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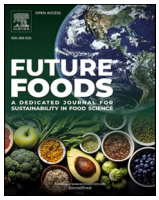

By-product of green pea canning as a substrate for *Propionibacterium freudenreichii* fermentation to fortify bread with vitamin B12

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ABSTRACT

This study focused on upcycling the industrial by-product of canned green peas for the production of vitamin B12 by *in-situ* fermentation and subsequent bread fortification. The by-product, rich in protein (24.3 g/100 g dry matter, DM) and dietary fibre (33.2 g/100 g DM), was fermented with *Propionibacterium freudenreichii* DSM 20271, resulting in a significant vitamin B12 production (1374–1535 ng/g DM). The fermented material was then incorporated into wheat bread at two levels (15 and 20 % on the dough weight), aiming to address the deficiency of vitamin B12 in strictly plant-based diets. From 40 to 70 g of fortified bread provided the recommended daily allowance of vitamin B12 (2.4 μg/day for adults), along with minimal losses in volume development and no significant differences in texture when compared to the controls. The addition of non-fermented or fermented pea by-product batter in breads increased only the content of soluble conjugated and insoluble bound *p*-coumaric acid. The study underscores the potential of using food-grade by-products for enhancing the nutritional value of plant-based products, while also contributing to food-waste reduction.

1. Introduction

It has been estimated that approximately one-third (about 1.3 billion metric tons) of global food production is wasted worldwide annually (FAO, 2011). FAO (FAO, 2019) defines food loss and waste (FLW) as "the decrease in quantity or quality of food along the food supply chain", resulting from decisions and actions taken by suppliers (food loss) and by consumers, retailers, or food service providers (food waste). On average, about 14 % of current food production is lost, with the greatest contribution from roots, tubers, and oil-bearing crops (25.5 % loss), followed by fruit and vegetables (21.6 %), meat and animal products (12 %), and cereals (8.7 %) (FAO, 2019). Approximately 17 % of food is wasted by households (67 %), food services (26 %) and retailers (13 %), corresponding to 931 million metric tons in 2019 (UNEP, 2021). Food diverted to feed, seeds or industrial purposes is considered lost or wasted only if originally destined for human consumption. Inedible parts, though not considered food, cannot be deducted from "food loss" calculation (FAO, 2019). However, some inedible residues (e.g., fruit and vegetable pomace, seeds, or peels) may be considered as non-food side-streams (i.e., waste), when disposed of, or source of food, when utilized. In fact, the Directive, 2008 distinguishes "waste", intentionally discarded substances/objects (art. 3), from "by-products", resulting from a production process without being its primary aim and certainly employed in a subsequent stage (art. 5). Upcycling of food-grade by-products has emerged as a major strategy to dispose of effluents and take advantage of valuable macro- (i.e., dietary fiber and protein above all) or micro-nutrients (especially antioxidants and bioactive compounds) (Plazzotta and Manzocco, 2019). However, the re-use of by-products as food implies appropriate segregation, storage, and/or stabilization to prevent microbial proliferation, an often-overlooked aspect in current studies, as well as investigation of possible contaminants (e.g., heavy metals, pesticides, dioxin, PCBs, pathogens and their toxins) (Socas-Rodríguez et al., 2021). In the European Union, by-products employable as ingredients must have a solid history of safe use; otherwise, they come under the *novel food* regulation, being authorized only after safety assessment (Regulation).

Legume processing generates high volumes of waste, encouraging the food industry to adopt novel strategies for their exploitation, although currently they are merely incorporated into traditional formulations or, more rarely, used for protein extraction (Estivi et al., 2024;

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Tassoni et al., 2020). Various dried by-products from legume processing (pods, hulls, broken seeds) or their extracted or purified fractions were successfully experimented for protein or dietary fiber enrichment of bakery products such as bread (Belghith Fendri et al., 2016b; Ni et al., 2020; Niño-Medina et al., 2019), cake (Belghith Fendri et al., 2016a), crackers (Mousa et al., 2021) or biscuits (Tiwari et al., 2011).

