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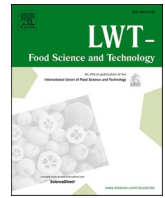
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Lactic and propionic acid bacteria starter cultures for improved nutritional properties of pea, faba bean and lentil

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ABSTRACT

Increasing plant-based food consumption as a sustainable and health-oriented alternative to meat is pivotal. Pulses are rich in proteins, minerals, and vitamins; however, they also contain antinutritional compounds, impairing their nutritional value. This study addresses this challenge through the development and application of four distinct microbial consortia in pulse-based fermentations, featuring lactic acid bacteria or a combination of lactic and propionic acid bacteria. Microbial starters significantly reduced galacto-oligosaccharides in all pulse materials, concurrently degrading vicine and convicine in faba beans, while the impact on tannins in faba beans and lentil was moderate. Fermentation with lactic acid and propionic acid bacteria consortia exhibited notable vitamin B12 production, and the effect on the content of phenolic compounds of the studied pulses was also evidenced. Additionally, genomic analyses discerned distinctive profiles among the samples, elucidating the microbial community dynamics shaping fermentation outcomes. The results of this study proved how fermentation can advance the development of pulse-based products with improved nutritional and sustainability attributes.

1. Introduction

A dietary shift toward plant-based foods has been evidenced during the past decade both for promoting health and for environmental reasons. Scientific research has shown that a plant-based diet can reduce the risk of many diseases, such as type 2 diabetes, heart disease, and certain types of cancer (Rosenfeld, Juszczak & Wong, 2023). The increased interest in plant foods has also led to the introduction of new plant-based alternatives for consumers.

Fermented products usually serve as stable and important sources of proteins, vitamins, minerals and other nutrients (Tamang et al., 2020). The live microorganisms, mainly belonging to lactic acid bacteria (LAB), propionic acid bacteria (PAB), yeasts, and moulds, produce a variety of components that can contribute to the flavor, aroma, color, texture,

safety, improved shelf life, as well as health properties of the product.

The use of plant-based raw materials in diets may be limited by the presence of antinutritional factors, which may cause flatulence, bind minerals, and decrease protein bioavailability. Antinutrients include e. g., protease inhibitors, phytate, oxalate, lectins, tannins, saponins, amylase inhibitors, and oligosaccharides (Kahala, Mäkinen & Pihlanto, 2021; Popova & Mihaylova, 2019). Galacto-oligosaccharides (GOS) are a group of compounds present especially in pulses and are important for gastrointestinal health. They are known to have both antinutritional and prebiotic properties (Martínez-Villaluenga, Frias & Vidal-Valverde, 2008). These compounds cannot be digested in the human small intestine, leading to anaerobic fermentation by microbes in the large intestine causing e.g. abdominal pain, flatulence, and diarrhoea. This discomfort is one limiting factor for consumers to increase the

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consumption of pulses. In fermentation, some components of the raw material are known to be degraded by the enzymes secreted by the microorganisms or those present on the plant-based raw materials (El Hag, El Tinay, & Yousif, 2002). It is well established that fermentation can enhance the nutritional quality of raw materials, like protein digestibility, the bioavailability of group B vitamins and minerals, such as magnesium, iron and zinc, antioxidant activity and have an effect on polyphenolic compounds (Ali, El Tinay & Abdalla, 2003; El Hag et al., 2002; K arlund et al., 2020). The pyrimidine glycosides vicine and convicine are present in the faba bean and are known to cause favism, an acute haemolytic anemia, in people with an inherited deficiency of glucose 6-phosphate dehydrogenase (G6PD) (Luzzatto & Arese, 2018). Partial or complete elimination of antinutrients, such as vicine/convicine, GOS, tannins, trypsin inhibitors and phytates by fermentation has been reported by several groups (Granito et al., 2002; Kahala et al., 2023; Osman, 2011).

People consuming mainly plant-based food are at higher risk for developing B12 deficiency since only a few plant-based foods contain considerable amounts of vitamin B12 (Watanabe, Yabuta, Tanioka, & Bito, 2013). Some bacteria are known to produce vitamin B12, and some fermented plant-based foods have been reported to contain this vitamin. Currently, the only known food-grade microbe known to synthesize an active form of vitamin B12 is *Propionibacterium freudenreichii* (Hugenholtz, 1993; Piwowarek, Lipińska, Hać-Szymańczuk, Kieliszek, & Ścibisz, 2018). Traditionally, *P. freudenreichii* is known for the formation of flavor and “eyes” in Swiss-type cheeses (Langsrud & Reinbold, 1974). Therefore, *P. freudenreichii* is an attractive microorganism for in situ B12 fortification of plant-based foodstuffs through fermentation. Special requirements such as strict anaerobic conditions, slow growth or nutrient requirements have hampered the use of *P. freudenreichii* as a vegetable starter, and case-dependent optimization is required for the successful production of active B12 in various plant-based materials (Chamlagain et al., 2018; Xie et al., 2021).

In this study, four microbial consortia, two with lactic acid bacteria and two with a combination of lactic and propionic acid bacteria, were utilized in pulse-based fermentations with the aim to reduce the content of galacto-oligosaccharides, vicine/convicine, tannins, produce vitamin B12 and affect the content of the bioactive compounds of the products.

2. Materials and methods

2.1. Plant materials

Peas (*Pisum sativum*) and faba beans (*Vicia faba* L.) were harvested in 2019 in Southwest Finland and purchased from Yp aj an Mylly (Yp aj a, Finland). Green lentils (*Lens culinaris*) were purchased from the local supermarkets (GoGreen, Canada).

2.2. Microorganisms and culture conditions

The selection of microbial consortia for fermentations was based on the results obtained from pea and faba bean cultivations, using 13 single LAB strains belonging to genera *Lactiplantibacillus*, *Limosilactobacillus*, *Levilactobacillus*, *Weissella*, *Pediococcus*, *Leuconostoc*, and *Lactococcus*, recently published in Kahala et al. (2023). Four different microbial mixtures containing three to five strains were designed for the fermentation of faba bean, oat, and lentil: starters 31, 51, 34 and 44 (Table 1.), of which starter 31 has been previously described in fermentation of faba bean-oat mixture (Kahala et al., 2023). Selection criteria were the ability of the strains to decrease the level of antinutrients, such as galacto-oligosaccharides and vicine/convicine, that are frequently found in pulses. In the single strain cultivations in pea and faba bean, reported in Kahala et al. (2023), high reduction levels of raffinose, stachyose and verbascose were obtained with several strains. *Pediococcus pentosaceus* (AM174) and *Leuconostoc mesenteroides* (PM173) selected in this study were among the best performing strains. Furthermore,

Table 1

The microbial mixtures selected for fermentations.

	Species	Strain	Origin
Starter 31	<i>Levilactobacillus brevis</i>	B2 M1A	Luke, isolated from pulses
	<i>Pediococcus pentosaceus</i>	AM174	Luke, isolated from pulses
	<i>Limosilactobacillus fermentum</i>	TM314 ^a	NMBU, isolated from soybean/soybean-maize blends
Starter 34	<i>Propionibacterium freudenreichii</i>	P18	NMBU, Laboratory of microbial gene technology (LMG)
	<i>Lactiplantibacillus plantarum</i>	MD20	Luke, isolated from cereals
Starter 44	<i>Pediococcus pentosaceus</i>	AM174	Luke, isolated from pulses
	<i>Propionibacterium freudenreichii</i>	P18	NMBU, LMG
	<i>Pediococcus pentosaceus</i>	AM174	Luke, isolated from pulses
	<i>Limosilactobacillus fermentum</i>	TM314	NMBU, isolated from soybean/soybean-maize blends
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	AM172B	Luke, isolated from pulses
Starter 51	<i>Lactiplantibacillus plantarum</i>	MD20	Luke, isolated from cereals
	<i>Leuconostoc mesenteroides</i>	PM173	Luke, isolated from pulses
	<i>Limosilactobacillus fermentum</i>	TM314	NMBU, isolated from soybean/soybean-maize blends
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	AM172B	Luke, isolated from pulses
	<i>Pediococcus pentosaceus</i>	AM174	Luke, isolated from pulses

^a Previously named LF314.

