

New fermented plant-based ingredients in sourdough breads enhanced nutritional value and impacted on gut microbiota

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ABSTRACT

Two new recipes of baker's yeast bread fortified with fermented apple by-products and avocado or walnut were designed resulting in enhanced profiles of total free amino acids, in vitro protein digestibility (IVPD) and predicted glycemic index (pGI). Concurrently, pools of lactic acid bacteria and yeast were screened to select the most promising starters for sourdough preparation. The type II sourdough with *Lactiplantibacillus plantarum* CR1, *Furfurilactobacillus rossiae* CR5 and *Saccharomyces cerevisiae* E10 had the highest acidification and total free amino acids value, while resulting in bread with the highest IVPD and the lowest pGI.

Sourdough breads were manufactured with the new recipes. They had improved protein digestibility and starch hydrolysis, and enhanced content of dietary fiber, phenolics and unsaturated free fatty acids. The impact of new fortified sourdough breads on colon microbial ecosystems was investigated by the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). The intake promoted the short-chain fatty acids synthesis and expanded several bacterial taxa with potential to exert beneficial activities. The synergic combination of sourdough fermentation and fortification with fermented plant-based matrices (including by-products) was a suitable strategy to promote better nutritional and digestibility properties in breads, and to promisingly address the modulation of gut microbiota.

1. Introduction

Digestibility of food combined with the capability to positively modulate the gut microbial ecosystem represent one of the most recent ambitious attributes requested for healthier lifestyles, becoming part of a broader concept of human health (Costantini et al., 2022), where

nutritional value pertains to balanced dietary patterns aimed at preventing disease onset (P. Wang et al., 2022). To reach the broadest part of the worldwide population, staple food (e.g., bread and pasta) fortification with high nutritional ingredients is one of the best safe and sustainable strategies. Among all, plant-based matrices and by-products were recently re-considered as capable in boosting the above health

Abbreviations: Apple by-products, (AB); Simulator of Human Intestinal Microbial Ecosystem, (SHIME®); lactic acid bacteria, (LAB); dough yield, (DY); total titratable acidity, (TTA); hydrolysis index, (HI); predicted glycemic index, (pGI); in vitro protein digestibility, (IVPD); total free amino acid, (TFAA); essential amino acid, (EAA); essential amino acid index, (EAAI); biological value, (BV); protein efficiency ratio, (PER); nutritional index, (NI); liquid chromatography-high-resolution mass spectrometry, (LC-HRMS); liquid chromatography system, (UHPLC); mediterranean diet adherence score, (MDS); stomach and small intestine, (ST/SI); proximal colon, (PC); distal colon, (DC); simulated salivary fluid, (SSF); short-chain fatty acids, (SCFA); partial least-squares discriminant analysis, (PLS-DA); National Institute of Standards and Technology, (NIST); principal component analysis, (PCoA); linear discriminant analysis, (LDA); linear discriminant analysis effect size, (LEfSe); Phenylalanine, (Phe); Tyrosine, (Tyr) Histidine, (His); Lysine, (Lys); methanol/water/hydrochloric acid-soluble extracts, (MWH-SE); Water/salt-soluble extracts, (WSE).

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features in leavened baked goods (Ranasinghe et al., 2022; Tlais et al., 2020). Moreover, baked goods are ideal food systems to accommodate fortification since dietary fiber and other biogenic compounds, that are typically present in plant-based matrices by-products, positively affect the digestibility of glycemic carbohydrates (e.g., starch). Bread fortification with such non-conventional matrices combines waste re-cycling and dietary recommendations while differentiating the market. The effects of such fortification will depend on the plant species, part of the plant and industrial processing (Mateos-Aparicio, 2021).

Apple (*Malus* sp.) is among the most widely consumed and processed fruits resulting in a substantial volume of by-products (Rabetafika et al., 2014). Numerous studies have investigated the addition of raw apple pomace as a fortifying agent in food, showing increased content of dietary fiber and phenolic compounds, lowering of the glycemic index, and increased antioxidant activity without adversely affecting the sensory profile (Antonic et al., 2020).

Avocado (*Persea americana* Mill) is also a fruit that garnered significant attention, and it is widely recognized for its great relevance in the human diet owing to its diverse array of nutrients, mainly due to significant content of monounsaturated fatty acids, notably oleic acid, along with linoleic, palmitoleic, and linolenic acids (Bhuyan et al., 2019; Filannino et al., 2020). Previous studies have investigated the potential of avocado to functionalize various products (Machado et al., 2022; Wekwete and Navder, 2008). The inclusion of avocado pulp in daily diet has the capacity to regulate the ratio of unsaturated to saturated fatty acids, improving cardiovascular health and body weight control. Additionally, its fiber content positively affected the status of the colonic microbiota and promoted the production of beneficial short-chain fatty acids (SCFA) (Dreher et al., 2021).

Walnuts (*Juglans regia* L.) are claimed for health-promoting properties and their bioactive compounds play a key role in multiple bodily activities. They are the richest source of polyunsaturated fatty acids among all the edible nuts (Pycia and Ivanišová, 2020). Regular supplementation of walnuts is positively correlated with the modulation of gut microbiota, resulting in prebiotic benefits and improvement of microbiome composition and diversity (Bamberger et al., 2018).

Nevertheless, the addition of plant-based matrices and by-products in baked goods leads to losses of acceptability, because of the worsening of sensory and rheological attributes (Cantatore et al., 2019). The fermentation has proven to be an effective tool not only for enhancing the nutritional profile (Tlais et al., 2020), the rheology and sensory characteristics of plant-based substrates (Di Cagno et al., 2013), including food by-products, but also for their exploitation for the fortification of leavened baked goods (Cantatore et al., 2019; Filannino et al., 2020).

Therefore, the use of fermented plant-based substrates to fortify leavened baked goods can represent an integrated suitable approach addressing both the need to reuse by-products and the demand for consuming foods with greater nutritional value (Amoah et al., 2020). While these plant-based matrices have been individually studied as fortifying ingredients (Antonic et al., 2020; Pycia and Ivanišová, 2020), their combination was not explored before. Based on this background, the aim of this study was to assess the effect of sourdough breads formulated with fermented apple by-product, avocado, and walnut, on bread digestibility and colon microbiota. To this purpose, we first fermented the plant-based matrices and determined the optimal fortification percentages for bread making. Concurrently, pools of lactic acid bacteria and yeasts were screened to select the most promising starters for sourdough preparation. Subsequently, sourdough breads fortified with fermented plant-based ingredients were manufactured and characterized based on key nutritional features, antioxidant activity, and digestibility indices. Finally, since the ingested food reflects on the status of gut microbiota, which is closely connected with host health (Zhang et al., 2018), the impact of the fortified sourdough breads on colon microbial ecosystems was investigated through the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), the reliable in vitro

model allowing to exclude interference from other dietary habits and host-dependent physiological factors (Polo et al., 2023). The impact on microbiota composition and metabolic answer was discussed.

2. Material and methods

2.1. Raw plant-based ingredients

Apple by-products (AB), avocado and walnut were used as raw plant-based ingredients. AB were kindly provided by the Pan Surgelati Srl (Laives, BZ, Italy), while avocado and walnut were achieved from local market. AB (peel and core) were homogenized by a blender and underwent heat treatment at 90 °C for 20 min, followed by cooling to 25 °C (approximately 2–3 h). Avocado was peeled and deseeded, while walnuts were opened, shelled, and the edible part was collected. Both ingredients were homogenized with a Classic Blender (MB 550, Microtron, Italy) for 60 sec at maximum speed (14 000 rpm). Sterile water was also added (10%, w/v) to homogenized walnut.

2.2. Microorganisms and culture conditions

The resulting AB puree was fermented with *Lactiplantibacillus plantarum* AF15 (final cell density at the inoculum, ca. 7 Log CFU/g) at 30 °C for 72 h. The avocado and walnut purees were inoculated individually with *L. plantarum* AVEF17 to a final cell density of ca. 7 Log CFU/g. The incubation was at 30 °C for 24 and 48 h for avocado and walnut puree, respectively. After fermentation, pH and cell density of presumptive LAB were determined by a pH meter (SensION™+ PH3, Hach, Italy) with a food penetration probe, and plate count method in modified MRS agar (Oxoid, Basingstoke, Hampshire, UK), respectively (Arora et al., 2022). All three raw matrices without the microbial inoculum were used as the control. Both fermented and raw purees underwent a freeze-drying to obtain the relative non-conventional plant-based flours.

2.3. Screening of plant-based flours as new no-conventional ingredients in bread making

Non-fermented (i.e. raw, NF) and fermented (F) plant-based flours from AB, avocado (AVO), and walnut (WAL) were individually added to soft wheat flour (15% of weight on flour weight, w/fw) for the manufacturing of six fortified wheat breads (15%AB-NF, 15%AVO-NF, and 15%WAL-NF, for not fermented, and 15%AB-F, 15%AVO-F, and 15%WAL-F for fermented non-conventional flours, respectively). Other six breads were made by combining the different fermented non-conventional flours as follows: 5% of AB and 10% of avocado or walnut (5%AB-F+10%AVO-F and 5%AB-F+10%WAL-F, respectively), 7.5% of AB and 7.5% of avocado or walnut (7.5%AB-F+7.5%AVO-F and 7.5%AB-F+7.5%WAL-F, respectively), and 10% of AB and 5% of avocado or walnut (10%AB-F+5%AVO-F and 10%AB-F+5%WAL-F, respectively). Breads and recipes of non-conventional flours are summarized in Supplementary Table 1. All doughs were prepared by mixing flour (62.5% w/w), water (37.5% w/w), salt (1.5%, w/w) and 2% baker's yeast (w/w). The leavening was at 30 °C for 1.5 h. The baking was at 220 °C for 30 min. A baker's yeast wheat bread (BYB) was manufactured with only soft wheat flour (without the addition of plant-based ingredients) and the same recipe, as a control. pH, total titratable acidity (TTA), total free amino acid (TFAA), in vitro protein digestibility (IVPD), and predicted glycemic index (pGI) were determined and used as criteria to select the best plant-based ingredient and most promising recipes.

2.4. Screening and selection of type II sourdoughs

Five strains of LAB (*L. plantarum* SD108B9, *L. plantarum* CR1, *Levilactobacillus brevis* 9F, *Furfurilactobacillus rossiae* 2A and *F. rossiae* CR5) and two yeasts (*Saccharomyces cerevisiae* SD108E3, and *S. cerevisiae* E10)

belonging to the culture collection of the Free University of Bozen/Bolzano were combined to prepare eight type II sourdough (SD) formulations (SD1 - SD8) (Supplementary Table 2). Water and soft wheat flour 00, dough yield (DY = $(100 \text{ g} / \text{weight flour}) \times 100$) of 160, were mixed and inoculated with LAB and yeast to a final cell density of ca. 7 (for SD1 – SD6) or ca. 6 (for SD 7 and SD8), and ca. 6 Log CFU/g (for all), respectively (Supplementary Table 2). The incubation was at 25 or 30 °C for 12, 16 or 24 h. Subsequently, the breads were prepared by mixing 20% of the previously fermented dough with flour (50% w/w), water (30% w/w), and salt (1.5%, w/w), and incubated at 30 °C for 4 h. BYB was used as a control. pH, TTA and TFAA concentrations were analyzed in sourdoughs, while pH, TTA, IVPD and pGI were determined in resulting breads, and used as criteria to select the most suitable starters. The final selected sourdough was refreshed at 20% with flour and water (DY of 160) and further incubated at 30 °C for 24 h, resulting in mature sourdough type IV.

