sorghum-based breads, whereas fSB-DSM, fSB-T118, and fSB-T114 exhibited higher fructose contents (Table 2). Although no specific dietary threshold for fructose has been established and symptom severity is generally dose-dependent (Wilder-Smith et al., 2013), fructose did not exceed 0.27 g/100 g. This low concentration suggests that residual fructose is unlikely to pose a concern for individuals consuming fSB-breads.

The addition of sorghum alone also led to a significant increase ($P < 0.05$) in free amino acids, from 197 to 244 mg/kg (Table 2). A further increase of approximately 18% compared to SB was observed in fSB-DSM. Lower free amino acid content was observed in fSB-T118 and fSB-T114 (210 and 160 mg/kg, respectively), contrary to a previous study (Verni et al., 2024), where *W. cibaria* T114 and T118 exhibited consistent proteolytic activity. This is likely due to the fermentation

Table 1

Viscosity, expressed as Pa·s, of sorghum doughs fermented with *Leuc. pseudomesenteroides* DSM20193 (DSM 20193), *W. cibaria* T114 (T114), *W. cibaria* T118 (T118), *W. paramesenteroides* AI7 (AI7) at 25 or 30 °C for 16 or 24 h, with 5 or 10% sucrose. Ct, unfermented dough.

	24 h – 25 °C Sucrose 5%	24 h – 30 °C Sucrose 5%	16 h – 25 °C Sucrose 5%	16 h – 25 °C Sucrose 10%
DSM20193	2.842 ± 73^a	2.276 ± 54^a	10.818 ± 401^a	11.635 ± 350^a
T118	1.927 ± 32^b	1.812 ± 62^b	4.215 ± 145^c	8.278 ± 354^b
T114	913 ± 57^c	1.294 ± 24^c	5.200 ± 156^b	7.122 ± 18^c
AI7	1.920 ± 29^b	1.887 ± 23^b	2.868 ± 202^d	2.952 ± 39^d
Ct	1.024 ± 21^c	1.091 ± 30^d	1.512 ± 203^e	1.075 ± 14^e

^{a-e}Values with different superscript letters in the same column mean significant differences ($P < 0.05$).

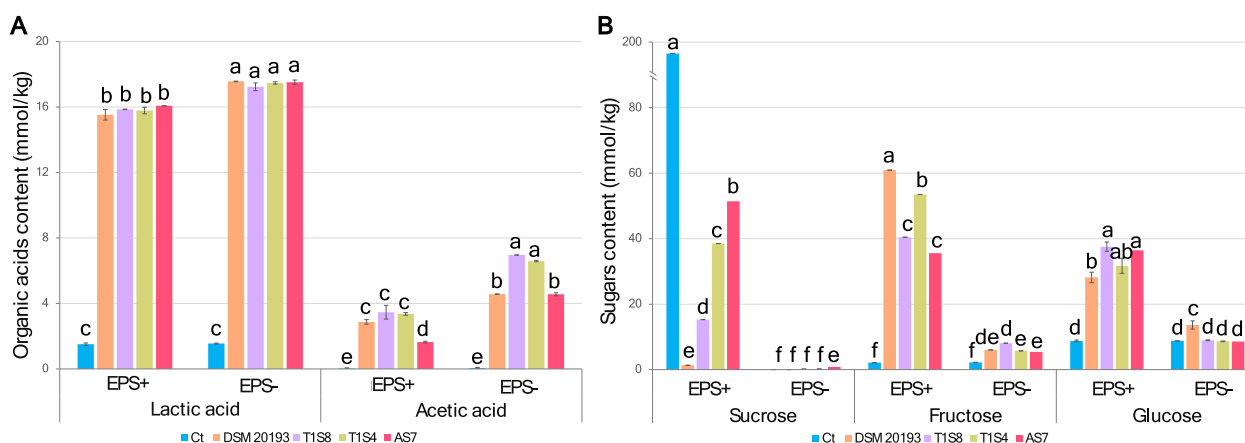


Fig. 1. Concentration (mmol/kg) in sorghum doughs of A) organic acids and B) sugars before (Ct) and after fermentation with *Leuc. pseudomesenteroides* DSM20193 (DSM 20193), *W. cibaria* T114 (T114), *W. cibaria* T118 (T118), *W. paramesenteroides* AI7 (AI7) at 25 °C for 16, with (EPS+) or without (EPS-) 10% sucrose. ^{a-f}Values with different superscript letters within the same parameter mean significant differences ($P < 0.05$).

Table 2

Biochemical and nutritional characterization of gluten-free breads. CtB, corn bread; SB, corn bread with raw sorghum; fSB-DSM, fSB-T118, fSB-T114, corn bread with sorghum fermented with *Leuc. pseudomesenteroides* DSM20193, *W. cibaria* T118, and *W. cibaria* T114, respectively.