Levilactobacillus brevis (B2M1A) appeared to be effective in GOS reduction especially in faba bean fermentations, also *Limosilactobacillus fermentum* (TM314) and *Lactococcus lactis* subsp. *lactis* (AM172B) were shown to degrade GOS but were less efficient. Vicine and convicine degradation was highest in the cultivation of faba bean with *Lactiplantibacillus plantarum* (MD20) but also *Pediococcus pentosaceus* (AM174) was shown to reduce the levels of vicine and convicine. (Kahala et al., 2023). These strains were selected for the microbial consortia and the mixtures were designed not only for the ability to degrade antinutrients but also with the aim to have balanced growth with desired pH decrease. The best performing four mixtures were selected for further studies, with the aim for a harmonious and efficient fermentation process, leveraging the strengths of each microorganism for the desired outcomes. *Propionibacterium freudenreichii* P18 was selected to study its ability to produce vitamin B12 in pulse-based fermentations.

2.3. Starter preparation

Lactic acid bacteria were cultivated in de Man Rogosa Sharpe (MRS) broth (Becton, Dickinson and Company, France) overnight at 30 °C. Cells were centrifuged at 5500 g for 3 min at 20 °C, washed with sterile tap water, and then suspended in sterile tap water at a volume equivalent to 1/10 of the initial volume. *L. plantarum* MD20 strain was shown to have the growth rate higher compared to other LAB strains, therefore, to limit the excessive growth over other bacteria 1% inoculum size was selected. For the other LAB, the inoculum size was 2%, which is typical for their optimal performance in the starter culture. The cell counts of the individual LAB strains in the beginning of fermentation varied from 5.9 to 7.3 log cfu/g and for *P. freudenreichii* P18 the variation was from 8.4 to 9.2 log cfu/g.

Propionibacterium P18 was cultivated anaerobically in sodium lactate broth (SLB) for 3 days at 30 °C, then refreshed and incubated 24 h in the same medium at 30 °C. The SLB broth was prepared as described by  stlie, Vegarud, and Langsrud (1995). The cells were centrifuged 5500g for 3 min at 20 °C, washed with sterile tap water and then suspended in sterile tap water at a volume equivalent to 1/10 volume of the initial volume. P18 was added as a 20% inoculum to the final fermentation

volume of the pulse-water mixture. To support better vitamin B12 production, higher inoculum size for propionibacteria was used. It was based on the slower growth rate of the propionibacteria compared to LAB. This is also supported by the results of Xie et al. (2019).

2.4. Fermentations

2.4.1. Pretreatments and fermentation conditions

The pulses' pretreatment protocol for fermentations containing LAB starters included rinsing, overnight soaking, crushing, and boiling with water. The dry matter was adjusted to 13% for lentil and to 19% and 18% for faba bean and pea, respectively. A cooking processor (KitchenAid Artisan Cook Processor, 5KCF0104, Kitchen Aid, MI, USA) was used for grinding and heat treatment of the seeds. The process is presented as a flow chart in Fig. 1A. The heat treatment parameters of the cook processor were adjusted for each pulse medium separately. Pre-heating using temperature set at 125 °C was continued until boiling, around 15 min, depending on the raw material. The device's heating element temperatures are indicated in the process chart. Fermentations with LAB starter mixtures were performed at 30 °C for 2 days.

The pretreatment and fermentation conditions with LAB and PAB differed from LAB fermentation (Fig. 1 B). The pretreatment protocol included grinding the raw material into flour Ø1mm using a laboratory mill (Laboratory Mill 120, Perten Instruments, Stockholm, Sweden), adding water to adjust the dry matter to around 10 % and boiling. Lower dry matter was selected since the fermentation process included an aeration phase, which was easier to perform in lower viscosity medium. The parameters were adjusted to each raw material as shown in Fig. 1B. Fermentations with starters containing LAB + PAB were incubated for 3 days at 30 °C anaerobically, followed by one day of aerobic incubation at room temperature. All the fermentations were carried out in triplicate. Two different controls were used in the study, one for LAB fermentations with the same pretreatment as the pulse material (Control1) and the other for LAB + PAB fermentations (Control2). The controls were stored in cold to prevent unwanted contaminant growth.

The success of the fermentation process was confirmed by determining the pH, growth of LAB and PAB, and absence of enterobacteria, yeasts, and moulds. At the end of the fermentation, samples were collected for analyses, including sugars, galacto-oligosaccharides, phenolic compounds, tannins, and organic acids. Samples for vitamin B12 were collected from the fermentations containing starter with LAB and PAB. Samples of faba bean were also collected for vicine/convicine analyses.

2.5. Microbiological analyses

Collected samples (10 g) in 90 ml Ringer's solution (VWR, Darmstadt, Germany) were homogenized (230 rpm, 30 s) with Stomacher 400 Circulator (Seward, UK) and serial dilutions were prepared in Ringer's solution. Lactic acid bacteria were enumerated using MRS Agar (Difco, Becton-Dickinson). Colonies were counted after incubation at 30 °C for 2–4 days. Propionibacteria were enumerated on sodium lactate agar (SLA), made by adding 1,5% (w/v) agar to SLB broth incubated anaerobically at 30 °C for 5 days (Anaerocult A, Merk, Millipore, Burlington, MA US). Lactic acid bacteria also form colonies on SLA, so the counts were estimates based on the morphology of P18.

To confirm the hygienic quality of the product, analysis of enterobacteria on Violet Red Bile Glucose agar (VRBGA, Neogen, UK) incubated at 37 °C for 24 h and yeasts and moulds on Dichloran Rose Bengal Chloramphenicol agar (DRBCA, Becton, Dickinson and Company, France) at 25 °C for 3–5 days (Neogen, USA) were performed.

2.6. Chemical analyses

The dry matter content was determined by drying the samples at 105 °C for 18 h, until a constant final weight was reached.

2.6.1. Carbohydrates

The extraction of carbohydrates was done according to Kahala et al. (2023). Briefly, the sample was extracted with 30 mL of distilled water

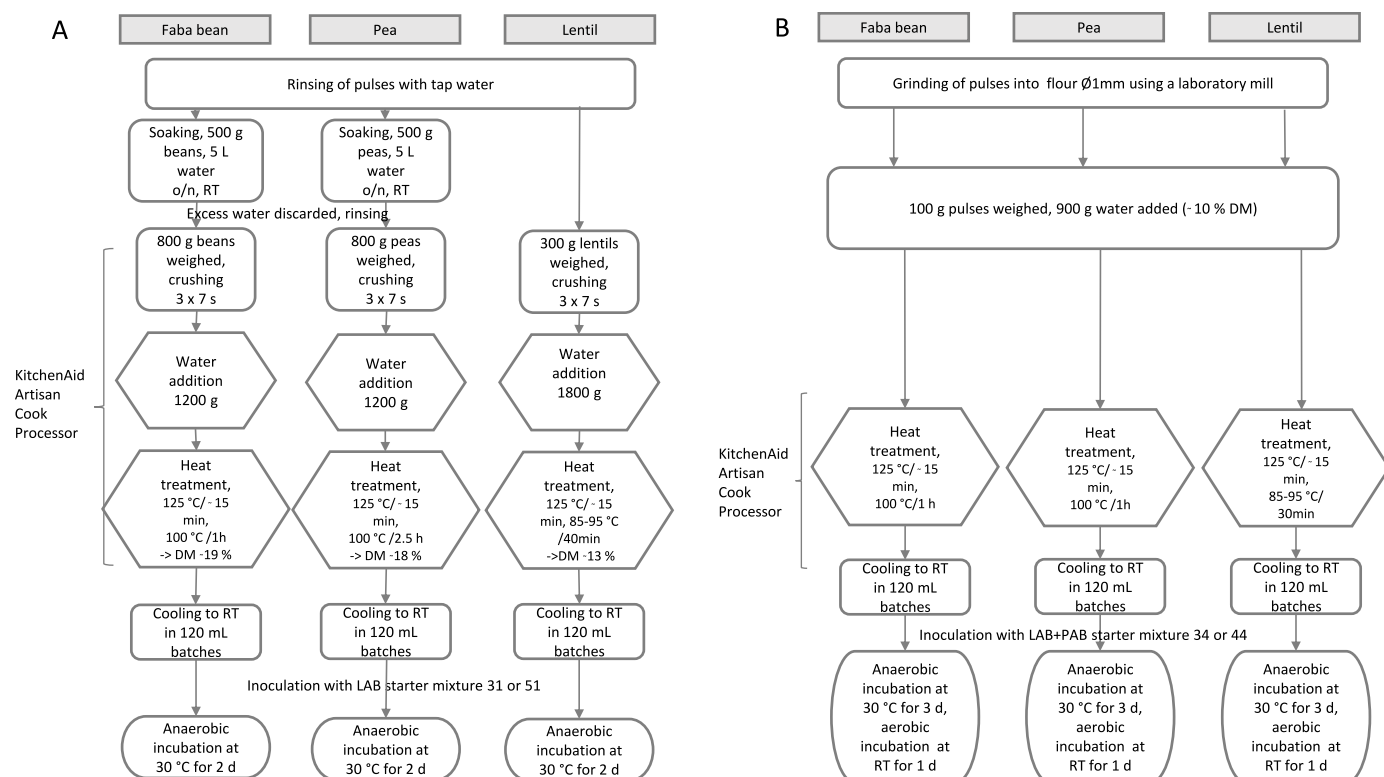


Fig. 1. Flow chart of the pretreatments and the fermentations with LAB (A) and LAB + PAB (B) for each raw material.

first in an ultrasonic water bath for 10 min followed by a water bath with agitation (80 rpm) at 80 °C for 1 h. After centrifugations and Carrez treatments, the sample was analysed by high-performance ion chromatography (HPIC). Carbohydrates were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex Integrion, Thermo Fisher Scientific, Waltham, MA, USA) using a Dionex CarboPacPA210–4 µm column (2 × 150 mm) and 12 mM KOH as the mobile phase (0.2 mL/min) at 30 °C.