2.5. Bread preparation

Two sourdough breads fortified with selected fermented plant-based ingredients were manufactured at the pilot plant of the Free University of Bolzano. Apple-by product and walnut sourdough bread (WA-SDB) was made by mixing soft wheat flour (85%, w/fw), fermented AB flour (7.5% w/fw), and fermented walnut flours (7.5%, w/fw). Apple-by product and avocado sourdough bread (AA-SDB) was manufactured by mixing soft wheat flour (85%, w/fw), fermented AB flour (7.5%, w/fw), and fermented non-conventional avocado flour (7.5%, w/fw). The final formulas consisted of these mixtures (50%, w/w) combined with mature sourdough type IV (20% w/w), water (30%, w/w), and salt (1.5%, w/fw), and further incubated for 30 °C for 4 h. BYB was used as a control. All breads were baked at 220 °C for 30 min. The workflow to design the new sourdough breads fortified with fermented plant-based ingredients

is shown in Fig. 1.

2.6. Biochemical and nutritional characterisation

Sourdough and breads were characterized based on pH, TTA, pGI, IVPD and TFAA. The pH value was determined using pH meter (Model 507, Crison, Milan, Italy) with a food penetration probe. TTA was assessed as reported by Arora et al. (2022). The degree of starch digestion was expressed as a percentage of potentially available hydrolyzed starch according to Costantini et al. (2022). The pGI was determined through quantification of the hydrolysis index (HI) according to Capriles and Aréas (2013) using the equation proposed by Goñi et al. (1997). A reference sample (white bread) was used as a control to estimate the hydrolysis index (HI = 100). The estimation of IVPD was carried out according to the method described by Akeson and Stahmann (1964) as modified by Rizzello et al. (2014). The IVPD% was expressed as the ratio of the digested protein fraction to the total protein fraction [IVPD = (digested protein/total protein) × 100]. The supernatant containing the digested fraction was used for the quantification of proteins (Bradford, 1976), peptides (Church, 1983), and TFAA (Doi et al., 1981).

Nutritional indices and protein quality were determined in WA-SDB, AA-SDB, and BYB breads. The digested protein fraction from IVPD analysis, after the freeze-drying process, underwent acid and basic hydrolysis. Acid hydrolysis was performed with HCl 5.7 M, under a nitrogen steam and incubated at 110 °C for 24 h, followed by amino acid quantification by Amino Acid Analyzer Biochrom 30+ (Verni et al., 2021). Tryptophan content was quantified following basic hydrolysis with NaOH 0.075M (Winterlin et al., 1981). The essential amino acid index (EAAI), estimating the quality of the protein under investigation, was calculated using the essential amino acid (EAA) content (Oser, 1959) and based on the following equation:

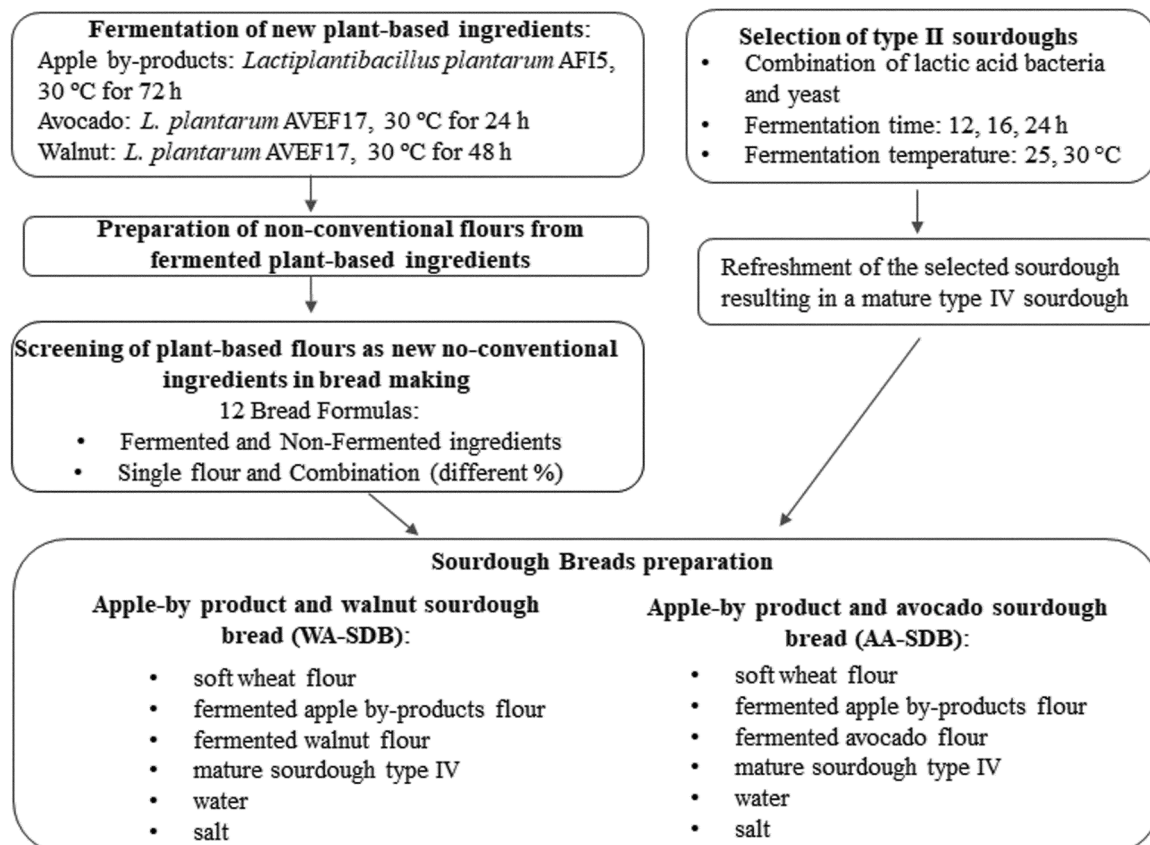


Fig. 1. Process flow applied in this study for obtaining the final sourdough breads fortified with selected fermented plant-based ingredients.

$$EAAI = \sqrt{\frac{(EAA_1 * 100)(EAA_2 * 100)(\dots)(EAA_n * 100)[\text{sample}]}{(EAA_1 * 100)(EAA_2 * 100)(\dots)(EAA_n * 100)[\text{reference}]}}$$

The Biological Value (BV), indicating the utilizable fraction of the sample protein (Oser, 1959), was calculated using the equation: $BV = ([1.09 \times EAA \text{ index}] - 11.70)$.

The Protein Efficiency Ratio (PER), estimating the nutritional quality of a protein based on the amino acid profile after hydrolysis, was determined according to the equation of Ihekoronye (1988)

$$PER = -0.468 + (0.454 * [\text{Leucine}]) - (0.105 * [\text{Tyrrosine}]).$$

The Nutritional Index (NI), evaluating qualitative and quantitative changes in the sample proteins, was calculated according to the equation of (Chang and Hayes, 1978):

$$NI = (EAA * \text{Protein } (\%) / 100).$$

The sequence of limiting EAA, estimating the amount of protein required to provide the minimal EAA pattern for adults, was calculated as described by Sindayikengera and Xia (2006).

Dietary fibers were determined through the AOAC 991.43 (2005)

2.7. Quantification of free fatty acids

Lipids were extracted from WA-SDB, AA-SDB, and BYB using the method designed by Masuero et al. (2021), and the resulting extractions were analysed by Ultra-high-performance liquid chromatography high-resolution mass spectrometry (LC-HRMS) using an Ultimate 3000 RSLCnano system coupled to a Q-Exactive Plus™ Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For the chromatographic separation and detection of fatty acids, the operating conditions reported in Fiorino et al. (2023) were employed with slight modifications. A Supelco Ascentis Express C18 column (150 × 2.1 mm ID, 2.7 µm particle size) was utilized for chromatographic separations of lipid fractions. The following binary gradient program was applied: 0–50 min, linear gradient from 70% to 100% solvent B; 50–60 min, isocratic at 100% B; 60–65 min, return to 80% B, followed by a 20 min equilibration time (solvent A = water + 2.5 mM ammonium acetate; solvent B = methanol + 2.5 mM ammonium acetate). The flow rate was set at 0.3 mL/min, and the column temperature was maintained at 31 °C. Chromatographic peaks corresponding to the major fatty acids identified in WA-SDB, AA-SDB and BYB (detected as [M–H][−] ions) were determined by comparing their retention times with those obtained from corresponding standards. Subsequently, quantification was carried out by applying calibration curve equations. For instrument control and data processing, the Xcalibur™ v.3.1 software (Thermo Fisher Scientific, Waltham, MA, USA) was utilized in conjunction with the Q-Exactive Plus™ spectrometer.

2.8. Analysis of free phenolic compounds

Phenolic compounds were extracted from WA-SDB, AA-SDB, and BYB using methanol/water/hydrochloric acid-soluble extracts (70:30:0.1, v:v) (MWH-SE) as described by Tlais et al. (2021), with few modifications. Specifically, 5 mL of the MWH-SE recovered by centrifugation was filtered (0.22 µm), subjected to nitrogen steam and then resuspended in 500 µL of CH₃OH. A targeted LC-MS/MS analysis of 45 free phenolic compounds was conducted according to an updated version of the method previously designed and validated by Tlais et al. (2021), by using an UHPLC Dionex 3000 (Thermo Fisher Scientific, Dreieich, Germany) equipped with a Waters Acquity HSS T3 column (1.8 µm, 100 mm × 2.1 mm) (Milford, MA, USA) and coupled to a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Germany) with an electrospray source. Target phenolics were detectable under multiple reaction monitoring modes, and the compounds were identified based on their reference standard, retention

time, qualifier, and quantifier ion. The chromatographic system was managed, and data acquisition was conducted using Xcalibur software version 4.1 (Thermo Fisher Scientific, Germany).

2.9. Individual free amino acids and derivatives

Water/salt-soluble extracts (WSE) from lyophilized breads were prepared according to the method described by Weiss et al. (1993), using a 1:4 ratio of sample to Tris–HCl buffer (50 mM, pH 8.8). The samples were resuspended in the buffer and held at 4 °C for 1 hour under stirring conditions (150 rpm), followed by centrifugation at 20,000 × g for 20 minutes. The resulting supernatant was collected for analysis. WSEs were analyzed to determine the profile of individual free amino acids through a Biochrom 30+ Series Automatic Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK), equipped with a Li-cation exchange column (4.6 mm × 200 mm internal diameter), as described by De Pasquale et al. (2021).