	CtB	SB	fSB-DSM	fSB-T118	fSB-T114
pH	5.7 ± 0.2 ^a	5.7 ± 0 ^a	4.6 ± 0.2 ^b	4.7 ± 0.1 ^b	4.7 ± 0.1 ^b
TTA (mL)	3.2 ± 0.1 ^d	6.6 ± 0.8 ^c	10.2 ± 0.4 ^a	8.6 ± 0.2 ^b	8.1 ± 0.3 ^b
Sucrose (g/100 g)	0.69 ± 0.03 ^b	0.36 ± 0.05 ^c	0.88 ± 0.05 ^a	0.94 ± 0.01 ^a	0.97 ± 0.04 ^a
Glucose (g/100 g)	1.45 ± 0.12 ^a	1.01 ± 0.09 ^b	0.57 ± 0.09 ^d	0.87 ± 0.00 ^c	0.89 ± 0.04 ^{bc}
Fructose (g/100 g)	0.06 ± 0.01 ^c	0.05 ± 0.00 ^c	0.16 ± 0.01 ^b	0.27 ± 0.03 ^a	0.14 ± 0.02 ^b
TFAA (mg/kg)	197 ± 12 ^c	244 ± 7 ^b	289 ± 21 ^a	210 ± 14 ^c	160 ± 5 ^d
HI (%)	98.1 ± 4.8 ^a	76.3 ± 3.8 ^b	62.1 ± 2.8 ^c	65.6 ± 3.6 ^c	64.2 ± 2.5 ^c
pGI	93.5 ± 5.4 ^a	77.3 ± 3.3 ^b	73.7 ± 2.9 ^b	75.4 ± 3 ^b	74.8 ± 2.7 ^b

^{a-d}Values with different superscript letters in the same row mean significant differences ($P < 0.05$).

conditions, which in the present study were optimized for EPS production (16 h at 25 °C versus the 24 h at 30 °C of the previous study), thus not sufficient to allow adequate proteolysis.

As previously mentioned, gluten-free products are often characterized by a high glycemic index, which measures the ability of foods to raise blood glucose levels during digestion (Romao et al., 2021). Therefore, the glycemic response potential of the experimental breads was evaluated using an *in vitro* system simulating starch digestion. As expected, the control bread made only with corn flour showed a starch hydrolysis index of 98%. The addition of sorghum flour significantly reduced ($P < 0.05$) starch hydrolysis and, consequently, the predicted glycemic index (Table 2). This is due to the high fiber content provided by sorghum which i) increase digesta viscosity, thus slowing gastric emptying and reducing postprandial glucose response; ii) reduce food particle breakdown; and iii) lower the activity of amylolytic enzymes (Zhou et al., 2022). A further significant reduction in starch hydrolysis index was observed in fSB-DSM, fSB-T114, and fSB-T118. This effect could likely attributed to fermentation. Although the mechanisms are not fully understood, key factors include biological acidification with organic acids production (which slow gastric emptying), and possible increases in resistant starch and soluble fiber (Gobbetti et al., 2019). It should be noted that, although the predicted glycemic index was

estimated using an equation originally developed using gluten-containing bakery products, it has been widely adopted as an indirect estimator of glycemic index in *in vitro* studies for gluten-free bread (Capriles & Arêas, 2013). Nevertheless, since gluten-free bread possesses a distinct starch-protein matrix, the estimated pGI values should be interpreted as comparative indicators among formulations rather than absolute predictors of *in vivo* glycemic responses.

3.4.2. Technological and sensory characterization

The addition of sorghum (fermented and non-fermented) caused notable color differences to both crust and crumb (Table 3). Specifically, sorghum addition significantly ($P < 0.05$) reduced lightness and increased the red (a) and blue (b) indices compared to CtB. In fSB-breads

Table 3

Technological characterization of gluten-free breads. CtB, corn bread; SB, corn bread with raw sorghum; fSB-DSM, fSB-T118, fSB-T114, corn bread with sorghum fermented with *Leuc. pseudomesenteroides* DSM20193, *W. cibaria* T118, and *W. cibaria* T114, respectively.

	CtB	SB	fSB-DSM	fSB-T118	fSB-T114
Specific volume (cm ³ /g)	1.78 ± 0.18 ^c	2.34 ± 0.12 ^b	3.17 ± 0.15 ^a	2.67 ± 0.22 ^{ab}	2.96 ± 0.13 ^a
Crust color					
L	73.77 ± 4.03 ^a	66.14 ± 2.15 ^{ab}	63.51 ± 3.40 ^b	60.21 ± 3.11 ^b	63.51 ± 4.38 ^b
a	-1.42 ± 0.90 ^c	0.97 ± 1.75 ^b	4.66 ± 1.69 ^a	5.27 ± 1.85 ^a	3.89 ± 2.05 ^a
b	17.03 ± 1.72 ^b	17.02 ± 4.17 ^b	24.67 ± 3.45 ^a	25.24 ± 3.21 ^a	21.07 ± 3.14 ^a
ΔE	23.53 ± 1.65 ^c	30.95 ± 2.72 ^b	37.66 ± 4.70 ^a	40.62 ± 4.59 ^a	35.39 ± 5.49 ^{ab}
Crumb color					
L	71.49 ± 0.13 ^a	59.61 ± 3.50 ^b	64.89 ± 1.24 ^b	63.29 ± 3.36 ^b	60.48 ± 5.39 ^b
a	-3.66 ± 0.12 ^c	-1.46 ± 0.17 ^b	-1.04 ± 0.01 ^a	-0.87 ± 0.06 ^a	-0.71 ± 0.22 ^a
b	9.87 ± 0.24 ^b	13.03 ± 0.46 ^a	13.44 ± 0.48 ^a	14.17 ± 0.59 ^a	13.36 ± 0.49 ^a
ΔE	22.98 ± 0.01 ^b	34.92 ± 3.10 ^a	30.16 ± 1.33 ^a	31.94 ± 2.80 ^a	34.22 ± 4.82 ^a
Textural profile analysis					
Hardness (N)	59.06 ± 2.48 ^a	46.47 ± 2.81 ^b	30.56 ± 1.66 ^c	32.34 ± 1.46 ^c	33.75 ± 1.42 ^c
Cohesiveness	0.297 ± 0.01 ^b	0.305 ± 0.01 ^b	0.35 ± 0.01 ^a	0.335 ± 0.01 ^a	0.295 ± 0.01 ^b
Springiness	0.584 ± 0.03 ^c	0.76 ± 0.03 ^b	0.98 ± 0.04 ^a	0.89 ± 0.03 ^a	0.86 ± 0.03 ^a

^{a-c}Values with different superscript letters in the same row mean significant differences ($P < 0.05$).

crusts, *a* and *b* values were significantly higher than in SB, likely due to a more intense Maillard reactions between sugars and free amino acids during baking.