Recent developments in food science and bioengineering have highlighted the potential of fermentation-based processes to enhance the sustainability of food systems. Microorganisms are increasingly being employed as bio-factories for the production of bioactive compounds and biomaterials with applications across the food, agriculture, and pharmaceutical sectors (İncili et al., 2025; Terefe, 2022). These strategies align with broader circular bioeconomy principles, including the upcycling of by-products (Estivi et al., 2024) and the employment of alternative non-animal protein sources (Amiri et al., 2025). Fermentation of plant-based foods has been shown to improve protein quality, reduce antinutritional factors, and enhance the bioavailability of essential micronutrients. In addition, emerging evidence suggests gut-modulating effects linked to microbiota regulation (Cakmak et al., 2025). In parallel with the growing demand for vegan and vegetarian food products, consumer awareness of the link between diet and health has driven a steady increase in demand for plant-based functional foods enriched with bioactive compounds (Abedinia et al., 2025; Kumar et al., 2017; Willett et al., 2019). However, the deficiency of vitamin B12 intake poses a major risk in strictly plant-based diets, naturally devoid of it (Ball, 2006). In fact, while the other B vitamins are also supplied by plant-based foods, vitamin B12 is uniquely present in animal-based foods, at least in considerable amounts (Watanabe and Bito, 2018). Moreover, the vast variety of commercial B12 supplements from algae, often containing the inactive pseudo vitamin, contributes to misleading consumers (van den Oever and Mayer, 2022). Although vitamin B12 is synthesized only by certain bacteria and archaea and then accumulates in animal tissues, it has also been detected in some mushrooms, such as black trumpet, golden chanterelle, and shiitake, as well as in certain plants like sea buckthorn, sidea couch grass, and elecampane. However, its presence in these sources is erratic and likely due to contamination or symbiosis with B12-producing bacteria (Martens et al., 2002; Watanabe, 2007; Watanabe and Bito, 2018). Among non-animal food, fermented soy and other legume products are considered the richest dietary sources of vitamin B12 (Xu et al., 2017) along with green (*Enteromorpha* sp.) and purple (*Porphyra* sp.) lavers and *Chlorella* sp. microalgae (Watanabe, 2007).

In-situ fermentation by *Propionibacterium freudenreichii* DSM 20271 has been recognized as a feasible strategy for vitamin B12 fortification of cereal and legume ingredients (Xie et al., 2021), DSM 20271 is a well-known and well-characterised bacterial strain with extensive safety records, proven efficacy, and genomic profiling (Koskinen et al., 2015). Although vitamin B12 fortification using microbial fermentation was studied in cereal-based side-products (i.e., wheat and rice bran) (Chamlagain et al., 2024; Xie et al., 2021, 2019), to the best of our knowledge no attempts have been made in valorizing industrially-generated legume by-products. Hence, the main aim of this study was to investigate the industrial by-product of canned green peas as a potential matrix for production of vitamin B12 by *in-situ* fermentation and subsequent bread fortification. As a secondary aim, phenolic compounds, abundant in the green pea by-product, were quantified and their fate in enriched breads was followed to determine the possible effect of fermentation. The optimization of the sensory properties of the enriched breads will be addressed in a future dedicated study.

2. Materials and methods

2.1. Materials

2.1.1. Standards and reagents

Cyanocobalamin was obtained from Supelco (Bellefonte, PA, USA). Phenolic standards were obtained from (Sigma-Aldrich, St. Louis, MO, USA), and included: apigenin (CAS 520–36–5), quercetin (CAS 117–39–5), *p*-coumaric acid (CAS 501–98–4), ferulic acid (CAS 537–98–4), catechin (CAS 154–23–4), *p*-hydroxybenzoic acid (CAS 99–96–7), caffeic acid (CAS 331–39–5), and syringic acid (CAS 530–57–4). All solvents used in the analyses—including acetonitrile (CAS 75-05-8), methanol (CAS 67-56-1), trifluoroacetic acid (TFA, CAS 76-05-1), diethyl ether (CAS 60-29-7), ethyl acetate (CAS 141-78-6), and formic acid (CAS 64-18-6)—were of HPLC grade and purchased from Merck (Darmstadt, Germany). Additional analytical-grade reagents were also purchased from Merck (Darmstadt, Germany), including sodium hydroxide (CAS 1310–73–2), acetic acid (CAS 64–19–7), propionic acid (CAS 79–09–4), butyric acid (CAS 107–92–6), succinic acid (CAS 110–15–6), lactic acid (CAS 50–21–5), sulfuric acid (CAS 7664–93–9), hydrochloric acid (CAS 7647–01–0), anhydrous sodium sulphate (CAS 7757–82–6), and sodium cyanide (CAS 143–33–9). Ultrapure water (hereafter referred to as Milli-Q water) was produced using a Milli-Q Plus system (Millipore Corporation, Bedford, MA, USA), equipped with a 0.22 μm final filter and delivering water with a resistivity $\geq$18.2 MΩ·cm.