2.10. Antioxidant in vitro assay

The antioxidant activity was evaluated in the methanol-soluble extract (MSE) of each bread (BYB, AA-SDB, and WA-SDB). The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was quantified using the Antioxidant Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. The assay involves the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, resulting in the oxidation of ABTS and the production of the green-colored radical cation (ABTS⁺), which was detected spectrophotometrically at 405 nm. Trolox, a water-soluble vitamin E analog, was employed as the reference antioxidant. The results were expressed as Trolox Equivalent Antioxidant Capacity (mM Trolox eq./g) (TEAC).

2.11. In vitro static digestion simulation

WA-SDB, AA-SDB breads and BYB control bread underwent an in vitro digestion according to the consensus method developed within a large European framework (COST Action InfoGest) with the modifications (Brodtkorb et al., 2019; Koirala et al., 2022).

The process involved oral, gastric, and small intestinal phases. The breads were grounded with a Classic Blender (MB 550, Microtron, Italy) and diluted 1:2 ratio (dry, w/w) with a simulated salivary fluid (SSF, pH 7) solution containing KCl (15.1 mM), KH₂PO₄ (3.7 mM), NaHCO₃ (13.6 mM), MgCl₂(H₂O)₂ (0.15 mM), and (NH₄)₂CO₃ (0.06 mM). Amylase (1500 U/mL of SSF) and CaCl₂ 0.3 M solution (0.75 mL and 41.98 µL, respectively) were added to the mixture. The final product was mixed at 37 °C for 2 min. Subsequently, the oral bolus was diluted with a simulated gastric content. The stomach phase incubation began with the addition of 82.02 mL of gastric juice (0.04 M KCl and 0.24 M NaCl), followed by 6.07 mL of 2% pepsin solution (≥400 U/mg) and 0.68 mL of lecithin solution (0.17 mM). During the incubation (at 37 °C for 2 h) a pH gradient from 5.5 to 2.0 was applied adding 0.5 M HCl through a SHIME® apparatus controlled by the TwinShime v2 software (version 4.0.43, Prodigest, Gent, Belgium). Finally, the gastric chyme (140.0 mL) was mixed with 50.0 mL of a simulated pancreatic juice containing NaHCO₃ (0.19 M) and 8.0 g/L of Oxgall (Difco™ Oxgall, BD, Franklin Lakes, NJ, USA), and added with 4.5 mL of trypsin solution (50.0 mg of trypsin 5000 USP-U/mg in 5.0 mL of 1.00 mM HCl), 5.6 mL of chymotrypsin (100.0 mg chymotrypsin ≥ 1000 USP-U/mg in 10.0 mL of 1.0 mM HCl), 180.0 mg of lipase (type II, 100–500 U/mg), 300.0 mg of hog α-amylase (50 U/mg) and 350.0 µL of 0.3 M CaCl₂. The entire mixture (200.0 mL) underwent a further 3-hour incubation at 37 °C under static dialysis, using a 14 kDa membrane in 400 mL of dialysis solution (0.04 M NaHCO₃, pH 7). The digested suspension was used to run a SHIME® experiment.

2.12. Experiment through SHIME®

The experimental setup comprised of three identical SHIME® units running in parallel. Each unit consisted of three double-jacketed bioreactors maintained at 37 °C under anaerobic conditions, representing the stomach and small intestine (ST/SI), proximal colon (PC) and distal colon (DC), respectively (Koirala et al., 2022). The PC and DC bioreactors were filled with 500 and 800 mL of adult L-SHIME® PDNM001B growth medium (ProDigest, Gent, Belgium), respectively. The pH in the PC and DC bioreactors ranged from 5.6 to 5.9 and from 6.6 to 6.9, respectively, and was computer-controlled using 0.5 M HCl and 0.5 M NaOH to mimic the physiological conditions of the colon. The ST/SI reactors were maintained at pH 2. Before starting, PC and DC bioreactors of all SHIME® units were simultaneously inoculated with 5% (v/v) of fresh fecal slurry from a selected and representative donor (Da Ros et al., 2021). For the selection, a homogeneous group of sixty-one healthy individuals aged between 19 and 50 were considered as candidates. Preliminary nutritional questionnaires were administered to assess their adherence to Mediterranean diet, as described by Gutiérrez-Díaz et al. (2016). Based on the answers, the Mediterranean Diet Adherence Score (MDS) was calculated, ranging from 0 (indicating the lowest adherence) to 8 (indicating the highest adherence) (Gutiérrez-Díaz et al., 2016). The cut off 4 was used to assess satisfactory MDS. Forty individuals had MDS > 4 and their fecal samples were collected as described by Polo et al. (2023) and analyzed for microbiota composition and SCFA profiles. The selection of the donor was based on the clustering of fecal microbiota abundances of the 40 recruited individuals, aggregated at family level, together with SCFA data. Clustering was performed with the Manhattan distance matrix and Ward D2 method. Partial least-squares discriminant analysis (PLS-DA) was conducted considering the adherence to MD as an independent variable and OTU abundances as features for the model. The contribution of each feature was further explored and annotated with the explanatory independent variable level. All statistical analyses were performed in R programming version 4.04 (R Foundation, Vienna, Austria). Each volunteer gave written informed consent to the study and the study protocol was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethical Committee of the Free University of Bolzano (17 September 2019), registration number: Mi4FO Cod 2020_06). After inoculum, a static incubation of 12 h was first performed to allow the initial microbiota adaptation. Then, a stabilization period (3 weeks) allowed the evolution of the stable colonic microbiota within colon bioreactors, followed by a steady state period (2 weeks) representing the experimental base line. During these 5 weeks, ST/SI bioreactors were fed with 140 mL of adult SHIME® feed PDNM001B (ProDigest, Ghent, Belgium), three times per day (every 8 hours). In every feeding cycle, the feed resisted 45-minute in the ST/SI bioreactors where 60 mL of pancreatic juice (12.5 g NaHCO₃, 6 g dehydrated bile extract and 0.9 g pancreatin per liter) were added. The content of ST/SI was finally transferred to the colon bioreactors. Subsequently, a 1-week treatment phase started, during which the feeding of colon bioreactors was supplemented with 100 g/day (33.3 g, three times per day) of pre-digested WA-SDB, AA-SDB or BYB (one for each SHIME® unit). During the simulation, all bioreactors were continuously stirred, flushed once a day with sterile N₂ and monitored for constant volume and pH stability. Samples of lumen (40 mL) were collected from both PC and DC bioreactors at the end of steady state period (T0), after three days of treatment (T1) and at the end (7 days) of treatment (T2) (Supplementary Figure 1). Lumen samples were stored at -80 °C until further analysis.

2.13. SCFA profile

Samples of lumen from the three units SHIME® bioreactors were analysed to determine the metabolomic profile of acetate, propionate and butyrate. Samples were centrifuged, and the supernatants were filtered with 0.2 µm. Extraction and analysis were performed as

described by Lotti et al. (2017). The extracts were injected into an Agilent 7890A gas chromatograph coupled to an Agilent 5975 quadrupole mass detector (Agilent Technologies Italia SpA, Cernusco sul Naviglio, Milano, Italy). For the chromatographic separation, a fused silica Stabilwax®- 42 DA column (30 m × 0.25 mm i.d. 0.25 mm) (Restek Corporation, Bellefonte, PA, USA) was used. Mass spectrometry detection was performed in full-scan mode (electron ionization of 70 eV, an ion source temperature of 250 °C, *m/z* values ranging from 40 to 300 Da, and an acquisition scan time of 0.2 s). The identification of acetic, propionic, and butyric acids was achieved by comparing their mass spectra with those reported in the National Institute of Standards and Technology (NIST) US Government library. All analyses were performed in triplicate.

2.14. DNA extraction and 16S rRNA sequencing

Seven mL of lumen samples were centrifuged (10,000 rpm for 10 min at room temperature), and genomic DNA was extracted from the resulting pellet using the FastDNA Spin Kit For Soil (MP Biomedicals, Irvine, CA, USA) following the manufacturer's instructions. Each sample was extracted in duplicate. The DNA concentration was quantified using Nanodrop One/One Spectrophotometer (Thermo Fisher Scientific, San Jose, CA, USA). DNA metabarcoding library preparation and sequencing were carried out by AllGenetics & Biology SL (www.allgenetics.eu). Briefly, the V4 region of the 16s rRNA gene was amplified with the following primers: Forward - F515 (5' GTGCCAGCMGCCGCGTAA 3'), Reverse - R806 (5' GGACTACHVGGGTWTCTAAT 3'). Paired-end sequencing was carried out on the Illumina MiSeq PE300 platform. Raw sequences underwent quality check with FastQC following trimming of adapters with fastp. The 16S rRNA sequences were processed with the DADA2 pipeline (v1.24.0) (Callahan et al., 2016). Taxonomy was classified to genus level with DADA2's RDP naive Bayesian classifier method with the Silva v138.1 database.

2.15. Statistical analysis

Chemical, biochemical and nutritional analyses were carried out considering three analytical replicates. Data were subjected to one-way ANOVA and the pairwise comparison of treatment means was obtained by Tukey's post-hoc analysis at *p*-value (*p*) < 0.05, while the comparisons between paired groups was performed through the Student's *t*-test, using the software R version 4.2.2 (R Development Core Team).

Statistical analyses of DNA data (in duplicate) were done using the phyloseq (McMurdie and Holmes, 2013) and vegan packages, and visualised with ggplot2 (Wickham, 2014). Alpha diversity (Shannon diversity index, SDI) was calculated on counts, and statistical differences in alpha diversity between groups were determined with Kruskal-Wallis and pairwise Wilcoxon tests, where *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg formula. Beta diversity was analysed by calculating Bray-Curtis dissimilarities on relative abundances agglomerated at genus level and filtered to keep only taxa > 0.001%. The results were visualized with a principal component analysis (PCoA) plot. A permutational multivariate analysis of variance (PERMANOVA) with Adonis2 was performed on Bray-Curtis dissimilarities to evaluate possible differences in community structure between the groups, while pairwise Adonis tests applying the Bonferroni correction was used to find which groups were different from each other. Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was used to determine taxa that were differently abundant between treatments and timepoints. Samples from treatment periods (T1 and T2) were grouped together for each respective treatment, while samples taken before treatment (all T0) were also grouped together to enhance sample size.

3. Results

3.1. Selection of plant-based ingredient combinations

The pH of raw avocado, walnut and apple by-products was 6.6 ± 0.2 , 5.6 ± 0.1 , and 3.6 ± 0.0 respectively. The fermentation significantly ($p < 0.05$) decreased the pH of avocado (4.9 ± 0.2) and walnut (4.6 ± 0.2), while being already acid substrate it did not change in AB (3.5 ± 0.2). Presumptive LAB cell density increased from < 2 to 9.1 ± 0.3 and 8.7 ± 0.7 Log CFU/g for avocado and walnut, respectively, while it increased ($p < 0.05$) from 6.2 ± 0.1 to 8.5 ± 0.3 Log CFU/g in AB.