Breads also differed in specific volume which in CtB was $1.78 \pm 0.18 \text{ cm}^3/\text{g}$. The addition of sorghum flour, likely due to its high fiber content

(which absorbs water and strengthens dough structure), increased specific volume by 32% (Table 3), whereas fermented sorghum, due to the EPS production, led to further significant increases, reaching up to $3.2 \text{ cm}^3/\text{g}$ in FSB-DSM.

The use of fermented sorghum also improved the rheological

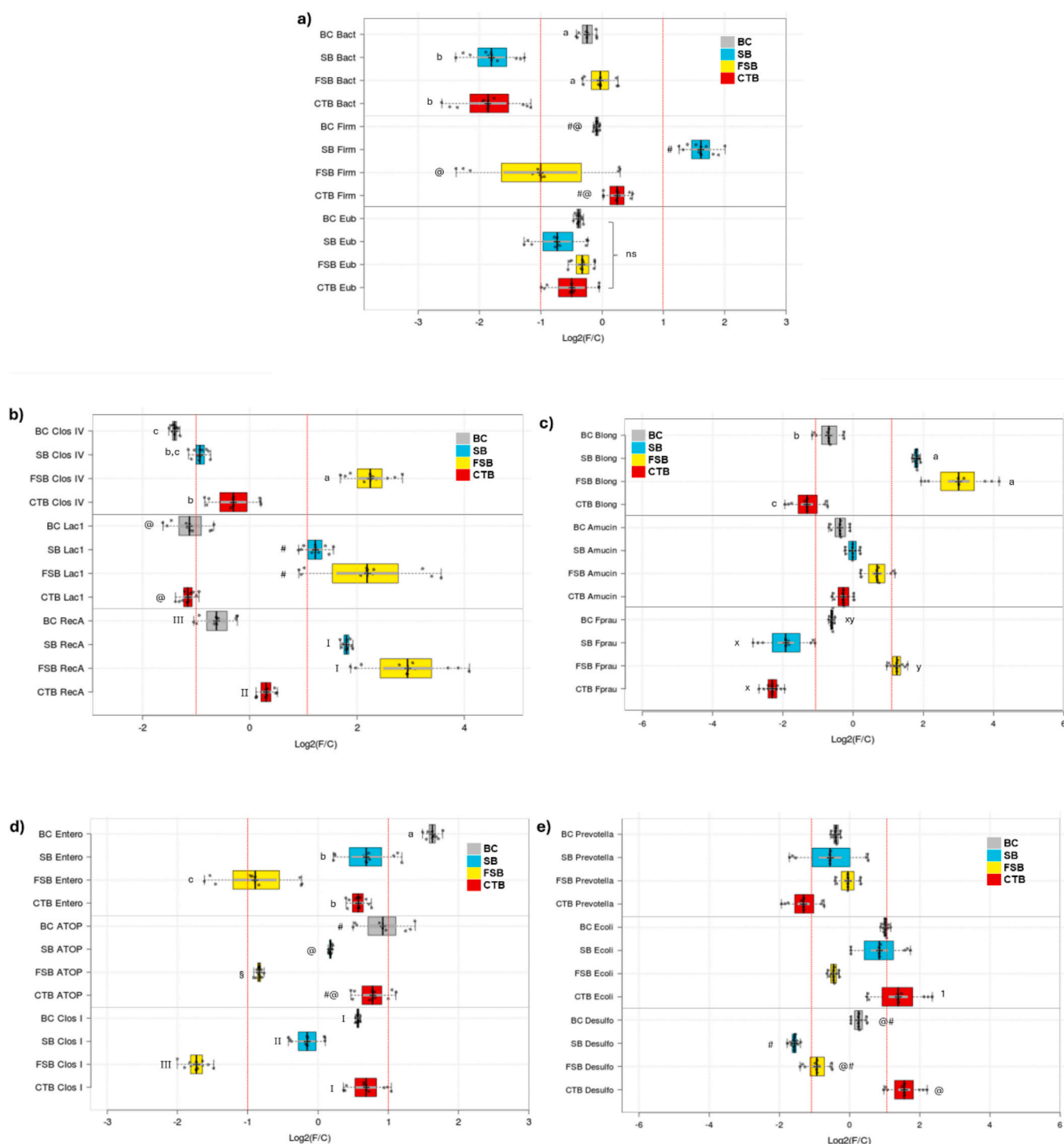


Fig. 2. Shifts as Log₂(F/C) of selected gut microbiota taxa in respect to time of *in vitro* fermentation. a) Core microbiota; b) beneficial groups; c) beneficial species; d) opportunistic groups; e) opportunistic species. CTB = bread made exclusively with corn flour; SB = bread made with corn flour and 25% sorghum flour; FSB = bread made with corn and 25% sorghum fermented with *Leuconostoc pseudomesenteroides* DSM 20193; BC = blank control (just GM without substrate addition); Eub = Eubacteria; Firm = Firmicutes; Bac = Bacteroidota; Clos IV = Clostridium group IV; Lac 1 = Lactobacillales; RecA = Bifidobacteriaceae; Blong = Bifidobacterium longum; Fprau = *Faecalibacterium prausnitzii*; Amucin = *Akkermansia muciniphila*; Entero = Enterobacteriaceae; Clos I = Clostridium group I; ATOP = *Collinsella aerofaciens*; Ecoli = *Escherichia coli*; Prevotella = *Prevotella* spp.; Desulfo = *Desulfovibrio* spp. Symbols, numbers or letters adjacent to a boxplot indicate significant differences by ANOVA followed by Duncan multiple range test with *P* < 0.05.