2.6.2. Organic acids

Organic acids were quantified by high-performance liquid chromatography (HPLC), using a modification of the method of Marsili, Ostapenko, Simmons, and Green (1981) as described by Dysvik et al. (2020), except that the samples were analysed through a cation-H-microguard precolumn (Bio Rad Laboratories, California, US) before the separation column. Standards included in the setup were the organic acids citric, alpha-ketoglutaric acid, orotic, pyruvic, succinic, lactic, formic, acetic, uric, propionic, and pyro-glutamic acids (MilliporeSigma, Burlington, Massachusetts, US).

2.6.3. Vitamin B12

Vitamin B12 was determined via liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). All work was carried out with minimal daylight and samples were protected from light. One gram of sample was weighed and 5 mL of 5 mM ammonium acetate (aq, Merck Life Science, Oslo Norway) was added. The mixture was sonicated for 30 min after which the sample was centrifuged, and the supernatant was transferred into a 2 mL vial for analysis.

Instrumentation was an Acquity UPLC system (Waters Norway, Oslo Norway) and a Xevo G2-S Q-TOF (Waters Norway, Oslo Norway). Separation was done on an Acquity BEH-C8 column (100 × 2.1 mm, id., Waters Norway, Oslo Norway) at 45 °C. The injection volume was 5 µL. The mobile phase was (A) 5 mM ammonium formate (Merck Life Science, Oslo Norway) in LCMS-grade Ultra water (HECO Laboratoriestyr, Oslo Norge), and (B) LCMS-grade Ultra methanol (HECO Laboratoriestyr, Oslo Norge). The mobile phase flow (0.45 mL/min) was initiated with a 2-min hold at 3% of phase B followed by a gradient increase to 25% of phase B from 2 to 8 min. A final wash step at 99% of phase B followed (for 3 min) before returning to initial conditions (3% B) for the next injection/sample.

Analytes were detected in positive mode after electrospray ionization, and quantification was performed on extracted ion chromatograms for the double-charged molecular ion (M+2H)²⁺ of each analyte. Detected ions were at $m/z = 664.8$ (hydroxycobalamine), $m/z = 678.3$ (cyanocobalamine), $m/z = 790.3$ (adenosylcobalamine) and $m/z = 672.8$ (methylcobalamine). Quantification was against freshly prepared standards (Merck Life Science, Oslo Norway) in 5 mM Ammonium Acetate (aq) at 1, 5, 10, 50 and 100 ng/mL of each analyte.

2.6.4. Phenolic and other UV absorbing compounds

For the determination and quantitation of phenolic and other UV-absorbing semi-polar compounds, freeze-dried samples (5 g) were extracted twice with 80% methanol (50 mL) in an ultrasonic bath for 30 min. The samples were centrifuged, and the supernatants were evaporated to dryness in a rotary evaporator. For analyses, samples were redissolved in methanol (2 mL) and analysed by Agilent 1100-series high-performance liquid chromatography equipped with a diode array detector (HPLC-DAD) (Agilent, Santa Clara, CA, USA). Phenomenex Kinetex® C18 (150 × 3.0 mm; 5 µm i.d.; 100 Å) was used in an analytical column at 35 °C. The mobile phase consisted of 0.05 M phosphate buffer (A) at pH 2.4 and methanol (B) with the following gradient: 5–60% B in 45 min; 60–98% B in 10 min, 98–100% in 10 min, and at 100% for 20 min. The flow rate was 0.6 mL/min. The chromatograms were obtained at 245, 280 and 370 nm wavelengths. For identification purposes, UV spectra were recorded at 190–600 nm. Based on the in-house UV spectral libraries and combined with the retention times, some

compounds were identified, while others were putatively identified. Quantitation of the main components was done at 280 nm by gallic acid (Sigma-Aldrich, Burlington, MA, USA) and for tryptophan, coumaric acid, caffeic acid and ferulic acid by authentic standards (Sigma-Aldrich) The flavonoids, identified as such by their typical UV-spectra, were quantitated as quercetin (Sigma-Aldrich) at 370 nm. Lenticin (L-Tryptophan betaine) was quantitated as tryptophan and using a conversion factor 1.20 based on the MW-ratio. The samples were prepared as three replicates. For further identification, the samples were analysed by ultrahigh-performance liquid chromatography quadrupole-time-of-flight tandem mass spectrometry (UHPLC-QTOF-MS/MS). QTOF equipment and analytical conditions as in Karonen and Pihlava (2022).

2.6.5. Tannins

Condensed tannins (CT, proanthocyanidins) were determined by HPLC-DAD-FLD after thiolytic degradation. Freeze-dried samples were ground and weighed (20–30 mg) into 1.5 mL Eppendorf vials and 1 mL of depolymerization reagent (3 g cysteamine (MilliporeSigma, Munich, Germany) dissolved in 56 mL of methanol (VWR International, Helsinki, Finland) acidified with 4 mL of 13 M HCl (Merck, Darmstadt, Germany) was added. After 60 min incubation at 65 °C the samples were transferred into an ice bath, and cold samples were filtrated into HPLC vials and analysed on an Agilent 1290 Infinity UHPLC device (Agilent, Santa Clara, CA, USA) as described by Korkalo et al. (2020). Catechin, epicatechin, galocatechin, epigallocatechin (MilliporeSigma, Munich, Germany), and procyanidin B2 (Extrasynthese, Lyon, France) were used as reference standards for quantification. The samples were prepared as three replicates.

2.6.6. Analysis of vicine and convicine in faba samples

Vicine and convicine were analysed according to Kahala et al. (2023). Briefly, the samples (1 g) were extracted with 10 mL of ultrapure water in a magnetic stirrer for 1 h, centrifuged and further treated as described by Kahala et al. (2023). Samples were analysed by an Agilent 1100 series high-performance liquid chromatography with a diode array detector (HPLC-DAD). As an analytical column Atlantis T-3 (2.1 × 150 mm, 3 µm) (Waters Corp., Milford, MA, USA) was used with a gradient of 50 mM phosphate buffer and methanol at 0.2 mL/min. Quantification of vicine (Sigma-Aldrich, St. Louis, MO, USA) and convicine was done at 280 nm and for the identification purposes spectrum from 190 to 450 nm was recorded.

2.7. Genomic analysis

All seven isolates used in this study were analysed for whole genome sequencing. Genomic DNA was extracted from 1 mL of overnight culture using the DNeasy Powerfood microbial kit (Qiagen, Düsseldorf, Germany). Genomic DNA was normalized to 0.2 ng/µL after quantification using the Qubit 3.0 fluorometer (Life Technologies, Waltham, MA USA). According to the manufacturer's instructions, the sequencing library was prepared using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, California, USA). Sequencing was performed using the Illumina MiSeq (Illumina, San Diego, California, USA) and V3 chemistry. Raw sequences were quality filtered using Trimmomatic (Bolger, Lohse & Usadel, 2014) and de novo assembled using Shovill pipeline (<https://github.com/tseemann/shovill>). Contigs shorter than 1000 bp and with coverage <3 were removed prior annotation step. All the genomes used in this study were annotated using the Prokka pipeline (Seemann, 2014) and MicrobeAnnotator (Ruiz-Perez, Conrad, & Konstantinidis, 2021). Genomes sequenced as part of this study are available at DDBJ/ENA/GenBank under the BioProject PRJEB70875.