Different percentages of plant-based ingredients (7.5, 10 and 15%) and their combinations were used for making a baker's yeast wheat bread. pH, TTA, TFAA, IVPD, and pGI were determined and used as criteria to select the best plant-based ingredient and most promising recipes (Table 1). The effect of the fermentation was also considered. Differences in pH and TTA were found among breads, with values ranging between 5.7 ± 0.0 (15%AVO-NF) and 4.0 ± 0.0 (15%AB-F) and between 6.1 ± 0.4 (15%AB-NF) and 3.1 ± 0.3 (15%AVO-NF) mL of 0.1 M NaOH/10 g of dough, respectively. None of the formulas resulted in the best performance for all criteria. Overall, the fermentation had the best effect and combining the data, 15%WAL-F, 7.5%AB-F+7.5%AVO-F and 7.5%AB-F+7.5%WAL-F were the samples having a good profile for both IVPD (62.8 ± 0.8 , 63.1 ± 1.9 , and 68.5 ± 0.6 %, respectively), TFAA (301 ± 2 , 218 ± 3 , and 214 ± 3 mg/kg, respectively) and pGI (68.2 ± 1.5 , 67.7 ± 1.5 and 67.5 ± 0.3 , respectively). With the aim of combining the promising properties of different plant-based ingredients, the combinations 7.5%AB-F+7.5%AVO-F and 7.5%AB-F+7.5%WAL-F were chosen as recipes for the next steps.

3.2. Optimal microbial combination for making type II sourdough

Eight sourdoughs were made with different microbial combinations (Supplementary Table 2), and fermentation conditions. pH, TTA and TFAA concentrations were analyzed in sourdoughs, while the resulting breads were characterized based on their acidity and digestibility indexes (IVPD and pGI) to select the most suitable starters (Supplementary Table 3).

All sourdoughs made with soft wheat flour showed higher ($p < 0.05$) acidification compared to the control (BYB), with SD4 showing the lowest pH value (3.9 ± 0.0), which reflected on the pH of the bread SD4-B (4.4 ± 0.0). The TTA values were consistent with the observed pH. SD4 also resulted in the highest value of TFAA (878.9 ± 5.9 mg/kg). The bread SD4-B had the highest IVPD% (75.7 ± 0.7) and the lowest pGI (60.4 ± 0.6). Based on these results, SD4 sourdough was selected for further characterizations.

3.3. Characterization of the sourdough breads fortified with fermented plant-based ingredients

The sourdough breads fortified with selected combinations of fermented plant-based ingredients are shown in Supplementary figure 2. The fortification affected the biochemical and nutritional characteristics (Table 2). The sourdough bread made with the combination of fermented avocado and apple by-products (AA-SDB) had the lowest pH (4.3 ± 0.1) ($p < 0.05$) followed by the combination of fermented walnut and apple by-products (WA-SDB) (4.5 ± 0.0). Accordingly, TTA values showed the opposite trend. pGI was significantly ($p < 0.05$) lower in WA-SDB (53.6 ± 0.3) followed by AA-SDB (62.2 ± 0.4), and BYB (73.4 ± 0.9). Total dietary fiber content in WA-SDB and AA-SDB (10.4 ± 0.2 and 10.3 ± 0.7 % dm, respectively) was higher ($p < 0.05$) compared to BYB. WA-SDB resulted in the highest ($p < 0.05$) IVPD, followed by AA-SDB. Threonine and cysteine were identified as the primary and secondary limiting amino acids, respectively, in both fortified sourdough and control breads. Samples differed from each other in the third limiting amino acid: phenylalanine (Phe) and tyrosine (Tyr) in WA-SDB,

Table 1

pH, total titratable acidity (TTA) (mL of NaOH/pH 8.3), total free amino acid (TFAA) (mg/kg), *in vitro* protein digestibility (IVPD%) and predicted glycemic index (pGI) of baker's yeast breads manufactured with different percentages and/or combinations of non-conventional ingredients.

Ingredients and combinations of ingredients added to bread recipe (percentage, weight/ flour weight)	pH	TTA mL NaOH 0.1 M/10 g	TFAA mg/kg	IVPD %	pGI
15%AVO-NF	5.7 ± 0.0 ^a	3.1 ± 0.3 ^d	256 ± 5 ^c	54.8 ± 0.2 ^e	72.7 ± 0.5 ^b
15%AVO-F	5.3 ± 0.0 ^c	4.1 ± 0.3 ^b	372 ± 2 ^a	57.0 ± 0.3 ^{ed}	69.6 ± 1.8 ^{bcde}
15%WAL-NF	5.6 ± 0.0 ^b	3.3 ± 0.1 ^{cd}	217 ± 2 ^d	59.7 ± 0.7 ^{dc}	71.5 ± 1.2 ^{bcd}
15%WAL-F	5.3 ± 0.0 ^c	4.0 ± 0.1 ^{bc}	301 ± 2 ^b	62.8 ± 0.8 ^b	68.2 ± 1.5 ^{def}
15%AB-NF	4.5 ± 0.1 ⁱ	6.1 ± 0.4 ^a	76 ± 3 ^g	68.0 ± 0.3 ^a	76.1 ± 0.6 ^a
15%AB-F	4.0 ± 0.0 ⁱ	6.0 ± 0.5 ^a	88 ± 12 ^{fg}	68.5 ± 0.2 ^a	69.0 ± 1.4 ^{cdef}
5%AB-F+10%AVO-F	5.0 ± 0.0 ^d	4.6 ± 0.3 ^b	242 ± 4 ^c	61.5 ± 0.5 ^{bc}	72.4 ± 0.9 ^{bc}
5%AB-F+10%WAL-F	5.0 ± 0.0 ^e	4.4 ± 0.1 ^b	227 ± 2 ^d	61.9 ± 2.1 ^{bc}	70.4 ± 1.5 ^{bcde}
7.5%AB-F+7.5%AVO-F	4.8 ± 0.0 ^f	5.4 ± 0.1 ^a	218 ± 3 ^d	63.1 ± 1.9 ^b	67.7 ± 1.5 ^{ef}
7.5%AB-F+7.5%WAL-F	4.8 ± 0.0 ^g	5.6 ± 0.2 ^a	214 ± 3 ^d	68.5 ± 0.6 ^a	67.5 ± 0.3 ^{ef}
10%AB-F+5%AVO-F	4.7 ± 0.0 ^h	5.7 ± 0.1 ^a	128 ± 4 ^e	62.2 ± 0.6 ^{bc}	67.2 ± 1.8 ^{ef}
10%AB-F+5%WAL-F	4.6 ± 0.0 ⁱ	5.9 ± 0.1 ^a	118 ± 6 ^e	66.1 ± 1.5 ^a	65.6 ± 0.8 ^f
BYB	5.6 ± 0.0 ^b	2.8 ± 0.2 ^d	92 ± 9 ^f	63.1 ± 0.2 ^b	73.4 ± 0.9 ^b

In the same column, values with different superscript letters differ significantly ($p < 0.05$) based on one-way ANOVA with Tukey-Kramer post hoc comparison. The data are the means of three independent analyses ± standard deviations ($n = 3$). In the list of ingredients and combinations of ingredients, AB-F means fermented non-conventional apple by-products flour, AB-NF means not fermented non-conventional apple by-products flour, AVO-F means fermented non-conventional avocado flour, AVO-NF means not fermented non-conventional avocado flour, WAL-F means fermented non-conventional walnut flour, and WAL-NF means not fermented non-conventional walnut flour. BYB is the baker's yeast control bread, without any additional ingredient.

histidine (His) in AA-SDB and lysine (Lys) in the control sample. To estimate the quality of the food proteins, the EAAI and BV index were evaluated. WA-SDB and AA-SDB had the highest ($p < 0.05$) values compared to control for both indexes. AA-SDB had the lowest ($p < 0.05$) PER index (45.0 ± 1.0). The NI was significantly ($p < 0.05$) higher in WA-SDB (2.2 ± 0.1) followed by AA-SDB (2.0 ± 0.1). Among primary fatty acids, α -linolenic, linoleic, palmitic, and oleic acids were identified in both fortified sourdough breads. Fortifying the sourdough bread with fermented plant-based ingredients led to a significant ($p < 0.05$) increase in all identified free fatty acids. The most abundant was linoleic acid, with the highest ($p < 0.05$) concentration in WA-SDB (186.3 ± 0.4 ug/g dm), while WA-SDB revealed the highest ($p < 0.05$) values of

Table 2

pH, total titratable acidity (TTA) (mL of NaOH/pH 8.3), predicted glycemic index (pGI), dietary fibers (% dm), *in vitro* protein digestibility (IVPD%), essential amino acid index (EAAI), biological value (BV) index, protein efficiency ratio (PER), nutritional index (NI), sequence of limiting essential amino acids (EAA), and free fatty acids (ug/g dm) profile of the three different breads.

	pH	TTA mL NaOH 0.1 M/10 g	pGI	Dietary fibers % dm	IVPD %	EAAI	BV index	PER	NI	Sequence of limiting EAA
WA-SDB	4.5 ± 0.0 ^b	4.5 ± 0.3 ^a	53.6 ± 0.3 ^c	10.4 ± 0.2 ^a	78.8 ± 0.8 ^a	77.1 ± 2.0 ^a	72.2 ± 1.9 ^a	50.6 ± 1.3 ^a	2.2 ± 0.1 ^a	Threonine, Cystine, Phenylalanine + Tyrosin
AA-SDB	4.3 ± 0.1 ^c	4.5 ± 0.1 ^a	62.2 ± 0.4 ^b	10.3 ± 0.7 ^a	74.0 ± 0.8 ^b	74.5 ± 1.7 ^a	69.4 ± 1.6 ^a	45.1 ± 1.0 ^b	2.0 ± 0.1 ^b	Threonine, Cystine, Histidine
BYB	5.8 ± 0.0 ^a	2.8 ± 0.1 ^b	73.4 ± 0.9 ^a	8.0 ± 0.3 ^b	63.1 ± 0.2 ^c	66.6 ± 3.3 ^b	61.6 ± 1.6 ^b	52.5 ± 1.0 ^a	1.7 ± 0.1 ^c	Threonine, Cystine, Lysine
	Linolenic acid (ug/g dm)		Linoleic acid (ug/g dm)		Palmitic acid (ug/g dm)		Oleic acid (ug/g dm)			
WA-SDB	69.5 ± 0.9 ^a		186.3 ± 0.4 ^a		161.2 ± 0.7 ^a		68.2 ± 2.8 ^b			
AA-SDB	11.7 ± 0.2 ^b		92.1 ± 0.3 ^b		141.9 ± 3.4 ^b		85.4 ± 2.5 ^a			
BYB	6.3 ± 0.2 ^c		75.8 ± 1.1 ^c		122.4 ± 3.2 ^c		51.5 ± 2.7 ^c			

For each parameter, values in the same column with different superscript letters differ significantly ($p < 0.05$) based on one-way ANOVA with Tukey-Kramer post hoc comparison. The data are the means of three independent analyses ± standard deviations ($n = 3$). WA-SDB, sourdough bread made with soft wheat flour added with fermented non-conventional walnut flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw). AA-SDB, sourdough bread made with soft wheat flour added with fermented non-conventional avocado flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw). BYB, soft wheat baker's yeast bread (control sample).