2.1.2. Preparation of *P. freudenreichii* coltures

P. freudenreichii subsp. *freudenreichii* DSM 20271, the whole-genome-sequenced type strain of the species (Koskinen et al., 2015) was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). This strain, originally isolated from cheese, has been extensively used for vitamin B12 production in plant-based matrices (Kantanen et al., 2024; Xie et al., 2019), food production side streams (Kruk et al., 2024), and food applications without significantly altering the sensory profile (Loivamaa et al., 2025). *P. freudenreichii* DSM 20271 was maintained as 15 % glycerol stocks at −80 °C. For routine cultivation, the strain was grown on yeast extract-lactate (YEL) medium (Malik et al., 2011). Initially, the strain was cultured on solid YEL medium supplemented with 1.5 % agar (Difco, Becton, Dickinson and Company, Sparks, MD, USA) for four days at 30 °C under anaerobic conditions (Anaerocult, Merck, Darmstadt, Germany). Colonies from the agar plates were then transferred to liquid YEL medium and incubated for three days at 30 ± 0.1 °C under microaerobic conditions. Following incubation, cells were harvested by centrifugation (7600 × *g*, 10 min at 20 °C) and resuspended in 10-fold less volume of sterile Milli-Q water prior to inoculation.

2.1.3. Pea canning by-product

The by-product of a canned pea production line—consisting of pods, leaves, hulls, and faulty, broken, or spotted seeds, strained of excess water from hydraulic transport—was sampled on three different days at the Casalasco Società Agricola SPA plant (Gariga di Podenzano, Italy), dried at 55 ± 1 °C for 24 h in a Venticell 55 ventilated oven (Medcenter Einrichtungen Gmbh, Planegg, Germany), milled at 8000 rpm in a ZM200 ultra centrifugal mill (Retsch, Haan, Germany) equipped with a 0.5 mm sieve, and stored at −20 °C until use. An equal mix of three by-product lots was used in the trials.

2.1.4. Fermentation of pea by-product

The final procedure to produce the fermented B12-vitamin-rich materials, outlined in the flow chart in Fig. 1, was fine-tuned in preliminary trials with incremental adjustments and. In the first two trials, a batter was prepared by mixing the powdered by-product and water at a 20 % w/w ratio. The batter was transferred into three 50-mL falcon tubes and inoculated as detailed below. In the second trial, the pH value was adjusted to 6.00 ± 0.05 prior to inoculation. The pasteurization conditions (70 ± 1 °C for 20 min, employing 15 % w/w ratio batter and native pH) were tested and established in the third preliminary trial.

For baking trials, 680 g of a 15 % (w/w) batter was prepared by mixing the powdered by-product with deionized water. The batter was then neutralized to pH 6.00 ± 0.05 with 10 M NaOH (Chamlagain et al.,

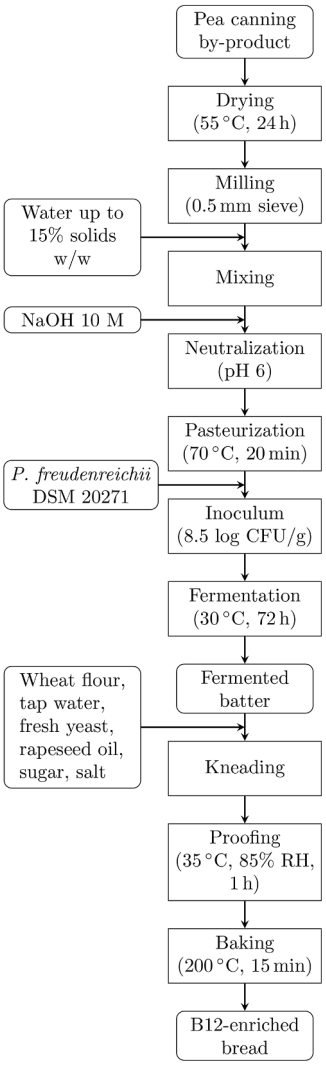

Fig. 1. Flow chart of the final procedure for producing the fermented batter and the enriched breads.

2024), transferred into a 1 L-sized polyethylene bottle, pasteurised at 70 ± 1 °C for 20 min in a water bath, and inoculated (10 %) with *P. freudenreichii* DSM 20271 (Chamlagain et al., 2016; Xie et al., 2019) to achieve an initial cell count of approximately 8.5 log CFU/g. After cells dispersion by mixing, six 30 g portions of batter were aseptically transferred into 50 mL falcon tubes, leaving 500 g batter in the bottle. The fermentation took place in an orbital shaker (Certomat H, Sartorious, Aubagne, France) set at 200 rpm and 30.0 ± 0.5 °C for 72 h (Chamlagain et al., 2024), positioning three tubes horizontally, while the remaining three and the bottle were kept vertically. Two independent batches (1 and 2) were fermented as explained above.