2.8. Statistical analysis

The fermentation process was carried out in three separate biological replicates, and each sample was analysed per triplicate. The results of all

studies are shown as mean \pm SD. For independent unpaired samples, p-values were calculated using Student's t-test (in R). All P values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg process, and adjusted $P < 0.01$ was considered significant.

3. Results and discussion

3.1. Fermentations with the selected microbial mixtures

Four different microbial mixtures (Table 1.), two of which contained *P. freudenreichii* strain (P18) to produce vitamin B12 in fermented product, were used in fermentations. All the starter mixtures grew well in pulse-based raw materials and a clear pH drop was detected (Table 2.). The LAB counts during the 48-h incubation reached up to 8.6–9.2 log cfu/g with all the mixtures. The pH decreased 1.4–2.7 units during LAB fermentation and the highest decrease was evidenced in lentil cultivations with starter 51. In LAB + PAB fermentations, the pH decreased by 1.5–2.3 pH units during the 96-h fermentation period, and the highest decrease was observed in lentils fermented with starter 44. The LAB numbers ranged from 8.6 to 9.1 log cfu/g. (Table 2.). Hygienic quality of fermented products was good. All the counts of enterobacteria, yeasts and moulds were under the detection limit.

Table 2

pH values, microbial counts on MRS agar, and dry matter after fermentation (2 days at 30 °C for starters 31 and 51 using control 1). pH values, microbial counts on MRS and SLA agar and dry matter after fermentation (3 days at 30 °C, 1 day at 20 °C for starters 34 and 44 using control 2). Heated, unfermented sample served as a control. All the fermentations were carried out in triplicate.

Material	Samples	pH	MRSA (log cfu/g)	SLA (log cfu/g)	DM (%)	
Faba bean	Control 1	6.4 \pm 0.07	<2.0 \pm 0.00		19.44 \pm 0.17	
	Starter 31	5.02 \pm 0.05	8.85 \pm 0.06		18.65 \pm 0.30	
	Starter 51	4.43 \pm 0.04	8.88 \pm 0.04		18.62 \pm 0.22	
	Control 2	6.36 \pm 0.03	<2.0 \pm 0.00	<2.0 \pm 0.00	9.31 \pm 0.40	
	Starter 34	4.86 \pm 0.01	9.10 \pm 0.34	9.6 \pm 0.12	10.55 \pm 0.43	
	Starter 44	4.71 \pm 0.01	8.88 \pm 0.20	<8.0 \pm 0.0 ^a	9.75 \pm 0.74	
	Pea	Control 1	6.26 \pm 0.01	2.38 \pm 0.25		17.94 \pm 0.04
		Starter 31	4.33 \pm 0.03	9.19 \pm 0.13		17.93 \pm 0.34
		Starter 51	4.27 \pm 0.04	8.61 \pm 0.49		17.84 \pm 0.35
		Control 2	6.57 \pm 0.05	<2.0 \pm 0.00	<2.0 \pm 0.00	9.57 \pm 0.46
		Starter 34	4.74 \pm 0.03	8.78 \pm 0.04	8.89 \pm 0.09	11.06 \pm 0.53
		Starter 44	4.55 \pm 0.02	8.62 \pm 0.10	8.94 \pm 0.04	11.46 \pm 0.30
Lentil		Control 1	6.67 \pm 0.01	2.14 \pm 1.22		12.98 \pm 0.59
		Starter 31	4.55 \pm 0.02	8.68 \pm 0.06		13.07 \pm 1.04
		Starter 51	3.96 \pm 0.12	8.75 \pm 0.08		12.74 \pm 1.70
		Control 2	6.79 \pm 0.07	<2.0 \pm 0.00	<2.0 \pm 0.00	9.78 \pm 0.44
		Starter 34	4.62 \pm 0.01	9.04 \pm 0.12	9.54 \pm 0.07	9.18 \pm 0.55
		Starter 44	4.51 \pm 0.02	8.80 \pm 0.02	<8.0 \pm 0.00 ^a	9.01 \pm 0.33

^a Indicative counts, both LAB and PAB grow on the SLA plates.

3.2. Carbohydrates

3.2.1. Sugars

Sucrose was the main sugar in all the pulses, and it degraded efficiently during the fermentation (Table 3.). Maltose was found in the control samples with the highest concentration (8.06 \pm 0.53 μ g/mg DM) in faba bean and it was found to be degraded by fermentation. As an exception to other pulses, boiled lentils contained a small amount of trehalose, up to 2.27 \pm 0.09 μ g/mg DM; however, it decreased during fermentation, especially with starter 44. Previously, a small amount of trehalose in green lentils has also been reported by Lee et al. (2017). Galactose was found in small amounts in all the studied pulses and reduction of the galactose content was evidenced by fermentation, except for lentils with starter 31. These findings indicated the ability of the selected microbes to use the sugars present in pulses for their growth.

3.2.2. Galacto-oligosaccharides (GOS)

Compared to other galacto-oligosaccharides, raffinose content was low in all raw materials. Pea flour had a higher content of raffinose than the other pulses. In lentil, stachyose content was highest and it was also the main GOS of lentils (Table 4.). Heat treatment resulted in a reduction of raffinose by 20.6% in the control 1 of faba beans, 47.7% in control 1 of peas, and 30.8% in control 2 of peas. Additionally, verbascose showed a decrease of 26.8% in pea control 1. Noticeably, the levels of other galacto-oligosaccharides remained constant across all pulse materials during the heat treatment.

Varney et al. (2017) established low-FODMAP cutoff values for every FODMAP carbohydrate. The cut-off value for e.g., fructan and α -galacto-oligosaccharides, which are found in grains, legumes, nuts, and seeds, is less than 0.3 g per meal. In our study, degradation of the main galacto-oligosaccharides was observed in all the studied fermented pulses. The overall amount of GOS (raffinose, stachyose and verbascose) in unfermented faba bean, pea, and lentils was 0.51, 0.59 and 0.57 g/100 g of product (control 1) and 0.27, 0.43, and 0.44 g/100 g of product (control 2) respectively. Following fermentation, almost all the raffinose, stachyose and verbascose degraded with all starter mixtures. In fermented products levels of raffinose were under 0.06 \pm 0.01, stachyose under 0.77 \pm 0.48 and verbascose under 1.94 \pm 0.82 μ g/mg DM (Table 4.).

The main enzyme metabolizing GOS was found to be α -galactosidase. However, in the fermented samples a new peak with a retention time 11.1 min appeared at the HPAED-PAD chromatograms. In our study, the peak was quantitated using raffinose as a reference (Supplementary Figure A). This oligosaccharide was identified at the Karlsruhe Institute of Technology (KIT) (by Dr. Daniela Schäfer, member of Prof. Mirko Bunzel's group), as a tetramer (manninotetraose) originated from the removal of fructose unit from verbascose by the β -fructofuranosidase activity. This enzyme is well known in bifidobacteria, but also some lactobacilli have been found to synthesize it (Liu et al., 2021; Nyssölä, Ellilä, Nordlund, & Poutanen, 2020).

As the raffinose family oligosaccharides are known to be the main soluble sugars in many pulses and are considered to cause gastrointestinal disorders, it is important to find ways to reduce their concentration. This study demonstrated that fermentation with both the LAB, and LAB combined with PAB can significantly reduce the content of the GOS. The selection of the starter strains is highly important when considering the effect on the GOS content during fermentation. Previous studies have indicated the reduction of GOS in faba bean by *L. plantarum* (Verni, De Mastro, De Cillis, Gobbetti, & Rizzello, 2019), in pea and lentils by adding *L. plantarum* and *L. brevis* (Curiel et al., 2015; De Pasquale, Pontonio, Gobbetti, & Rizzello, 2020) and in lentils by *L. acidophilus* and *L. fermentum* (Verni, Demarinis, Rizzello, & Baruzzi, 2020). In the study of Verni et al. (2019) a reduction of GOS was studied using *L. plantarum* in fermentation of thirteen faba bean accession, resulting in a marked decrease or complete degradation of verbascose, stachyose and raffinose. Interestingly, clear differences between the faba bean accessions

Table 3

Amounts of simple sugars in faba bean, pea, and lentil flour, heat-treated control, and fermented products. All the analyses were carried out in triplicate in all three parallel fermented faba bean, pea, and lentil samples. *Values significantly differ from their respective control ($p < 0.01$).