α-linolenic, linoleic, and palmitic acids. Only oleic acid was found to be higher ($p < 0.05$) in AA-SDB compared to WA-SDB.

Fifteen phenolic compounds belonging to various chemical classes were identified (Table 3), and the fortification significantly ($p < 0.05$) enhanced the release of all compounds (Table 3) when compared to BYB, where they were mostly absent. The combination of walnut and apple by-products (WA-SDB) led to the highest ($p < 0.05$) concentration of most phenolic compounds. In the latter, ellagic acid was the most prevalent (36.82 ± 5.69 ug/g dm), followed by gallic acid (5.25 ± 0.37 ug/g dm). Both fortified sourdough breads had no difference in the concentration of epicatechin. Myricetin and 4 ethyl-catechol were found only in AA-SDB.

The profiles of individual free amino acids and derivatives (mg/kg of fresh weight) of breads were presented in Supplementary Table 4. Asn and Arg were the most abundant in WA-SDB with concentrations of 73.57 ± 0.02 mg/kg and 64.75 ± 0.24 mg/kg, respectively, while Ala

and γ-aminobutyric acid (GABA) were the highest in AA-SDB (61.48 ± 2.02 and 60.70 ± 0.50 mg/kg, respectively). Most EAA were found in higher concentrations in fortified sourdough breads compared to the control, including Thr, Val, Ile, Leu, Phe, and Lys, with WA-SDB exhibiting higher levels of Ile, Leu, and Phe (19.68 ± 0.09 , 59.38 ± 0.07 , and 35.11 ± 0.47 mg/kg, respectively).

AA-SDB and WA-SDB did not differ significantly for antioxidant activity, while both exhibited higher ($p < 0.05$) ABTS radical scavenging activity (29.8 ± 1.3 and 28.4 ± 0.6 mM/g DW, respectively) compared to BYB (19.8 ± 0.9 mM/g DW).

3.4. Selected fecal donor with high adherence to mediterranean diet

Following the clustering analysis, the candidate donors displaying high adherence to the Mediterranean Diet (MDS from 5 to 8) grouped in two separate clusters. The fecal donor was randomly selected from volunteers belonging to the main cluster. The detailed gut microbiota composition of the selected donor is detailed in Supplementary Table 5.

3.5. SCFA profiles during SHIME® simulation

Before the treatment phase, no significant ($p < 0.05$) differences were observed in lumen samples among 3 SHIME® units both in PC and DC, for all examined SCFAs (Table 4). The acetic acid concentration at T1 increased ($p < 0.05$) compared to T0, both in PC and DC, with the only exception of DC fed with BYB. Overall, the feeding with both fortified sourdough breads AA-SDB and WA-SDB resulted in higher level ($p < 0.05$) compared to feeding with the control baker's yeast (BYB). At T2, acetic acid concentration decreased ($p < 0.05$) compared to T1 both in PC and DC fed with WA-SDB but it remained still higher ($p < 0.05$) than T0. Differently, in the SHIME® unit fed with AA-SDB, the level in PC did not vary compared to T1, while it decreased in DC to similar value than T0. In PC the levels at T2 did not differ among the different treatments, while in DC, the concentration after feeding with newly developed breads remained still higher ($p < 0.05$) compared to that found after the intake of control bread. Feeding with BYB resulted in a progressive increase ($p < 0.05$) of acetic acid levels in the PC over time, while it did not affect the concentration in DC.

At T1 (in DC) and at T2 (in both PC and DC), the feeding with AA-SDB and WA-SDB resulted in higher ($p < 0.05$) propionic acid levels ($p < 0.05$) compared to the intake of control bread, with WA-SDB resulting in the highest ($p < 0.05$) value at T2. The administration of BYB in PC did not change the concentration of propionic acid over

Table 3

Quantification of phenolic compounds (μg/g dm).

μg/g dm	WA-SDB	AA-SDB	BYB
Gallic acid	5.25 ± 0.37	0.08 ± 0.03 [*]	nd
Catechin	0.02 ± 0.01	nd	nd
Chlorogenic acid	0.52 ± 0.07 ^a	0.30 ± 0.01 ^b	0.15 ± 0.01 ^c
Procyanidin B2	0.29 ± 0.02 ^a	0.17 ± 0.01 ^b	0.08 ± 0.01 ^c
Caffeic acid	0.05 ± 0.02	0.08 ± 0.01 ^a	nd
Epicatechin	0.15 ± 0.02 ^a	0.14 ± 0.01 ^a	0.10 ± 0.01 ^b
Rutin	0.35 ± 0.06 ^a	0.23 ± 0.04 ^b	0.07 ± 0.01 ^c
Ellagic acid	36.82 ± 5.69	2.19 ± 1.05 [*]	nd
Hyperoside	0.37 ± 0.07	0.41 ± 0.08	nd
Ferulic acid	0.32 ± 0.08	0.34 ± 0.12	nd
Myricetin	nd	0.11 ± 0.02	nd
Phloridzin	0.87 ± 0.20	0.46 ± 0.07 [*]	nd
4 ethyl-catechol	nd	0.18 ± 0.05	nd
Quercetin	0.19 ± 0.12	0.13 ± 0.06	nd
Phloriten	0.50 ± 0.16	0.10 ± 0.01 [*]	nd

In the same row, values with different superscript letters differ significantly ($p < 0.05$) based on one-way ANOVA with Tukey-Kramer post hoc comparison, while values with asterisk differ significantly ($p < 0.05$) based on Student's *t*-test. The data are the means of three independent analyses ± standard deviations ($n = 3$). Nd means not detected. WA-SDB, sourdough bread made with soft wheat flour added with fermented non-conventional walnut flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw). AA-SDB, sourdough bread made with soft wheat flour added with fermented non-conventional avocado flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw). BYB, soft wheat baker's yeast bread (control sample).

Table 4

Acetic, propionic and butyric acids concentration (mM) in proximal (PC) and distal (DC) colon bioreactors before (T0), after three (T1) and seven (T2) days of treatment period.

Acetic acid (mM)						
	PC			DC		
	AA-SDB	WA-SDB	BYB	AA-SDB	WA-SDB	BYB
T0	25.1 ± 1.2 ^{a/b}	25.8 ± 1.2 ^{a/c}	26.5 ± 1.2 ^{a/c}	34.9 ± 1.2 ^{a/b}	34.9 ± 1.2 ^{a/c}	35.2 ± 1.2 ^{a/a}
T1	59.6 ± 1.7 ^{b/a}	67.9 ± 2.3 ^{a/a}	47.6 ± 0.6 ^{c/b}	42.9 ± 1.7 ^{b/a}	56.3 ± 1.3 ^{a/a}	34.9 ± 2.2 ^{c/a}
T2	54.9 ± 3.8 ^{a/a}	60.7 ± 2.3 ^{a/b}	56.0 ± 0.6 ^{a/a}	38.5 ± 0.6 ^{a/b}	40.0 ± 0.6 ^{a/b}	30.5 ± 2.9 ^{b/a}

Propionic acid (mM)						
	PC			DC		
	AA-SDB	WA-SDB	BYB	AA-SDB	WA-SDB	BYB
T0	9.8 ± 1.8 ^{a/b}	10.9 ± 1.8 ^{a/b}	10.9 ± 1.8 ^{a/a}	12.4 ± 0.8 ^{a/c}	13.4 ± 0.8 ^{a/c}	13.8 ± 0.8 ^{a/b}
T1	7.3 ± 1.3 ^{b/b}	9.8 ± 1.1 ^{b/b}	13.1 ± 1.1 ^{a/a}	20.0 ± 1.3 ^{a/b}	22.5 ± 0.6 ^{a/b}	17.1 ± 1.3 ^{b/a}
T2	20.3 ± 1.3 ^{b/a}	28.0 ± 2.5 ^{a/a}	10.5 ± 2.3 ^{c/a}	28.0 ± 0.6 ^{b/a}	32.0 ± 0.6 ^{a/a}	12.7 ± 0.6 ^{c/b}

Butyric acid (mM)						
	PC			DC		
	AA-SDB	WA-SDB	BYB	AA-SDB	WA-SDB	BYB
T0	8.4 ± 0.6 ^{a/c}	8.7 ± 0.6 ^{a/c}	8.7 ± 0.6 ^{a/c}	9.1 ± 1.1 ^{a/c}	9.8 ± 1.1 ^{a/c}	9.5 ± 1.1 ^{a/c}
T1	40.0 ± 0.6 ^{b/a}	33.8 ± 0.0 ^{c/a}	69.8 ± 1.1 ^{a/a}	40.3 ± 0.0 ^{a/a}	34.9 ± 0.0 ^{b/b}	39.2 ± 1.9 ^{a/b}
T2	14.9 ± 0.6 ^{c/b}	24.7 ± 1.7 ^{b/b}	65.4 ± 1.1 ^{a/b}	30.5 ± 1.1 ^{c/b}	46.1 ± 0.6 ^{a/a}	45.0 ± 0.6 ^{a/a}

The Three-SHIME® units were fed with sourdough bread made with soft wheat flour added with fermented non-conventional avocado flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw) (AA-SDB), sourdough bread made with soft wheat flour added with fermented non-conventional walnut flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5 %/fw) (WA-SDB), and soft wheat baker's yeast bread as control sample (BYB), respectively. For each colon tract (PC1–3 and DC1–3), one-way ANOVA with Tukey-Kramer post hoc comparison was performed independently for each sampling point (to compare the three SHIME® units at the same time), and for each SHIME® unit (to compare the temporal evolution within the same bioreactor). For each colon tract, values in the same row with different regular superscript letters differ significantly ($p < 0.05$), and values in the same column with different bold superscript letters differ significantly ($p < 0.05$). The data are the averages of three standard deviations of independent analyses ($n = 3$).

time, while it increased ($p < 0.05$) in DC only at T1.

The concentration of butyric acid showed an increase ($p < 0.05$) in all colon tracts during the feeding period. Specifically, the feeding with control bread resulted in the highest levels ($p < 0.05$) in PC both at T1 and T2. In DC, the feeding with AA-SDB manufactured an initial increase ($p < 0.05$) of butyric acid concentration at T1 followed by a decrease ($p < 0.05$) at T2, even if the final value was still higher than T0. Differently, the feeding with WA-SDB and BYB resulted in a gradual increase ($p < 0.05$) during time, displaying the highest levels at T2.