properties of fSB-breads, compared to SB and especially CtB, showing up to 48% lower hardness, namely the force required to cause the first deformation of the bread; and higher ($P < 0.05$) springiness, that is ability to return to the original shape after compression (Table 3).

Sensory analysis revealed that the main differences among samples were related to crust and crumb color (due to sorghum addition) and to sour taste and aroma (due to fermented sorghum). However, breads made with fermented sorghum also exhibited better chewiness and elasticity compared to CtB and SB (Fig. S1). In general, breads containing fermented sorghum, which showed higher sweetness likely due to residual fructose released from sucrase activity, were more appreciated in terms of aroma and overall acceptability. This demonstrates that tailored fermentation resulting from a proper starter selection process, as well as processing condition and bread formulation, represents a proof of concept for the design of new innovative and healthy gluten-free bread.

Moreover, given that only *Leuc. pseudomesenteroides* DSM20193 is considered safe for human consumption and *Weissella* species have still not been granted the QPS status (Fusco et al., 2023), only fSB-DSM was selected for the study of bread eubiotic potential using the MICODE system. CtB and SB were used as references to respectively evaluate the effect of sorghum flour and the fermentation process. Nevertheless, although the standardized INFOGEST digestion protocol was applied prior to colonic fermentation to simulate upper gastrointestinal digestion and to provide the insoluble fraction expected to reach the colon, the static *in vitro* approach cannot fully reproduce nutrient absorption, host epithelial interactions, or dynamic gastrointestinal transit. Consequently, the observed microbiota responses should only be interpreted as indicative of the potential *in vivo* responses.

3.5. Eubiotic potential of the functional bread

3.5.1. Modulation of gut microbiota groups

After fermentation of fSB-DSM, *Bacteroidota* abundance ($2.06\text{E}+09 \pm 5.58\text{E}+08$ cells/mL) did not change, whereas *Firmicutes* abundance ($3.92\text{E}+09 \pm 4.19\text{E}+08$ cells/mL) changed only slightly. Similarly, total bacterial abundance ($5.54\text{E}+10 \pm 6.79\text{E}+09$ cells/mL) remained stable suggesting that fSB-DSM is capable of maintaining the *Firmicutes* to *Bacteroidota* (F/B) ratio close to that of a healthy baseline, indicating its eubiotic potential (Fig. 2a). This stability is particularly noteworthy when compared to previous studies. For instance, Mariat et al. (2009) observed that the F/B ratio in healthy adults typically hovers around 1.9, while significant deviations from this ratio have been associated with dysbiosis and various health conditions (Mariat et al., 2009). Our results show that fSB-DSM fermentation does not disrupt this balance, which may be beneficial for maintaining gut homeostasis. In fact, the core microbiota was not perturbed by any fermentation, except for underrepresentation of *Bacteroidota*, maybe due to a reduced bioavailability of dietary fibers (Simpson & Campbell, 2015). Considering health-promoting bacterial GM taxa (Fig. 2b), compared to CtB and SB, fSB-DSM fermentation fostered *Clostridium* group IV, increasing from a mean value of $3.31\text{E}+07$ cells/mL at the baseline to $2.16\text{E}+08$ cells/mL at the endpoint of fermentation. SB and fSB-DSM fostered *Lactobacillales*, oppositely than CtB fermentation. In detail, in fSB-DSM fermentation the abundance of *Lactobacillales* increased from $9.40\text{E}+04$ cells/mL at the baseline to $9.86\text{E}+05$ cells/mL at the endpoint. SB and fSB-DSM also fostered *Bifidobacteriaceae*, which in fSB-DSM colonic fermentation increased from $1.19\text{E}+05$ cells/mL at the baseline to $1.76\text{E}+06$ cells/mL at the endpoint. These results suggest that fSB-DSM promotes the colonization of beneficial bacteria and brings prebiotic potential. Also, when the quantification of taxa was done at the species level (Fig. 2c), *Bifidobacterium longum* growth was supported by FSB with a high mean value, passing from $3.43\text{E}+04$ cells/mL at the baseline to $5.30\text{E}+05$ cells/mL at the endpoint. The modulation observed may be partly attributed to the intrinsic composition of sorghum, which is rich in dietary fiber and phenolic compounds known to exert selective pressures

on gut microbiota (Yang et al., 2022). Sorghum phenolic compounds, which include phenolic acids and flavonoids, can reach the colon where they are metabolized into bioactive metabolites, contributing to the stimulation of beneficial taxa such as *Bifidobacterium* and species belonging to the former *Lactobacillus* genus (Domínguez-Avila et al., 2021; Yang et al., 2022).