Material	Samples	Sugars ($\mu\text{g}/\text{mg}$ of DM)					Maltose
		Trehalose	Galactose	Glucose	Sucrose	Fructose	
Faba bean	Flour		0.19 ± 0.01	0.25 ± 0.06	23.99 ± 1.51	0.29 ± 0.11	
	Control 1	0.58 ± 0.19	0.73 ± 0.15	0.67 ± 0.16	19.37 ± 0.15	0.59 ± 0.27	
	Starter 31	*	0.38 ± 0.25*	0.10 ± 0.06*	0.06 ± 0.06*	*	
	Starter 51	*	0.38 ± 0.28	0.12 ± 0.09*	*		1.82 ± 0.05
	Control 2		0.44 ± 0.04	0.58 ± 0.04	24.34 ± 1.99	0.35 ± 0.04	
Pea	Flour		0.74 ± 0.03	0.28 ± 0.19	33.72 ± 3.22	0.30 ± 0.07	
	Control 1		1.90 ± 0.11	0.55 ± 0.06	24.26 ± 0.50	0.76 ± 0.17	
	Starter 31		0.07 ± 0.01*	0.22 ± 0.02	0.23 ± 0.13*	0.16 ± 0.04*	0.13 ± 0.02
	Starter 51		0.14 ± 0.09*	0.26 ± 0.14*	0.38 ± 0.18*	0.36 ± 0.40	
	Control 2		2.12 ± 0.21	0.27 ± 0.04	33.25 ± 2.53	0.48 ± 0.02	
Lentil	Flour		0.49 ± 0.04*	0.25 ± 0.01	17.6 ± 2.36	0.28 ± 0.05	
	Control 1	2.25 ± 0.31	0.93 ± 0.10	0.53 ± 0.07	15.8 ± 1.60	0.62 ± 0.11	2.61 ± 1.05
	Starter 31	1.83 ± 0.23	3.46 ± 3.00	0.46 ± 0.26	0.29 ± 0.03*	0.07 ± 0.01*	
	Starter 51	1.43 ± 0.13*	0.05 ± 0.04*	0.08 ± 0.07*	0.68 ± 0.03*	0.06 ± 0.01*	
	Control 2	2.27 ± 0.05	1.83 ± 0.09	0.7 ± 0.08	15.92 ± 0.4	0.60 ± 0.01	0.46 ± 0.18

Table 4

Level of galacto-oligosaccharides (GOS) in faba bean, pea and lentil flour, heat-treated control and fermented samples. All the analyses were carried out in triplicate in all three parallel fermented samples. *Values significantly differ from their respective control ($p < 0.01$).

Material	Samples	GOS ($\mu\text{g}/\text{mg}$ of DM)		
		Raffinose	Stachyose	Verbascose
Faba bean	Flour	1.60 ± 0.01	5.69 ± 0.17	19.07 ± 0.67
	Control 1	1.27 ± 0.05	6.64 ± 0.15	18.49 ± 0.52
	Starter 31	*	0.33 ± 0.03*	0.18 ± 0.17*
	Starter 51	*	0.19 ± 0.03*	0.12 ± 0.16*
	Control 2	1.5 ± 0.12	6.56 ± 0.47	20.9 ± 1.45
Pea	Flour	7.78 ± 0.39	17.21 ± 0.70	20.93 ± 0.81
	Control 1	4.07 ± 0.15	13.41 ± 0.28	15.31 ± 0.46
	Starter 31	0.06 ± 0.00*	0.32 ± 0.32*	0.83 ± 0.53*
	Starter 51	0.05 ± 0.01*	0.77 ± 0.48*	1.94 ± 0.82*
	Control 2	5.38 ± 0.35	17.9 ± 1.13	21.28 ± 1.45
Lentil	Flour	2.98 ± 0.30	29.14 ± 3.39	10.86 ± 1.14
	Control 1	3.30 ± 0.49	29.33 ± 4.09	11.55 ± 1.88
	Starter 31	*	0.92 ± 0.40*	
	Starter 51	*	1.03 ± 0.23*	
	Control 2	2.80 ± 0.09	30.36 ± 0.58	11.47 ± 0.21

were evidenced. In the production of baked goods enriched with legume flour, good results in reducing the GOS content have been achieved in several studies using sourdough technology, also with added LAB strains (Curiel et al., 2015; Galli, Venturi, Pini, Guerrini, & Granchi, 2019).

3.3. Organic acids

The content of organic acids was analysed in the pulses and the results are shown in Table 5. All pulses contained citric acid ranging from 3.20 to 7.35 g/kg DM, with the lowest content in faba bean and the highest in pea, see Table 5. Pulses fermented with starters 31, 51 and 34 showed a complete degradation of the citric acid during fermentation. However, no citrate degradation was seen by starter 44. In citrate-degrading starters, one or more of the following bacteria *L. brevis*,

Table 5

Amount of organic acids in peas, lentils and faba beans after fermentation with different starters where starters 31 and 51 were fermented for 2 days at 30 °C and starters 34 and 44 for 3 days at 30 °C, 1 day at 20 °C. The values reported are the mean values ± standard deviation of three replicates. Heated, unfermented samples served as a control.

Material	Samples	Organic acids (g/kg DM)			
		Citric acid	Lactic acid	Acetic acid	Propionic acid
Faba bean	Control 1	3.20 ± 0.23	–	–	–
	Starter 31	–	25.27 ± 1.46	4.28 ± 0.09	–
	Starter 51	–	32.15 ± 1.06	3.89 ± 0.05	–
	Control 2	3.86 ± 0.15	–	–	–
	Starter 34	–	–	10.76 ± 0.44	17.87 ± 0.98
Pea	Control 1	5.02 ± 0.18	–	–	–
	Starter 31	–	34.64 ± 0.30	3.89 ± 0.09	–
	Starter 51	–	33.54 ± 1.22	4.46 ± 0.16	–
	Control 2	7.35 ± 0.43	–	–	–
	Starter 34	–	–	13.75 ± 1.47	25.18 ± 1.06
Lentil	Control 1	4.31 ± 0.62	–	–	–
	Starter 31	–	25.63 ± 1.11	2.99 ± 0.23	–
	Starter 51	–	36.45 ± 7.43	3.83 ± 0.70	–
	Control 2	3.6 ± 0.43	–	–	–
	Starter 34	–	–	12.88 ± 1.16	24.83 ± 2.01

^a Not detected.

L. plantarum or *Le. mesenteroides* were present, being able to degrade citrate. Citrate can be degraded to acetic acid, diacetyl, acetoin, 2,3 butanediol and CO₂, where diacetyl is associated with a buttery flavour technologically important in many dairy products (Hugenholtz, 1993).

Starters 31 and 51 contained both homofermentative and heterofermentative LAB, and they produced lactic acid in the range 25.27–36.45 g/kg DM. In general, starter 51 produced more lactic acid than starter 31, except for pea. Low amount of lactic acid (7.58 g/kg DM) was found only in the pea samples fermented with starter 44, whereas no lactic acid was found in the other samples fermented with LAB and PAB (starters 34 and 44). Propionic acid bacteria fermented lactic acid into acetic acid, propionic acid and CO₂.

Acetic acid can be produced by heterofermentative LAB, citrate-degrading bacteria and by PAB. The level of acetic acid was considerably lower in fermented samples using starters 31 and 51, ranging from 2.99 to 4.6 g/kg DM compared to starters containing PAB. In the LAB + PAB fermented samples the level of acetic acid ranged from 9.80 to 14.19 g/kg DM with the highest levels in lentils fermented with starter 44 and the lowest in peas fermented with starter 44.

Propionic acid produced by starters 34 and 44 ranged from 17.87 to 31.5 g/kg DM with the highest level in lentils with starter 44 and the lowest in faba bean with starter 34. As with the acetic acid production, the highest levels of propionic acid were measured in pulses fermented with starter 44, except in peas where the highest levels were found with starter 34. The production of organic acids leads to the observed pH drop and, furthermore, to an increased safety of the products.