3.6. Colon microbiome evolution

The PCoA plots (Fig. 2a) depicted the beta diversity of microbial communities across different samples, with distances between points reflecting Bray-Curtis dissimilarities. In PC, samples at T1 and T2 are grouped in a separate cluster compared to samples at T0 suggesting a modulation of the microbiome due to the treatment. In DC the grouping was less pronounced: at T0 no significant differences were found among SHIME® units. However, a modulation within each unit was observed

during treatment, with significant differences between T0 and T1, while no significant changes were found between T0 and T2. The alpha diversity, as determined by the Shannon index of the microbiome, revealed significant differences among different time points within each SHIME® unit: significant differences were found between both T0 and T1, and T0 and T2. On the contrary, no significant changes were present among SHIME® units at every single time point (Fig. 2b).

The 20 most abundant bacterial genera were identified in the three SHIME® units (Supplementary Figure 3). At the initial time point (T0), the predominant genera in the PC were *Bifidobacterium* (40–64%), *Bacteroides* (12–25%), *Centipeda* (7–9%), and *Enterococcus* (1.9–5%). Meanwhile, in DC, the most abundant genera were *Bifidobacterium* (24–47%), *Bacteroides* (21–35%), *Lachnoclostridium* (9–20%), and *Bilophila* (9–10%). *Bifidobacterium* increased over time after feeding with BYB in both PC and DC, while the intake of AA-SDB and WA-SDB resulted in an increase only in PC and DC, respectively. In both colon tracts, *Lachnoclostridium* decreased after the longer-term intake (T2) of both fortified breads (although it was increased in the short term, T1), while it increased in PC and decreased in DC with BYB intake. *Centipeda* was absent after feeding with all breads in both PC and DC, although it was present at T0. The same trend was displayed by *Collinsella* after feeding with control breads and, only in DC, with WA-SDB. In PC, *Lactiplantibacillus*, that was absent at T0, became dominant at T2 after feeding with both fortified breads, while it increased in DC only after the intake of AA-SDB. After feeding with all breads, *Bacteroides* showed a decrease in PC, while in DC it had an initial decrease at T1 followed by an increase at T2. In DC, a reduction of *Bilophila* was observed over time with the administration of all three types of breads, while it was absent in PC.

LEfSe analysis revealed differential abundant taxa per colon tract and kind of treatment across all analyzed time points (Fig. 3). In PC bioreactors, *Bacteroides* was the most abundant taxon differentiating the time T0 compared to the treatment period (T1 and T2), in all SHIME® units, followed by *Centipeda*, *Enterococcus*, *Collinsella*, *Blautia*, *Klebsiella*, and *Proteus*. In the same colon tract, the short term (T1) intake of all breads significantly increased the abundance of *Lachnoclostridium*. At the same timepoint, the intake of AA-SDB also resulted in significantly higher abundance of *Bacillus*, while WA-SDB intake significantly increased *Pediococcus*. Differently, the feeding with BYB increased *Agathobacter*. The longer term (T2) intake of both AA-SDB and WA-SDB breads showed a significant increase in *Lactiplantibacillus* abundance. WA-SDB intake also increased *Agathobacter*. Differently the longer intake (T2) of BYB reflected in a significant increase of *Lactococcus*.

In DC, *Bilophila* and *Parabacteroides*, followed by *Proteus* and *Delftia*, were significantly more abundant at T0 than at T1 and T2. At T1, the intake of AA-SDB resulted in higher abundance of *Alistipes*, *Sutterella* and *Lachnospiraceae* taxa, while the intake of WA-SDB enhanced *Collinsella* and *Megasphaera*. The control bread (BYB) showed increased relative abundances of *Bifidobacterium*, *Agathobacter*, *Alistipes*, *Ochrobactrum* and *Sutterella* at T1. At longer intake period (T2), feeding with AA-SDB manufactured significantly higher abundance of *Lactiplantibacillus*, *Veillonella*, *Parasutterella*, *Anaeroglobus*, *Klebsiella*, *Intestinimonas* and *Lachnospiraceae* taxa, while the intake of WA-SDB resulted in significantly higher level of *Alistipes*, *Parasutterella*, *Sutterella* and *Lachnospiraceae* taxa, meanwhile BYB increased the relative abundances of *Parasutterella*, *Anaeroglobus*, *Lactococcus*, *Selenomonas* and *Lachnospiraceae* taxa.

4. Discussion

Purees of apple by-products, avocado and walnut were fermented with different *L. plantarum* strains. The acidification and the growth of strains during the avocado and walnut purees fermentation were coherent with previous studies (Filannino et al., 2020; Fiorino et al., 2023). Conversely, in apple by-products, although a growth of LAB cell density was found, the pH did not decrease after fermentation with *L.*

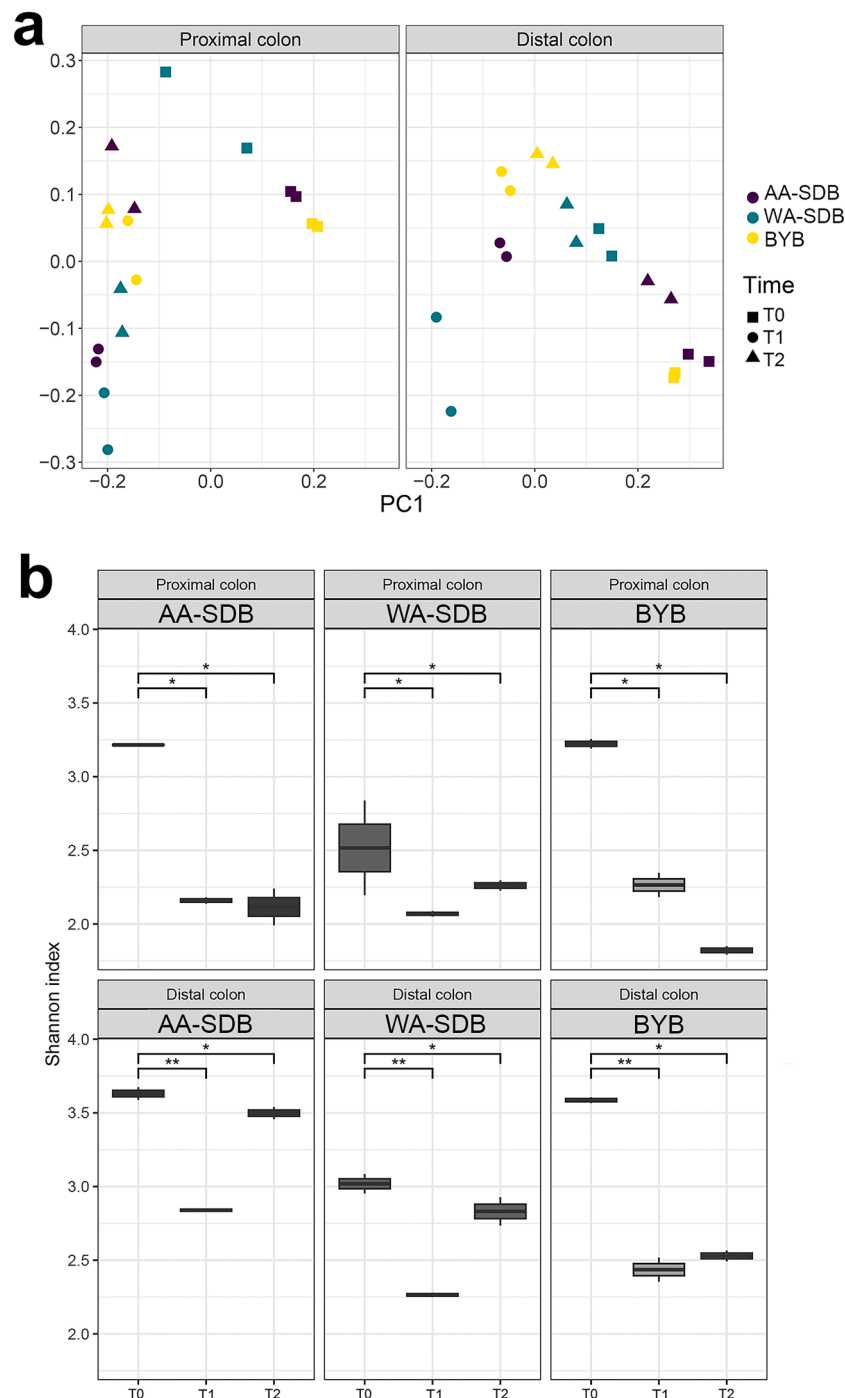


Fig. 2. Panel a: the principal component analysis (PCoA) diagrams constructed with relative genus abundances in bacterial communities in proximal and distal colon bioreactors before (T0), and after three (T1) and seven (T2) days of feeding with sourdough bread made with soft wheat flour added with fermented non-conventional avocado flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw) (AA-SDB), sourdough bread made with soft wheat flour added with fermented non-conventional walnut flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5 %/fw) (WA-SDB) and soft wheat baker's yeast bread (control sample, BYB). Panel b: alpha diversity of microbiota in proximal and distal colon at different time points (T0, T1, T2) determined with Shannon index for SHIME® units fed with AA-SDB, WA-SDB and BYB. Data are the means (n=2) of two independent analysis. The asterisk means $p < 0.05$; two asterisks indicate $p < 0.01$.

plantarum AFI5. This outcome can be attributed to the low initial pH, which acted as a stress factor (Papadimitriou et al., 2016) addressing *L. plantarum* to use other available energy sources (e.g., malic acid) (Gullón et al., 2008), and to adopt different metabolic pathways (e.g., malolactic fermentation) (Filannino et al., 2014) that did not reflect in further pH decrease (Liu, 2002).