Additionally, the impact on 2nd generation probiotics, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* (Jan et al., 2024) was also assessed. From our results, although no significant modulations were seen on the abundance of *A. muciniphila*, for any of the breads. On the contrary, *Faecalibacterium prausnitzii*, underrepresented after fermentation of SB and CtB, was overrepresented after fermentation with fSB-DSM. Specifically, in the latter its abundance increased from a mean value of $4.36\text{E}+07$ cells/mL at the baseline to $1.22\text{E}+08$ cells/mL at the endpoint. These results suggest that FSB fosters colonization of beneficial bacteria and brings prebiotic potential. A key contribution to the observed eubiotic effect may derive from, among other microbial metabolites, the EPS synthesized during sorghum fermentation, which act as soluble dietary fibers and potential prebiotic substrates (Gościński et al., 2024). Indeed, several studies focused on evaluating the prebiotic activity of dextran, the EPS produced by *Leuc. pseudomesenteroides* DSM20193 (Koirala et al., 2022). Dextran was found to have sustained activity particularly in the distal regions of the colon, promoting the growth of *Bifidobacterium longum*, *Bifidobacterium animalis*, *Faecalibacterium prausnitzii*, *Lactobacillus salivarius*, *Lactiplantibacillus plantarum*, and *Lactocaseibacillus rhamnosus* (Gościński et al., 2024; Sip et al., 2024). Moreover, some probiotic strains can use dextran to enhance the formation of biofilms that adhere to the mucosal surfaces of the gut, thus fostering their colonization and persistence in the gastrointestinal tract (Gao et al., 2022; Gościński et al., 2024).

Assessment of opportunistic taxa within the gut microbiota (GM) revealed several notable trends (Fig. 2d). Following fermentation of fSB-DSM, *Enterobacteriaceae* were underrepresented, although the reduction was not statistically significant. Similarly, *Collinsella aerofaciens* showed a non-significant decrease in abundance when exposed to fSB-DSM, although important in number as passed from a mean value of $9.24\text{E}+05$ cells/mL at the baseline to $5.28\text{E}+05$ cells/mL at the endpoint. Most notably, the *Clostridium* group I exhibited a marked reduction of nearly $2.0 \text{ Log}_2(\text{F/C})$, indicating a substantial limitation on growth. These findings suggest that fSB-DSM may exert a selective inhibitory effect on opportunistic or potentially pathogenic taxa, particularly *Clostridium* group I, which has been associated with pro-inflammatory states and metabolic disorders. This aligns with previous research showing that certain prebiotic substrates, such as galacto-oligosaccharides, can modulate gut microbiota composition by reducing the abundance of *Escherichia-Shigella* and other opportunistic taxa (Pi et al., 2022). Moreover, *in vitro* studies have demonstrated that dietary fibers and prebiotics can lead to a decrease in *Enterobacteriaceae* and *Clostridium* spp., depending on the substrate and fermentation conditions (Xie et al., 2024). Lastly, *Collinsella* reduction is a positive trait, as *Collinsella aerofaciens* is considered a biomarker of IBS (intestinal bowel syndrome) (Gargari et al., 2024), and its containment or reduction in the GM of healthy people vows to be a clear gut health predictor. Taken together, these results reinforce the hypothesis that fSB-DSM may contribute to gut eubiosis by limiting the colonization and persistence of opportunistic bacteria, with a particularly pronounced effect on *Clostridium* group I. Also, other outcomes at deeper taxonomic level remarked the sample by different functional strength (Fig. 2e).

For example, after fermentation of SB and fSB-DSM, *Desulfovibrio* spp. was reduced, although significantly just for the latter, whereas CtB fostered the growth of *Desulfovibrio* spp. The ability to counteract *Desulfovibrio* spp., is a beneficial feature, since this genus is able to breakdown mucus layer reducing the barrier effect. After fermentation, *Prevotella* spp. showed no modulation, except for reduction seen on the control CtB. *Prevotella* in non-western countries is associated with plant-rich diets, while in western countries is associated to inflammation and

drug metabolism (De Filippis et al., 2019). Containment of *E. coli* growth by fSB-DSM was also a desirable aspect, supporting fSB-DSM potential in limiting colonization of opportunistic bacteria and brings prebiotic potential. The reduction in opportunistic taxa in fSB-DSM, may be explained by the EPS provided with fermented sorghum as well as by competitive exclusion mechanisms driven by increased availability of fermentable substrates favoring beneficial microbes as already proposed for other studies (Gościński et al., 2024; Tingirikari et al., 2014). Additionally, metabolites such as organic acids produced during fermentation can lower the pH and create unfavorable conditions for proteolytic bacteria like *Enterobacteriaceae* and pathogenic bacteria, whereas sorghum phenolic compounds and their fermentation metabolites may also exert antimicrobial activity against specific opportunistic taxa, contributing to the observed microbial shifts.

3.5.2. Modulation of gut microbiota ecological indicators

In Table 4, the different ecological indicators are presented. The *Firmicutes/Bacteroidota* ratio is an indicator of eubiosis w/ values <2.00 (Magne et al., 2020). Fermentations of SB and fSB-DSM were able to maintain the eubiosis of colon microbiota for 48 h, because the ratio never raised above the threshold of 2.0. For example, fSB-DSM passed from F/B value of 1.43 at the BL to 1.69 at the endpoint of fermentation. In contrast, CtB reached a value that exceeded the threshold of 2.0 and was 2.1 times higher than fSB-DSM. This result denotes a clear functional efficacy of fSB-DSM. In a recent publication of enriched breads assessed on the GM using *in vitro* models and omic approaches, only the samples fermented with a specific functional sourdough were able to keep the F/B lower than the value of 2.0, suggesting that the functional strength is depending on sourdough processing and its extend are related to the type and quality of the sourdough employed (Nissen et al., 2024). Another ecological indicator that was assessed is the quantitative prebiotic index (qPI) (Nissen et al., 2021), that is an indicator of prebiotic activity that considers the ability of a substrate to foster some beneficials and *ibidem* reduce some detrimental taxa, reported to the total Eubacteria quantity. fSB-DSM in our experiments had the highest score, passing from a value of 0.10 at the BL to the value of 1.25 at the endpoint, that was 2.7 times higher than the runner-up sample SB. Still the qPI scores indicate that fSB-DSM is the best among all samples, suggesting that the sourdough fermentation is the principal booster that functionalizes a bakery product. These results are in line with the literature reports, in fact several articles indicate that sourdough brings superior performances and fine-tunes the GM either in *in vivo* or *in vitro* (Da Ros et al., 2021; Nieto et al., 2024).