3.4. Vitamin B12

Vitamin B12 levels were analysed in samples collected from fermentations with starters 34 and 44, containing LAB + PAB. The results showed a good level of vitamin B12. Starter 34 produced the highest level of cobalamins (AdoCbl and HOCbl) in all materials, although there were significant differences between the pulses (Table 6.). The level of produced vitamin B12 can be dependent on the nutrient content of the raw materials and the availability of certain nutrients required for B12 synthesis, such as cobalt, which is higher in faba bean compared to pea and lentil (<https://www.feedtables.com/>). No methylcobalamin (MeCbl) and cyanocobalamin (CNCbl) were detected in the samples. As shown in this work and comparing the results with Xie et al., 2021, different microbe compositions of the starter culture affect the vitamin B12 level achieved. In the faba bean product fermented with starter 34, total cobalamin production (183.2 ng/g AdoCbl and 24.7 ng/g HOCbl) corresponds to 1.9 µg of cobalamins in 100 g of the fermented product (dry matter approx. 10.7%). This can be considered substantial when compared to the EFSA's recommended Adequate Intake (AI) values for cobalamin, 4 µg/day for adults (EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA), 2015). This type of product can therefore represent a good additional source for vitamin B12 for vegetarians

Table 6

Concentrations of different forms of vitamin B12: AdoCbl, HOCbl, MeCbl^a and CNCbl^b content (ng/g) of freeze-dried fermented product. n.d = not detected. Heat treated sample served as a control. The values reported are the mean values ± standard deviation of three replicates.

		AdoCbl ng/g	HOCbl ng/g
Faba bean	Control 2	n.d	n.d
	Starter 34	183.2 ± 65.6	24.7 ± 0.2
	Starter 44	110.4 ± 14.1	19.2 ± 6.7
Pea	Control 2	n.d	n.d
	Starter 34	82.4 ± 8.5	n.d
	Starter 44	64.7 ± 14.0	n.d
Lentil	Control 2	n.d	n.d
	Starter 34	136.7 ± 41.3	19.1 ± 1.1
	Starter 44	89.7 ± 3.4	11.8 ± 2.1

^a MeCbl, and CNCbl were not detected on the samples analyses.

as well, as their diet usually requires a B12 supplement.

3.5. Impact of fermentations on phenolic compounds and other UV absorbing compounds

Pulses are known as good sources of phenolic compounds, which potentially exhibit health effects. The capacity of phenolic compounds to reach target locations and provide protective effects is determined by their bioaccessibility and bioavailability (Roasa, De Villa, Mine, & Tsao, 2021), which fermentation has also been reported to affect (Leonard, Zhang, Ying, Adhikari, & Fang, 2021). In this study, possible changes in profiles of the main phenolic and UV-absorbing compounds were investigated by comparing the HPLC chromatograms at 245, 280 and 370 nm of the fermented samples with the corresponding control sample. Some compounds were putatively identified by their UV-spectra, and some were confirmed by UHPLC-QTOF-MS/MS. The results are presented in Table 7.

In faba beans main peaks identified by intensity (mAU) at 280 nm were phenylalanine (Phe), tryptophan (Trp) and catechin (cat). The biggest peak was tentatively identified as dihydrophaseic acid hexoside (DHA-h) (C21H32O10) by the LC-MS/MS analysis as reported earlier by Kowalczyk et al. (2021). Of the other intense peaks at 280 nm, two eluting at 16.2 and 21.3 min were identified by UV-spectrum and LC-MS/MS as vicine and L-DOPA acylated with valeric/isovaleric acid, respectively. From the 370 nm chromatogram, two most intense peaks with a distinctive UV-spectrum profile of flavonoids (Flav A and B), were followed. An additional compound at retention time 16.4 min appeared in fermented samples. Based on the QTOF analysis it had the elemental composition C15H22O5 and is thus a free dihydrophaseic acid (DHA). Examples of the HPLC chromatograms and UV spectra are presented in Supplemental Fig. S1. The amount of Phe and flavonoids remained constant during the fermentations. Free Trp content was constant except with starter 34. The increase of DHA in fermented samples is explained by the decrease of DHA-h due to the cleavage of hexose unit by microbial activity (Galgano et al., 2021). Interestingly, while the amount of acylated vicine remained fairly constant during the fermentations, L-DOPA-valeric acid ester clearly decreased.

The main peaks identified in lentils by intensity (mAU) at 280 nm were Phe and Trp, lenticin, catechins and two kaempferol glycosides (Flav C and D). Flavonoids C and D were the most dominating peaks in the 370 nm chromatogram. Examples of the HPLC chromatograms and UV spectra are presented in Supplemental Fig. S2. The amount of Phe and lenticin, which is considered a sleep-inducing compound (Ozawa, Honda, Nakai, Kishida, & Ohsaki, 2008), remained constant during fermentations in lentils. Trp remained at a constant level, except with starter 34, with a decrease ca. 90%. Tryptophan has a role in the body to produce serotonin and melatonin as well as in the liver to produce niacin. The flavonoids, including catechins, remained constant during fermentations (Table 7.). Catechins and their derivatives are well known antioxidants, associated in prevention and treatment of chronic diseases (Fan, Sang & Jiang, 2017). The levels of the flavonoids were stable during fermentations. Interestingly, as in the case of faba fermentation, the starter 34 hydrolysed DHA-h partly to free dDHA.

In pea control, the identified main peaks at 280 nm were Phe, Trp, free ferulic acid (FA), p-coumaric acid (p-COU-c) and caffeic acid conjugate (CA-c). Also, DHA-h and late-eluting pisatin were present. At 370 nm, two major peaks of flavonoids (E and F) were followed. In fermentation with starter 34, an additional late eluting peak, Flav G, at 370 nm appeared. By the QTOF analysis it was shown to have the elemental composition C15H10O6, thus most likely being kaempferol or luteolin aglycon. Examples of the HPLC chromatograms and UV spectra are presented in Supplemental Fig. S3.

As with faba and lentil, content of phenylalanine did not decrease, while tryptophan decreased with starter 34. An interesting unknown compound (PFerm 9.0) appeared at 280 nm with starter 34 and to a lesser extent with starter 44. Unfortunately, identification of this

Table 7

Phenolic compounds found in faba bean, lentil and pea. The values reported are the mean values ± standard deviation of three replicates. Heated, unfermented samples served as a control. *Values significantly differ from their respective control (p < 0.01).

Materials	Samples	Phenolic compounds (mg/100g)										
		Phe	Trp	Cat A	DHA-h	VVA	DHA	L-DOPA VA	Flav A	Flav B		
Faba bean	Control 1	1.0 ± 0.2	6.0 ± 0.3	10.3 ± 1.1	11.0 ± 0.7	4.0 ± 0.3		1.8 ± 0.1	0.4 ± 0.0	0.2 ± 0.0		
	Starter 34	1.5 ± 0.0		7.7 ± 0.3		3.4 ± 0.0	14.0 ± 2.2	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.0		
	Starter 44	2.1 ± 0.1	4.5 ± 0.9	13.1 ± 1.5	13.4 ± 0.2	5.5 ± 0.2	1.0 ± 0.1		0.4 ± 0.0	0.3 ± 0.0		
	Control 2	1.5 ± 0.1	12.5 ± 1.1	11.4 ± 1.0	13.0 ± 0.5	5.1 ± 0.4		2.1 ± 0.3	0.4 ± 0.0	0.4 ± 0.0		
	Starter 31	1.2 ± 0.0	7.5 ± 0.7	5.9 ± 0.4	11.0 ± 1.1	4.1 ± 0.4	1.7 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	0.5 ± 0.0		
	Starter 51		7.3 ± 0.2	5.7 ± 0.2		3.2 ± 0.5	11.5 ± 2.0	0.3 ± 0.0	0.3 ± 0.1	0.5 ± 0.1		
			Phe	Trp	Lenticin	Cat	Cat A	DHA-h	Cat B	DHA	Cat C	Flav C
Lentil	Control 1	2.8 ± 0.2	8.9 ± 0.1	15.9 ± 0.3	29.2 ± 0.0	15.5 ± 0.2	5.0 ± 0.1			68 ± 1.6	6.7 ± 0.1	5.6 ± 0.1
	Starter 34	3.5 ± 0.1	1.2 ± 0.0*	17.0 ± 0.2	*	49.1 ± 1.4	*	12.1 ± 0.3*	4.9 ± 0.1*		6.8 ± 0.3	5.7 ± 0.3
	Starter 44	3.9 ± 0.3	5.4 ± 0.1*	17.4 ± 0.2	10.9 ± 0.3*	34.7 ± 0.4	0.2 ± 0.1*	6.9 ± 0.5*	5 ± 0.1*	33.5 ± 1.7*	7.9 ± 0.5	6.6 ± 0.4
	Control 2	2.3 ± 0.3	7.3 ± 1	16.7 ± 2.0	29.9 ± 3.7	13.5 ± 1.8	4.6 ± 0.7			56.1 ± 11.5	6.8 ± 1.2	5.8 ± 1.1
	Starter 31	3.0 ± 0.5	6.8 ± 1	17.5 ± 1.1	17.4 ± 2.3	37.4 ± 3.1	5.2 ± 0.5	6.1 ± 2.9	1.1 ± 0.4	84.1 ± 18.7	8.7 ± 0.1	7.5 ± 0.1
	Starter 51	2.5 ± 0.5	5.2 ± 0.2	16.5 ± 0.5		54.1 ± 3.3		8.8 ± 1.5	4.1 ± 0.2		8.7 ± 0.5	5.9 ± 0.4
			Phe	Trp	PFerm9.0	DHA-h	p-COU-c	CA-c	FA	Pisatin	Flav E	Flav F
Pea	Control 1	3.5 ± 0.3	7.5 ± 0.5		1.5 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	0.3 ± 0.0	0.6 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	
	Starter 34	3.6 ± 0.2	2.3 ± 0.1*	7.6 ± 0.8	*	1.4 ± 0.2	1.2 ± 0.3	*	0.8 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.2 ± 0.0*
	Starter 44	5.1 ± 0.2	6.4 ± 0.0	2.9 ± 0.1	1.4 ± 0.1	1.6 ± 0.0	1.2 ± 0.0	*	0.8 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	
	Control 2	4.8 ± 0.8	10.4 ± 3.1		1.1 ± 0.4	0.8 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
	Starter 31	5.2 ± 0.6	8.6 ± 1.1		1.2 ± 0.4	0.8 ± 0.1	0.4 ± 0.1		0.8 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
	Starter 51	4.7 ± 0.1	9.1 ± 0.2		0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.0		0.8 ± 0.1	0.5 ± 0	0.1 ± 0.0	0.1 ± 0.0