The preliminary screening of formulations with different percentages

of fermented non-conventional flours proved that, when used for baker's yeast bread manufacture, no combinations reached the best performance in all considered criteria. Nevertheless, the combinations of fermented apple-by product and avocado (7.5% AB-F + 7.5% AVO-F) and apple-by product and walnut (7.5% AB-F + 7.5% WAL-F) resulted as those that met most of the analyzed criteria. Indeed, they showed lower pH and pGI compared to the unfermented samples, among the highest

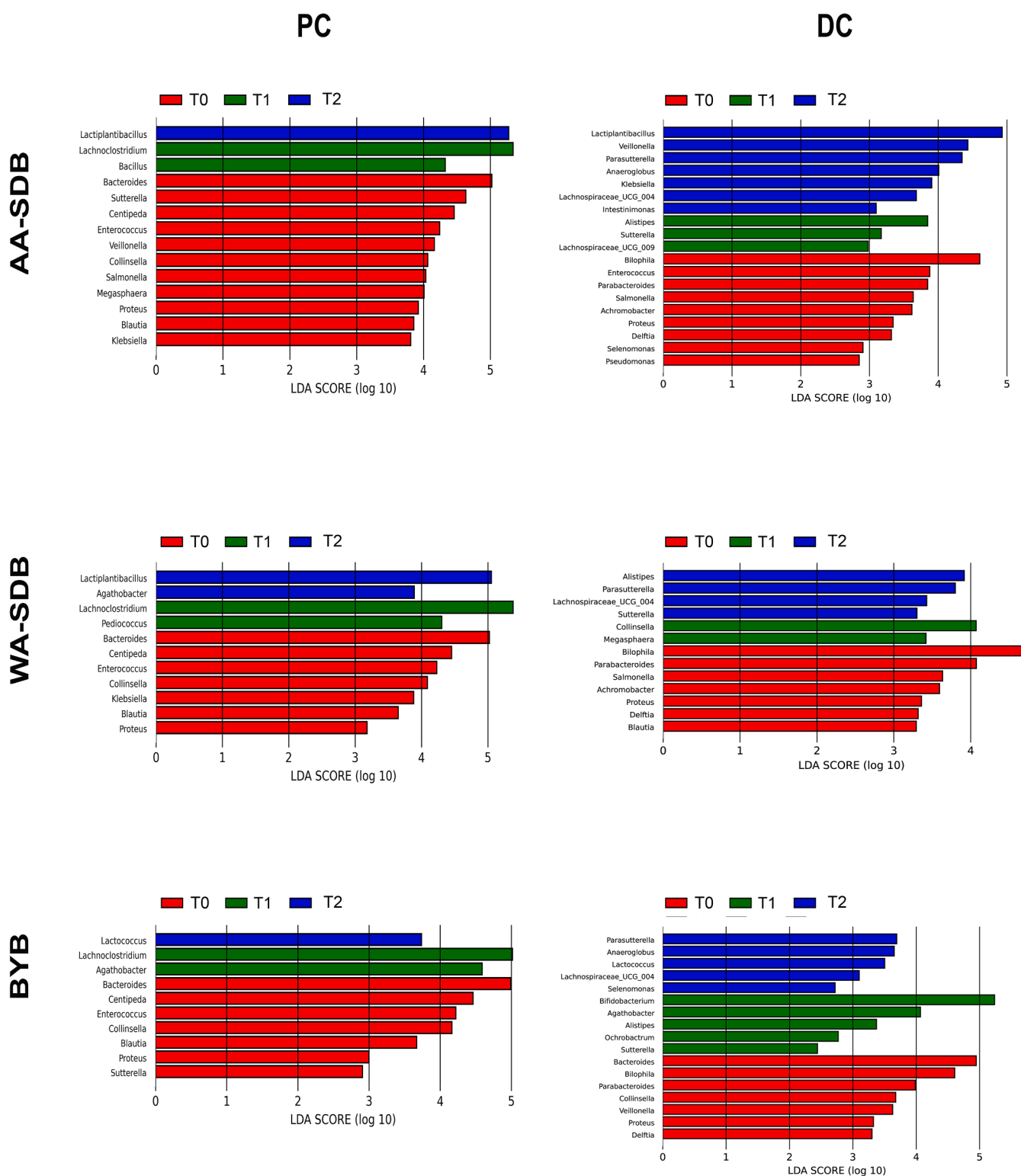


Fig. 3. Linear Discriminant Analysis (LDA) of differential abundance of genera in proximal (PC) and distal (DC) colon before (T0), and after three (T1) and seven (T2) days of treatment period. Graphs in the first e second column refer to PC and DC, respectively. The three rows represent the SHIME® units fed with sourdough bread made with soft wheat flour added with fermented non-conventional avocado flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5% w/fw) (AA-SDB), sourdough bread made with soft wheat flour added with fermented non-conventional walnut flour (7.5% w/fw) and fermented non-conventional apple by-products flour (7.5 %/fw) (WA-SDB), and soft wheat baker's yeast bread (control sample, BYB).

IVPD out of studied combinations, and, anyway, a significant increase of TFAA content compared to control. Differently, other combinations had the highest performance for some criteria but lower performance in others. Therefore, 7.5% AB-F + 7.5% AVO-F and 7.5% AB-F + 7.5%

WAL-F were chosen for the following steps. These outcomes also confirmed our hypothesis to combine different plant-based matrices as fortifying ingredients to enhance nutritional qualities.

The following screening on the sourdoughs prepared using different

microbial pools and various fermentation settings aimed at finding the conditions yielding the most favorable biochemical and digestibility characteristics (Arora et al., 2021). The microbial pool composed of *L. plantarum* CR1, *F. rossiae* CR5, and *S. cerevisiae* E10 yielded the best pH value and TFAA content in the sourdough, which led to a superior performance of acidity, IVPD and pGI values in the final bread. The high numbers of genes involved in carbohydrate and amino acid transport and metabolism harbored by these species may have contributed to the highest nutritional indices (De Angelis et al., 2014; Cui et al., 2021). Consequently, such microbial pool was chosen to prepare the sourdough for bread manufacture in the following steps.

Based on the preliminary outputs, two new soft wheat flour sourdough bread formulations were made: one added with both fermented walnut and AB flours, and one with both fermented avocado and AB flours. Overall, the sourdough fermentation and the fortification positively affected the nutritional potential of breads. The lower pH in WA-SDB and AA-SDB compared to the baker's yeast bread is due to the longer fermentation process, and the incorporation of fermented plant-based ingredients (Arora et al., 2021; Filannino et al., 2015; Cantatore et al., 2019; Fiorino et al., 2023). The increased IVPD for AA-SDB and WA-SDB compared to the control bread (ca. 17 and 25% higher, respectively) is in agreement with previous studies in both non-conventional (Rizzello et al., 2017) and cereal (Gobbetti et al., 2014) flours and reflects the proteolytic activity of *L. plantarum* favoring the digestion by pepsin (Pranoto et al., 2013; Gobbetti et al., 2014). Similarly, the peptidase activity exhibited by *F. rossiae* strains may have also positively influenced the increase in protein digestibility, although this capacity is strain dependent (Gänzle et al., 2008).

Also, the reduction of pGI (15% and 27% lower in AA-SDB and WA-SDB, respectively, compared to the control) confirms the potential of sourdough fermentation and/or fermented ingredients used for bread fortification and/or to enhance the rate of starch hydrolysis (Cantatore et al., 2019; Gobbetti et al., 2014). According to Harvard Medical School, WA-SDB and AA-SDB can be classified as low and moderate glycemic index foods, respectively (Arora et al., 2021). The differences in IVPD and pGI between WA-SDB and AA-SDB more likely depends on the different characteristics and composition of walnut and avocado flours. For instance, the gluten fractions, which constitute a significant portion of walnut proteins, have shown high in vitro digestibility, thereby positively influencing overall protein digestibility (Sze-Tao and Sathe, 2000). Conversely, since both avocado and walnuts can contain different carbohydrates profile (Boldingh et al., 2007; Pereira et al., 2008), their various hydrolysis index can justify the different pGI values (Cai et al., 2015).

The increased concentration of dietary fiber (ca. 29%) in both fortified breads is related to the addition of fermented apple by-product that is an excellent source of such compounds (Gómez and Martínez, 2018). Based on the reached content, they can be claimed as sources of fiber according to (European Commission, 2006). This finding is also consistent with the lower pGI values, since dietary fibers slow down the processes associated with starch digestion (Cantatore et al., 2019).

Considering the differences of IVPD among the samples, the EAAI, BV index, PER and NI were also investigated to identify which bread formulations could enhance the protein quality. While EAAI and BV improved in two sourdough fortified breads compared to the control, in agreement with previous similar investigations (Rizzello et al., 2019), PER was not dependent on the kind of fermentation (Costantini et al., 2022). However, among these indexes, only the NI combines qualitative and quantitative aspects, being a global predictor of the protein quality (Sozer et al., 2019), and it reached the highest values in WA-SDB and AA-SDB. Based on the estimation of limiting EAA sequence, all samples had threonine and cysteine as the first and the second limiting EAA, respectively. However, different breads differed in the third limiting EAA, which was Lys in baker's yeast bread since wheat proteins are mainly poor in it (Costantini et al., 2022). Phe, Tyr, and His were the third limiting EAA in WA-SDB and AA-SDB, respectively. This may be

attributed to the amino acid catabolic pathways of lactic acid bacteria, potentially involving histidine and aromatic amino acids (Fernández and Zúñiga, 2006). The profile of individual free amino acids confirmed the improved protein quality of fortified sourdough breads. The overall increase of most free amino acids in WA-SDB and AA-SDB compared to BYB can be attributed to fermentation conditions that enhanced the activity of endogenous proteases (Gobbetti et al., 2005). Specifically, the depolymerization of proteins during sourdough fermentation is associated with the high extracellular proteolytic activity of LAB species, with *L. plantarum* being recognized for its particularly potent protease activity. Furthermore, after sourdough fermentation, the pH reached an optimal range for aspartic proteases, the main proteases in wheat grains (Satılmış, et al., 2023; Gänzle, M. G., 2014). Among the detected free amino acids, GABA, that has well-established health benefits (including anti-hypertensive, anti-diabetic, anti-inflammatory, and gut-protective properties) (Ngo and Vo, 2019), had higher levels in both fortified sourdough breads, in agreement with previous literature on sourdough fermentation (Coda et al., 2010).

The fortified breads also exhibited higher concentrations of all analyzed free fatty acids, especially when walnut and avocado were added (Gangopadhyay, 2021). Oleic acid, that is a predominant fatty acid fraction in avocado (Cantatore et al., 2019) was most abundant in avocado and apple by-products bread. The overall higher concentrations of free fatty acids in sourdough bread samples compared to the baker's yeast bread can be attributed also to sourdough fermentation, since *L. plantarum* strains possess an intracellular system of lipases and esterases (Esteban-Torres et al., 2016).

Phenolic compounds play a crucial role in providing health promoting benefits (Verni et al., 2019) and are recognized as significant components in walnut (Ni et al., 2022) and avocado (Dreher et al., 2021). Consequently, fortified breads overall displayed high contents. WA-SDB showed a richer phenolic profile for most of the compounds, with notable concentrations of health-promoting ellagic and gallic acids (Landete, 2011; Kahkeshani et al., 2019). Moreover, the bacterial starters have also contributed to enhance the concentration of chlorogenic, caffeic and ferulic acids (Filannino et al., 2015). Indeed, the enzymes involved in lactic acid fermentation induce structural breakdown of the plant-based cell walls releasing various bioactive compounds including phenolics (Hur et al., 2014). Moreover, employing a microbial pool composed of both LAB and yeast in bread making can have a synergistic effect. While lactic acid fermentation positively impacts phenolics concentration, *S. cerevisiae* fermentation can increase their bioaccessibility (Andrade Barreto et al., 2023). The enhanced antioxidant potency observed in the fortified breads aligns with the trend observed for individual phenolic profiles. Indeed, the increased capacity to scavenge ABTS radicals reflects the ability of lactic acid fermentation by *L. plantarum* to increase antioxidant features (Pyo et al., 2006).