Another ecological indicator tested was the Bifidogenic effect, which indicates the ability of a substrate to foster bifidobacteria and reduce enterobacteria (Meyer & Stasse-Wolthuis, 2009). From this analysis, the results showed that fSB-DSM had the highest grade of bifidogenic effect, reaching a level that was 2.7 times higher than the runner-up sample SB and 9.2 times higher than the negative control CtB. Again, fSB-DSM had superior quality and the chemical products generated by sourdough action were able to foster bifidobacteria serving as prebiotic compounds. Such increase in this aspect is reported in other studies when a higher quality leavening process was applied, although not related to *in vitro* GM models. The “Anti-IBS Indicator”, instead, represents the strength of a substrate to induce beneficial lactobacilli to counteract IBS marker *Collinsella* (Gargari et al., 2024). fSB-DSM had anti-IBS capacity, because was able to increase such value from 0.16 to 1.32, that was 1.6 times higher than SB and 8.2 times higher than the value recorded by CtB. This indicator gives an evident message, that when fSB-DSM digestate is exposed to the GM there is a reduction of pro-inflammatory *C. aerofaciens* in respect to an increment in anti-inflammatory lactobacilli. Lastly, “*Clostridium* competition indicator” represents the strength of a substrate to induce beneficial clostridia and reduce colonization of opportunistic clostridia. fSB-DSM was able to double this attitude in 24 or 48 h, with a strength that was 1.6 times harder than the runner-up SB sample, and 4.4 times harder than CtB. Thus far, fSB-DSM triggered 5

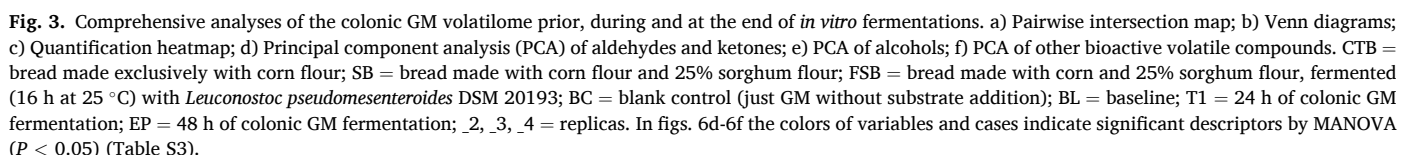
Table 4
Ecological indicators.

<i>Firmicutes/Bacteroidota</i>				
Sample	BL	24 h	48 h	Effect
CtB	1.39	2.23	3.60	☹️
SB	1.27	1.21	1.90	😊
fSB-DSM	1.34	1.56	1.69	😊
BC	1.40	2.89	4.22	😊
<i>Quantitative Prebiotic Index</i>				
Sample	BL	24 h	48 h	Effect
CtB	0.09	0.03	0.06	☹️
SB	0.12	0.13	0.45	😊
fSB-DSM	0.10	0.48	1.25	😊
BC	0.09	0.04	0.03	😊
<i>Bifidogenic effect</i>				
Sample	BL	24 h	48 h	Effect
CtB	0.31	0.28	0.23	☹️
SB	0.27	0.53	0.77	😊
fSB-DSM	0.36	1.19	2.13	😊
BC	0.21	0.20	0.05	😊
<i>Clostridium competition</i>				
Sample	BL	24 h	48 h	Effect
CtB	0.91	0.71	0.50	☹️
SB	1.15	0.93	1.35	😊
fSB-DSM	1.04	2.13	2.22	😊
BC	0.88	0.24	0.11	😊
<i>Anti-IBS indicator</i>				
Sample	BL	24 h	48 h	Effect
CtB	0.17	0.22	0.16	😊
SB	0.11	0.19	0.76	😊
fSB-DSM	0.16	0.28	1.32	😊
BC	0.13	0.00	0.01	😊

CtB = bread made exclusively with corn flour; SB = bread made with corn flour and 25% sorghum flour; fSB-DSM = bread made with corn and 25% sorghum fermented with *Leuconostoc pseudomesenteroides* DSM 20193; BC = blank control (only gut microbiota (GM) without substrate addition); BL = baseline; 24 h = 24 h of colonic GM fermentation; 48 h = 48 h of colonic GM fermentation.