Abbreviations: phenylalanine (Phe), tryptophan (Trp), catechin (Cat, A-C), dihydrophaseic acid hex (DHA-h), vicine-valeric acid (VVA), dihydrophaseic acid (DHA), L-DOPA-valeric acid (L-DOPA VA), flavonoids (Flav, A-G), ferulic acid (FA), caffeic acid conjugate (CA-c), p-coumaric acid conjugate (p-COU-c), unknown compound in fermented pea (PFerm 9.0).

compound was not successful by LC-MS/MS. CA-c and p-COU-c remain constant, but free FA disappeared in fermentations. As in faba and lentil fermentations, the flavonoid content appeared to remain constant. The emerging free aglycon by starter 34 is not originated from the main flavonoids, as their amounts did not decrease respectively. Also of interest is that fermentation didn't affect the amount of pterocarpan pisatin.

3.6. Tannins

Condensed tannins (CTs) in faba bean and lentil were mixtures of procyanidins and prodelphinidins (Table 8.) agreeing with previous studies (Jin, Ozga, Lopes-Lutz, Schieber & Reinecke, 2012). No CTs were detected in pea. It is well-known that CT contents in pulses depend on species and cultivars. While colour-flowered peas can have significant amounts of CTs, white-flowered cultivars are generally considered tannin-free (Jezierny, Mosenthin & Bauer, 2010). Similarly, also faba bean and lentils have cultivars with a wide range of tannin contents (Crépon et al., 2010; Mirali et al., 2016). Generally, the amounts determined in the current study are in good accordance with literature (Duc, Marget, Esnault, Guen, & Bastianelli, 1999; Jin et al., 2012; Zhang et al., 2015).

Table 8

Tannin concentrations measured in faba beans and lentils, average degree of polymerization (DP) and relative proportions of procyanidins and prodelphinidins (PC/PD). Faba beans and lentils were fermented with starters 31, 51, 34 and 44. Heat treated faba bean or lentil served as a control. The values reported are the mean values ± standard deviation of three replicates.

	Sample	Tannins (mg/100g)	DP	PC/PD
Faba bean	Control 1	445 ± 31	5.77 ± 0.14	55/45
	Starter 31	399 ± 57	5.47 ± 0.05	57/43
	Starter 51	355 ± 73	6.02 ± 0.12	55/45
	Control 2	512 ± 3	5.10 ± 0.20	59/41
	Starter 34	372 ± 45	4.99 ± 0.08	59/41
Lentil	Starter 44	327 ± 21	4.86 ± 0.27	60/40
	Control 1	567 ± 88	3.72 ± 0.27	71/29
	Starter 31	531 ± 44	3.76 ± 0.07	71/29
	Starter 51	533 ± 73	3.88 ± 0.18	71/29
	Control 2	578 ± 21	3.66 ± 0.14	70/30
	Starter 34	564 ± 15	4.11 ± 0.26	68/32
	Starter 44	573 ± 16	3.98 ± 0.16	68/32

Generally, the fermentation procedures had a minor effect on the tannins in faba beans and lentils (Table 8.). Only faba bean samples fermented with starters 34 and 44 had significantly lower ($p < 0.05$) tannin contents than the control sample. Proportions of structural units (PC/PD) and the average degree of polymerization (DP) were not affected by fermentation.

Degradation of tannins would be beneficial for protein bioavailability and mineral absorption since condensed tannins can form complexes with proline-rich proteins and may also chelate minerals present in raw material. Although fermentation with LAB is known to be one of the means for degrading tannins (Coda et al., 2015; Verni et al., 2019) only some of the starters used in this study had a reducing effect on the tannin levels.

3.7. Vicine and convicine

Concentrations of the two pyrimidine glycosides, vicine and convicine, were evaluated in faba bean samples. In control samples, the levels exceeded 5000 $\mu\text{g/g}$ for vicine and were around 3000 $\mu\text{g/g DM}$ for convicine. Fermentation was shown to reduce the levels significantly. Starter mixtures 51, 34, and 44 effectively reduced the vicine and convicine, leading to values between 1 and 30 $\mu\text{g/g DM}$ after fermentation (>98% of reduction), while mixture 31 showed a lower level of reduction (61.3% and 22.0% for vicine and convicine, respectively) (Fig. 2.). The level of DOPA (564.2 \pm 29.9 $\mu\text{g/g DM}$), detected with the same analysis protocol, remained stable (only decreasing 3.2 to a maximum of 11.3%) during the fermentation process.

Different approaches to lower glycoside concentrations in faba beans have been studied, e.g. boiling and roasting show some reduction (Corzo-Ríos et al., 2022). However, enzymatic treatments and fermentation have been proven to effectively reduce vicine, convicine, and their aglycones from foods (Kahala et al., 2023). Degradation of vicine and convicine has been demonstrated in the fermentations of faba bean with LAB, e.g., by *L. plantarum*. Significant reductions of the contents have been shown by Verni et al. (2019) and Rizzello et al. (2016). In our previous study, degradation up to 99% and 84% of vicine and convicine, respectively, was achieved using LAB strains with high β -glucosidase activity (Kahala et al., 2023). In this study, with a carefully selected consortium, e.g., starter 34, we achieved degradation of 99.5% and 98.8% of vicine and convicine, respectively.

3.8. Genomic sequencing

Whole genome sequencing and functional annotation of the genomes

was performed for all the strains used in the study. MicrobeAnnotator (Ruiz-Perez et al., 2021) was selected for functional annotation. These allowed the comparison of the genomes and their functional potential based on the KEGG modules. Since the isolates were used together as a consortium for performing the fermentations, the maximum value of module completeness was used for each bacterial consortium. A total of 67 KEGG modules had a completeness of over 80% in at least one microbial consortium (Fig. 3.). The microbial consortia were grouped in two clusters (starter 34 and 44 and starter 31 and 51). This was attributed mainly to the presence of the *Propionibacterium* P18 in the starters 34 and 44. As expected, the figure also shows the presence of the cobalamin biosynthesis for starters 34 and 44. Although both the starters 31 and 51 consisted of LAB only, clear differences in their profiles could be seen. Starter 31 was the starter with the lowest amount of completed KEGG modules and the one with the lowest metabolic potential.

3.9. Performance of selected microbial consortia in pulse fermentations

Some differences in the performance of the microbial consortia were observed, depending on both the starter composition and the raw material used for fermentation. The nutrient composition of the culture medium is possibly one important factor for the adaptation of microbes and successful fermentation in the medium. No big variation was found in terms of growth between the mixtures. All the starter mixtures performed well in reducing pH in all media to pH 5.0 or lower, and the colony counts evidenced their adaptability to pulse materials. In terms of antinutrients, all the starters performed very well in degrading the three main GOS in all the selected pulses, while in degrading vicine and convicine starters 51, 34 and 44 were the most efficient. The fermentations with starters 34 and 44 (LAB + PAB) resulted in more significant changes in the phenolic composition, but no firm conclusions can be drawn regarding their effect on the nutritional value of the products. Acid composition varied between the fermentations and the most significant differences could evidently be seen between the starters containing only LAB and LAB + PAB. It is evident that the propionic acid produced by starters 34 and 44 also affects the taste of the product, as well as what kind of products these starters are suitable for. Fermented plant-based yogurts incorporating cereal or legume proteins have entered in the market as dairy alternatives in recent years. LAB + PAB fermented product could be seen to fit into this category well, having also the advantage to produce vitamin B12.