The impact of feeding with three different types of bread on the gut ecosystem was accessed in vitro through the SHIME® model. To this purpose, a representative fecal inoculum was selected from a cohort of potential donors displaying high MDS (Gutiérrez-Díaz et al., 2016). The higher acetic and propionic acid levels in DC after the intake of both WA-SDB and AA-SDB, and in PC only in the short- or longer-term intake period, respectively, can be attributed to their dietary fiber content (Mateos-Aparicio et al., 2020; Thompson et al., 2021; Wang et al., 2021). Indeed, dietary fibers serve fermentable substrates of enzymatic pathways that produce acetate and propionate (Tan et al., 2014). These dynamics are promising as these SCFA mediate several healthy actions on host, such as positively impacting the immune system, maintaining a favorable environment for commensal bacteria, and controlling pathogens' growth (Martin-Gallausiaux et al., 2021). They also positively influence colonic mobility affecting absorption of water and electrolytes in the intestine, and contribute to anti-inflammatory responses (Wang et al., 2020; Al-Lahham et al., 2010). The increase of butyric acid levels after the consumption of WA-SDB and AA-SDB reflected the expansion of *Lachnospirillum* taxa, that include relevant butyrate producers (Zhou

et al., 2020). Moreover, its synthesis was also associated with walnut ingestion (Schlörmann et al., 2016).

The complex microbial communities harboring the human gut also play a pivotal role in modulating numerous aspects linked with physiological well-being (Hou et al., 2022). Since the delicate balance between beneficial and harmful effects on the host hinges upon the intricate interplay of microbiota composition/variety/relative abundance, and taxa distribution (Liu et al., 2022), the understanding these dynamics is essential for unraveling the impact of new fortified breads on gut ecosystems and, potentially, on the host health. In our experiment, we carried out a metatranscriptomics analysis on SHIME samples, and eventual evolution of gut microbiota during and after feeding with new breads was assessed through alpha and beta diversity indexes. The alpha-diversity index, which reflects microbial diversity within each SHIME® unit and colon tract, demonstrated a change of gut microbiota over time due to the bread intake. The feeding with all different breads induced, after one week of intake, a specialization in colon microbiota, with consequent overall reduction of alpha diversity in all colon tracts. This trend was already observed in previous in vitro studies, where species that were more suitable to the new feeding regime persisted while no-tailored species lowered, resulting in a decrease of the whole variety (Tlais et al., 2024). Indeed, the diet input together with the metabolic abilities of species harboring colon tracts address the modulation of communities, favoring bacteria species that can effectively metabolize the received substrata (De Paepe et al., 2020). Such evolution is reflected also in changes of relative genus abundances. Beta-diversity proved a reshape of bacterial communities structure in colon tracts in response to the feeding with different types of breads. In proximal colons, such evolution was pronounced already after a short-term intake. Indeed, although the relative genus abundances were similar at T0, they differentiated after feeding with the different breads. Before the treatment period, the bacterial community comprised several genera recognized among dominant taxa in human gut microbiota (Rinninella et al., 2019). However, the administration of breads reshaped the relative abundance of bacterial groups in both colon tracts, including several taxa that have the potential to exert relevant activities. This was more pronounced in the PC for all SHIME® units. The increase of *Lachnospiraceae* in PC after the short-term consumption (T1) of all breads was associated with a substantial synthesis of SCFA (Zhou et al., 2020). Also, *Agathobacter* species, increased with the administration of BYB (at T1) and WA-SDB (in PC at T2), are well known butyrate-producers (Iversen et al., 2022; Kircher et al., 2022) and the expansion of this genus mirrors the higher levels of butyrate detected after feeding with BYB and WA-SDB. *Lactiplantibacillus*, that increased after the longer-term intake of sourdough-fortified breads, was associated with probiotic effects in human clinical trials, and benefits to the overall fecal microbiota (Qin et al., 2022; López-García et al., 2023). The growth has probably been stimulated by the higher content of dietary fibers and polyphenols in AA-SDB and WA-SDB that exert a prebiotic action (Andrade et al., 2020; Lippolis et al., 2023). *Bacillus* genus, increased in PC after the consumption of AA-SDB, promotes a robust immune response (by producing antibiotics) and has linked to significant therapeutic effects such as normalization of feces, alleviation of abdominal pain, and reduction in intestinal dysbiosis (Suva et al., 2016). As the increase was observed only with AA-SDB consumption, a potential correlation with the consumption of fermented avocados can be hypothesized. *Lactococcus*, that increased after 7 days of feeding with BYB, was also already identified in human gut (Von Wright, 2019), and it is acknowledged for its sugar fermentation, resulting in lactate as the primary end-product of the glycolysis pathway (Teuber, 1995). Lactate is usually found in low concentrations in the healthy human colon, where it can exert both positive and negative effects depending on the balance between lactate production and utilization (Louis et al., 2022). Indeed, if on one hand other intestinal bacterial species can convert lactate into propionate or butyrate, on the other it can also serve as a growth substrate for certain pathogenic bacteria such as *Salmonella*

(Louis et al., 2022). *Pediococcus*, stimulated in PC by the intake of WA-SDB, can adhere to the gastrointestinal mucosa and establish stable colonization exerting beneficial metabolic activities such as the production of digestive enzymes and organic acids (Qi et al., 2021). Some strains were also classified as probiotic (Porto et al., 2017) since they can compete with the resident microbiome and antagonize harmful microorganisms by producing antimicrobial compounds, thereby regulating the immune response (Qi et al., 2021).

Among taxa emerged only in DC tracts, the increase of *Sutterella* at T1 with the consumption of both AA-SDB and BYB, and at T2 after the feeding of WA-SDB can be attributed to different factors. About the intake of AA-SDB and WA-SDB, it can be due to the increased supply of polyunsaturated fatty acids (Xu et al., 2022). Moreover, avocado is known for its capacity to support *Sutterella* growth (Henning et al., 2019). In case of BYB intake, the increase can be due to its lower fiber content that, in an observational study comparing fecal microbiota, was associated to an expansion of *Sutterella* population (Jang et al., 2019). *Parasutterella*, increased in DC with the long-term intake of all breads, is a core component of the human gut microbiota and it relies on amino acids intake, that overall was higher during the treatment period, to support its metabolic activities producing succinate as main fermentation end-product (Ju et al., 2019). *Lachnospiraceae*, that increased in DC after 7 days of feeding with all kinds of breads, are generally recognized as major producers of acetic and propionic acids, although strain-dependent differences exist (Abdugheni et al., 2022), and they can have contributed to their increased synthesis. *Alistipes* also increased after the intake of all three types of bread, and its role is controversial and varies depending on the host and the influenced bodily system (Parker et al., 2020). Indeed, although it was primarily found in the healthy human gut (Parker et al., 2020) and it is a propionate producer (Houtman et al., 2022), some studies associated it with a potential opportunistic pathogenic role in human diseases (Parker et al., 2020). However, our analysis was not resolute at the species level, thus preventing an exhaustive conclusion. *Bifidobacterium*, increased in DC with the intake of control bread, is well-known for its probiotic role utilizing non-digestible carbohydrates as source for fermentation (X. Wang et al., 2022). The presence of dietary fiber in the control sample likely provided a suitable substrate (Makki et al., 2018). However, although the fortified breads contained higher amount of fibers than control bread, its increase was mainly with the intake of BYB. This apparently contradictory evolution probably resulted from the complex competitions among different microorganisms with overlapping abilities to utilize such fibers (De Paepe et al., 2020). Indeed, ecosystems harboring similar microbial communities can display divergent responses to the different environmental input (Cantu-Jungles and Hamaker, 2020). Consequently, the modulation of colon microbiome following the intake of different breads varies depending on both the original gut ecosystem and the bread features. On the other hand, the administration of fortified breads enhanced the growth of other genera potentially able to ferment non-digestible carbohydrates, such as *Megasphaera*, that increased after the short-term intake of WA-SDB, and *Lactiplantibacillus*, that expanded after the administration of AA-SDB. Indeed, a positive correlation was already observed between the augmented relative abundance of these taxa and heightened dietary fiber intake, suggesting their involvement in fiber fermentation processes (Choi et al., 2023; Andrade et al., 2020). Moreover, *Megasphaera* is also known for its capacity to metabolize lactate to produce propionate as main SCFA (Shetty et al., 2013). *Veillonella* species, that increased only with the consumption of AA-SDB, are unable to ferment carbohydrates, but they use lactic acid (produced by other bacteria) converting it into propionate (Kolenbrander, 2006). The amino acids resulting from protein digestion by anaerobic bacteria further enhanced this metabolism (Kolenbrander, 2006). *Klebsiella*, increased at T2 after the consumption of AA-SDB, has not a univocal role in human health. Some strains are recognized as pathogens, while the majority can contribute to a balanced gut microbiota composition (Hakalehto, 2013). *Collinsella* increased during the feeding of WA-SDB

due to its capacity to metabolize polyphenols (Kemperman et al., 2013), that are more abundant in this bread. Conversely, the feeding with BYB enhanced *Ochrobactrum*, a genus found in human gastric environment (Kulkarni et al., 2017) that includes some species exhibiting opportunistic pathogenicity (Ryan and Tony Pembroke, 2020). Unfortunately, the identification did not reach the species level, thus avoiding a clear and precise interpretation and conclusion.

5. Conclusion

This study proposed novel formulations of sourdough bread fortified with combinations of fermented plant-based ingredients with high nutritional qualities, including exploitable plant by-product. Such synergic approach successfully enhanced nutritional and digestibility properties reflecting in increased levels of polyphenols and free fatty acids, lower pGI as well as enhanced protein quality and relative digestibility. Moreover, the resulting breads can be claimed as sources of fiber and had the capacity to promisingly address the modulation of gut microbiota and its metabolic answer. While the intake of both fortified sourdough breads reflected in potentially beneficial impact on gut ecosystem, the fortification with apple by-product and walnut (WA-SDB) coupled it with the best biochemical and nutritional profile. The outputs of the study suggest that the designed formulations of sourdough breads fortified with fermented apple by-products, avocado and walnut represent a promising strategy for both delivering baked goods with considerable nutritional values and health features, and valorizing food by-products.

Ethical statement

The authors ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The manuscript is in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. A statement reporting that the informed consent was obtained for experimentation with human subjects was included in the manuscript. The privacy rights of human subjects were always observed. The collection of data from consumers and the use of human fecal sample were approved by the Ethics Committee of the Free University of Bozen on 17.07.2019.

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CRediT authorship contribution statement

Federica Mastroiardo: Writing – original draft, Investigation, Formal analysis, Data curation. **Alice Costantini:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Andrea Polo:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Michela Verni:** Methodology, Formal analysis, Data curation. **Wilson José Fernandes Lemos Junior:** Methodology, Formal analysis, Data curation. **Ali Zein Alabiden Tlais:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Olga Nikoloudaki:** Validation, Methodology, Investigation, Data curation. **Lena Birgitta Marie Granhäll:** Validation, Methodology, Formal analysis, Data curation.

Marco Gobbetti: Writing – review & editing, Supervision. **Erica Pontonio:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Raffaella Di Cagno:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fufo.2024.100498](https://doi.org/10.1016/j.fufo.2024.100498).

Data availability

Raw sequence data are available in the SRA database under the accession BioProject ID PRJNA1134081

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