Notably, fSB-DSM samples clustered differentially as a function of fermentation duration, indicating an evolutionary trend in the flavor profile. Many fSB-enriched compounds had high intensities and were organized into different modules that include aldehydes, terpenes, and short-chain fatty acids. Considering the PCAs (Fig. 3d–f), SB samples showed enrichment in isopulegol, indicating the development of distinct bioactive signatures, while fSB-DSM samples were characterized by menthols. A similar pattern emerged in the analysis of the other volatile classes (Fig. 3f), with fSB-DSM characterized by propanoic acid and SB dominated by carvone. This latter compound is of high biological value, as possess potent antioxidant and antimicrobial traits, it is found in triticale bread but usually is not found in the sourdough processed bread (Galoburda et al., 2020). These results highlight fSB-DSM increased bioactive potential, which is most likely due to fermentation with *Leuc. pseudomesenteroides* and its metabolites, which increased the diversity of volatiles while also shifting the profile toward potentially beneficial metabolites such as propanoic acid (Jiang et al., 2022).

At the beginning of fermentation, the volatilome was composed of 72 VOCs. At the endpoint of fermentation fSB-DSM was the sole sample increasing speciation (Fig. 3a and b). The Jaccard index shows this trend: fSB-DSM has less overlap than SB and CtB, indicating an active change of the volatility profile over time. This finding is supported by a Venn diagram analysis (Fig. 3b), which shows 33 VOCs common to all substrates and fermentation stages, as well as distinct compounds that appeared, particularly in fSB-DSM, at later times. The total volatilome included 134 different VOCs distributed among 3 samples, 3 time points and 4 replicates. Regarding the volatilome, in the y axis of the heatmap 3 clusters are identified for the three samples. Additionally, the cluster for fSB-DSM is splitted in two sub-clusters related to time points (Fig. 3c). The heatmap revealed three distinct sample-specific groups, indicating that each fermentation condition produces a distinct volatile signature.



3.5.4. Quantitative modulation of gut microbiota main metabolites

Relatively to organic acids, their changes during GM fermentation were studied (Fig. 4a). Specifically, short chain and medium chain fatty acids (SCFA and MCFA), renowned to exert beneficial effects to the host (Gibson et al., 1996), and branched-chain fatty acids (BCFA), that represent an insult to the host mucosa were evaluated. After fermentation of fSB-DSM many beneficial fatty acids were produced increasing the baseline loads, thus indicating a large prebiotic effect of the substrate (Wang et al., 2020). After fermentation of fSB-DSM proteolysis-derived BCFAs are consumed, likely by the action of beneficial taxa of the microbiota (Wang et al., 2020). 9 out of 9 tested beneficial fatty acids were produced and increased their initial loads, 2 out of 2 detrimental organic acids were consumed and not produced by colonic microbiota on fSB-DSM fermentation. fSB-DSM fermentation produced a higher quantity of organic VOCs than SB and the control, suggesting that *Leuc. mesenteroides* sourdough brings good biotechnological improvements. Considering the main microbial metabolites of the class of indoles and phenols (Fig. 4b), indolizine, *N*-acetylindole and niacinamide, which are derived compounds beneficial to the host, as well as phenol, *p*-cresol, indole, and skatole, that are associated with negative effects on the host gut mucosa, were considered. After fermentation of fSB-DSM, health-related aromatics are produced, some were typical of *Leuc. mesenteroides* activity, e.g., niacinamide (Kim et al., 2022) and indolizine, that were not present in other bioreactors. Toxic aromatics are not produced during fermentation but are consumed from the baseline loads, eventually bioremediated by increased activity of bifidobacteria (Sakurai et al., 2019). The burden of 4 out of 4 toxic aromatic VOCs as phenol, *p*-cresol, indole and skatole was reduced due to transformation by beneficial taxa of the microbiota, once induced by fSB-DSM fermentation. Transformations of toxic dietary compounds and production of aromatic bioactives by colonic fermentation of digested fSB-DSM, brings to this substrate an important functional value. The higher viscosity and soluble fiber fraction associated with EPS may have also modulated substrate accessibility and fermentation kinetics in the colon environment, whereas, the increase in species like *Faecalibacterium prausnitzii* may be linked to cross-feeding mechanisms, where EPS degradation products are converted into short-chain fatty acids, particularly acetate, propionate, and butyrate (Razzak et al., 2026). The increase of SCFA in fSB-DSM, may also be linked to other compounds produced during sorghum fermentation. For instance, 1,2-propanediol, which was previously found in higher quantity in fermented sorghum compared to raw sorghum (Verni et al., 2024), has an important role in the establishment of trophic interactions among gut symbionts since it promotes the

production of SCFA, particularly propionic acid (Cheng et al., 2020). Overall, the results obtained in this study suggested a synergistic effect of the metabolites produced during sorghum fermentation in modulating the gut microbiota and metabolome and confirmed that the combination of sorghum and selected strains represents a promising strategy to develop functional gluten-free products with improved rheological and health-related properties. Among the three bread formulations tested during *in vitro* colonic fermentation, fSB-DSM demonstrated the highest functional strength, followed by SB and the control corn bread. fSB-DSM reached the top functional score due to a combination of beneficial microbiome and metabolome outcomes. Specifically, it induced a higher production of beneficial volatile organic compounds and showed positive modulation across all five ecological indicators evaluated. It also resulted in a lower generation of detrimental VOCs typically associated with proteolytic and dysbiosis. Furthermore, the abundance of pro-inflammatory and proteolytic bacteria, such as *Enterobacteriaceae* and *Atopobium*, was reduced, along with mucus-disrupting taxa like *Desulfovibrio*, indicating a healthier microbial environment. In parallel, fSB-DSM promoted an increased presence of well-known probiotic genera, including *Bifidobacterium* and *Lactobacillales*, as well as second-generation probiotics like *Faecalibacterium*, which are associated with anti-inflammatory functions. The volatilome analysis revealed a higher degree of chemical diversity (speciation) and a greater overall number of bioactive compounds compared to the other samples. These results suggest a synergistic interaction between sorghum components (soluble and insoluble fibers, as well as phenolic compounds) and fermentation-derived metabolites (mainly EPS but also organic acids and phenolic compounds derivatives), enhancing the overall prebiotic potential of the fermented sorghum bread. This synergy likely explains the superior performance of fermented sorghum bread compared to the non-fermented counterparts in modulating gut microbiota and metabolome.