For improving the nutritional content, particularly B12 levels, using a combination of LAB and PAB would generally be effective. These combined consortia can take advantage of LAB's fermentation benefits

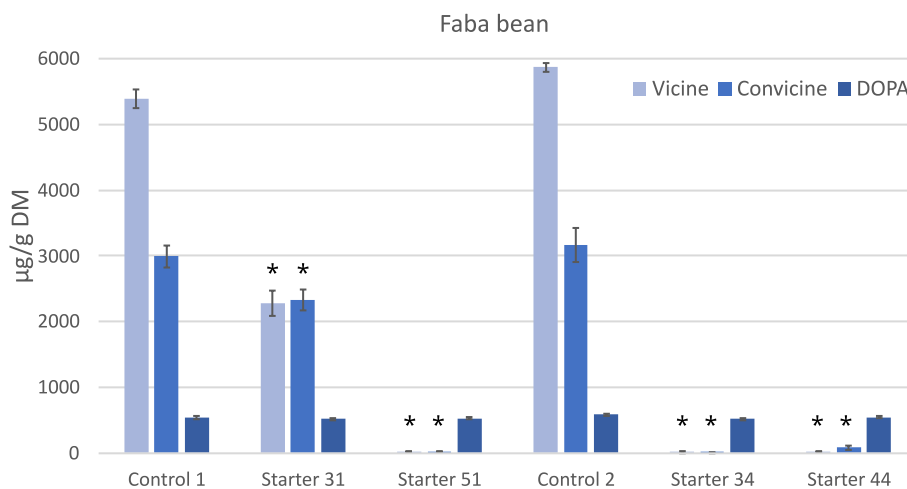


Fig. 2. Degradation of vicine and convicine by selected LAB starters (31, 51) and LAB + PAB starters (34, 44) in fermentations of faba bean. Heat treated raw material served as a control (separate controls for LAB and LAB + PAB fermentations). The values reported are the mean values \pm standard deviation of three replicates. *Values significantly differ from their respective control ($p < 0.01$).

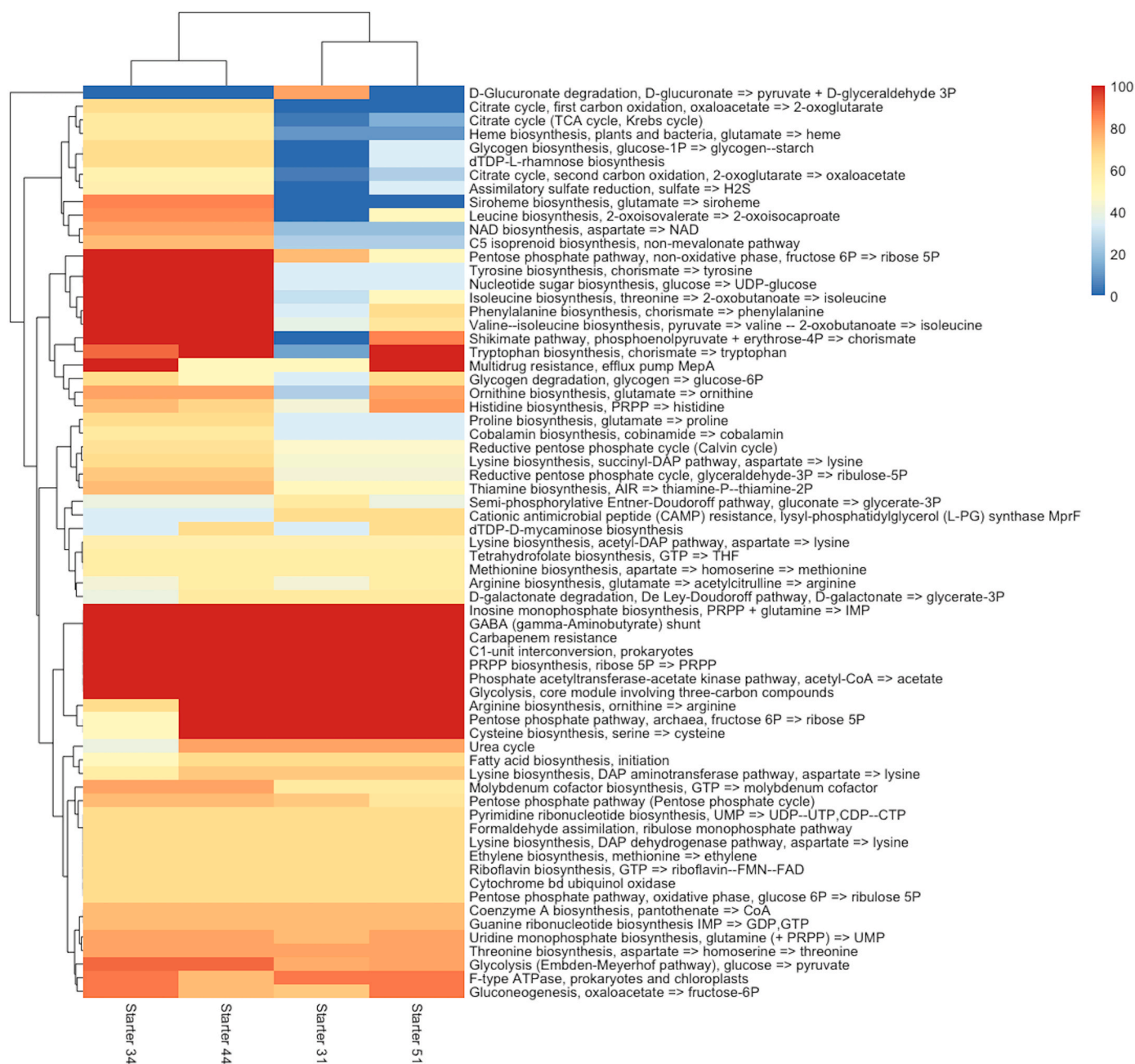


Fig. 3. Heatmap of KEGG modules present in the bacterial genomes grouped at consortium level. Only modules with at least >80% completeness in at least one consortium were kept in the analysis.

and PAB's B12 production. In this study, starter 34 performed better in vitamin B12 production in all the pulse materials, therefore, this combination could be recommended for product development for B12 enriched products. Good production of vitamin B12 was obtained, having potential to develop products for consumers, whose diets consists mainly of plant-based foods.

Both the LAB and LAB + PAB starters, in particular starters 51 (LAB) and 34 (LAB + PAB) have potential to be used for developing new plant-based products with enhanced nutritional quality. In case the production of vitamin B12 is not the main goal, the LAB fermentation protocol was practically simpler and can be recommended. Fermentation with the selected starters is an example of successful biotechnology to achieve nutritionally enhanced pulses, however, the microbial mixtures and the process need to be tailored to the specific nutrient and phytochemical profiles of the selected pulses to achieve optimal results.

4. Conclusions

The microbial consortia developed in the study were successfully applied in plant-based fermentations and showed capacity to enhance the nutritional quality of pulses. Both the reduction in vicine and convicine and the reduction in galactose-oligosaccharide contents were clearly evidenced. Furthermore, since only a few plant-based foods contain substantial amounts of vitamin B12, the production of vitamin B12 by fermentation is of particular interest.

This integrative approach provided a comprehensive understanding of the multifaceted effects of microbial activities, encompassing nutritional enhancements and genomic insights, advancing the development of pulse-based products with improved health and sustainability attributes.

As protein is one of the nutritionally important components of pulses,

the work has continued on studying the proteolytic changes and the release of active peptides during LAB and PAB fermentation. The results will be published soon. This will provide important additional information on the effect of fermentation on the nutritional properties of pulses.

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CRediT authorship contribution statement

Minna Kahala: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lucia Blasco:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Rina Bragge:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Davide Porcellato:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hilde Marit Østlie:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Thomas Rundberget:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jose Antonio Baz-Lomba:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Juha-Matti Pihlava:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jarkko Hellström:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Emilie Gullberg Jørgensen:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Vesa Joutsjoki:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition. **Tove Gulbrandsen Devold:** Writing – review & editing, Writing – original draft, Methodology. **Anne Pihlanto:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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