4. Conclusion

Based on these findings, fSB-DSM emerged as the most promising functional bread prototype and understanding how such products influence gut microbiota composition and activity could support the development of next-generation functional gluten-free foods with targeted health benefits. Further research could be focused on distinguishing the contribution of EPS from that of the other fermentation metabolites, i.e., by producing bread supplemented with isolated dextran. Moreover, future studies could aim to investigate fSB-DSM

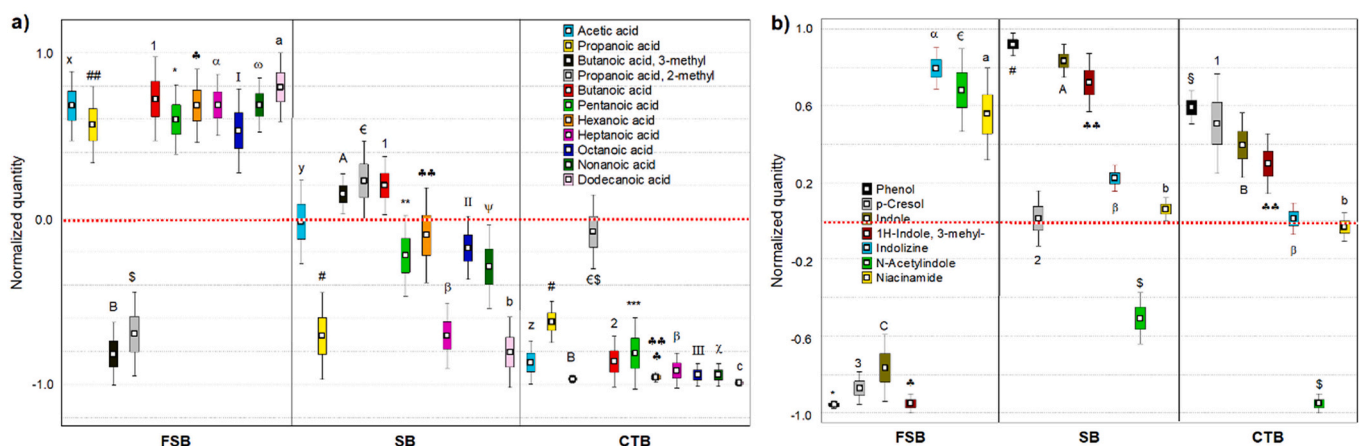


Fig. 4. Quantitative modulation of GM main metabolites after MICODE fermentation. CtB = bread made exclusively with corn flour; SB = bread made with corn flour and 25% sorghum flour; FSB = bread made with corn and 25% sorghum fermented with *Leuconostoc pseudomesenteroides* DSM 20193; BC = blank control (only gut microbiota (GM) without substrate addition); BL = baseline; 24 h = 24 h of colonic GM fermentation; 48 h = 48 h of colonic GM fermentation. Letters and symbols indicate statistically significant differences among a single compound by *post hoc* Tukey's test ($P < 0.05$).

impact in disease-specific models, such as celiac conditions, thus deepening our understanding of how food choices influence gut health and overall well-being. This study further highlights the value of NAMs as reliable and reproducible tools for investigating food–gut microbiota interactions in a human-relevant manner. The consistent integration of microbiota, volatilome, and ecological data supports the suitability of NAM-based strategies for functional food evaluation. Within this framework, the MICODE *in vitro* colon model proved to be a robust and informative platform, enabling a detailed and mechanistic assessment of gut eubiosis. The exclusive use of this methodology allowed the full replacement of *in vivo* experimentation, in line with the 3Rs principles, avoiding animal studies that would have required at least 30 laboratory mice. Overall, these findings support NAMs, and MICODE as advanced tools for predictive nutrition research without reliance on animal models.

Ethic statement

While the primary experimental research did not involve human or animal subjects, the sensory analysis component was conducted with human participants and received ethical approval from the Territorial Ethics Committee of Lazio Region (n. 0998/2024, Approval Date: 2024-11-06).

Studies in Human

This study was performed in compliance with relevant laws, regulatory frameworks and guidelines where the research took place.

This study was approved by the Territorial Ethics Committee of Lazio Region.

(Approval No. n. 0998/2024, Approval Date: 2024-11-06)

CRedit authorship contribution statement

Lorenzo Nissen: Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis. **Davide Addazii:** Formal analysis, Data curation. **Andrea Gianotti:** Supervision, Resources. **Andrea Torreggiani:** Formal analysis, Data curation. **Carlo Giuseppe Rizzello:** Supervision, Resources, Project administration, Conceptualization. **Michela Verni:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Data curation, Conceptualization.

Informed consent and patient details

Written informed consent to take part in the study and to publish the article has been obtained from all participants or their legal representatives. The privacy rights of participants have been observed.

Funding

This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU [PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR)-MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4-D.D. 1032 17/06/2022, CN00000022]. This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Carlo Giuseppe Rizzello reports financial support was provided by European Union Next-GenerationEU. Carlo Giuseppe Rizzello reports a relationship with European Union Next-GenerationEU that includes:

funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2026.120554>.

Data availability

Data will be made available on request.

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