2.1.5. Bread making

Bread making is outlined in the flow chart in Fig. 1. Refined wheat flour, rapeseed oil, fresh baker's yeast, salt and sugar used in breadmaking were acquired from a local supermarket (Helsinki, Finland). The batters fermented in the 1 L bottle were used for baking. Six lots of bread were baked with the straight-dough method, following the recipe of Edelmann et al. (2016) with some modifications (**Supplementary Table 1**): one lot of white bread (Control), one lot of white bread with the addition of 20 % of non-fermented by-product batter (Control-NF), two lots with 15 % enrichment of fermented pea by-product (1a and 2a; one from each of the two batches of fermented material) and two lots with 20 % enrichment (1b and 2b). All the ingredients were mixed at room temperature (22 ± 1 °C) in a BE10 mixer (Metos, Instrumentarium Ltd., Kerava, Finland) for 1 min at the lowest speed and then for 4 min at the middle speed. Three 150 g-pieces of dough were put in aluminium pans and proofed for 45 min in a proofing cabinet (Lillnord TopLine, Odder, Denmark) maintained at 35.0 ± 0.5 °C and 85 % ±2 % relative humidity. A dough piece (ca. 50 g) was sampled after proofing and immediately stored at −20 °C. The proofed-doughs were baked in a convection oven (Sveba Dahlen, Fristad, Sweden) set at 200 °C for 15 min with an initial 15 s-long steam injection. After cooling for 1 h, the breads were weighed. Three slices (25 mm thick and weighing approximately 30 g) were cut from the central part of each bread and crushed with a GM200 cutter mill (Retsch, Haan, Germany) at 7000 rpm for 30 s. The crushed breads were packaged in plastic bags and stored at −20 °C until further analyses.

2.2. Methods

2.2.1. Proximate composition

The moisture content of the by-product, breads, and doughs was determined gravimetrically according to the method 925.10 (AOAC, 1990). Proximate composition of the by-product was determined according to the AOAC methods (AOAC, 1990): protein by the Kjeldahl method 920.87 ($N \times 6.25$); fat by the Soxhlet method 920.39C; raw fiber by the method 962.09 and ash by the method 923.03. Results are expressed as g/100 g dry matter (DM).

2.2.2. Microbial counts

Presumptive lactic acid bacteria (LAB), propionic acid bacteria (PAB) and *Enterobacteriaceae* were enumerated in the pea by-product batter immediately after inoculation (0 h) and post-fermentation (72 h). Ten grams of batter were weighed, homogenised with 90 mL of sterile 0.9 % NaCl solution for 60 s in a stomacher bag using a BagMixer (Interscience, France), and then serially diluted. LAB counts were determined from appropriately diluted samples using Lactobacilli MRS Agar (Neogen, Auchincruive, UK) and the pour-plate method, followed by incubation of plates at 30 ± 0.1 °C under microaerobic conditions. For *Enterobacteriaceae*, Violet Red Bile Glucose Agar (VRBGA, Neogen) was utilized, and the plates were incubated at 37 ± 0.1 °C for 24–48 h. PAB counts were performed on yeast extract lactate (YEL) agar, employing anaerobic jars with an oxygen absorbent (Oxoid, Basingstoke, UK) at 30 ± 0.1 °C for 5 days. Results are expressed as log CFU/g fresh matter (FM).

2.2.3. pH and organic acids

The pH was measured with a Mettler Toledo (Greifensee, Switzerland) pH meter. The content of succinic, lactic, acetic, propionic and butyric acid was determined according to Chamlagain et al. (2018): 1 g of the fermented batter was diluted with 9 mL of ultrapure MilliQ water and, following centrifugation (6900 × *g*), the supernatant was filtered through 0.45 µm PTFE membrane (Millipore, Carrigtwohill Co., Cork, Ireland). 60 µL of the extract was injected in an high-performance liquid chromatography (HPLC) system equipped with an Hi-Plex H column (300 × 6.5 mm; Agilent, CA, USA) and a guard column (Hi-Plex H 50 × 7.7 mm; Agilent, CA, USA) thermostated at 40 °C and coupled to a 996 photodiode array detector (Waters, Milford, MA, USA) and a refractive index detector (2414, Waters). The mobile phase consisted of 10 mM sulfuric acid in water set at a flow rate of 0.5 mL/min. The identification of the individual acids was based on RI and UV detections, while for the quantification RI response was used with external standard curves of 0.12–40 µg per injection of each analyte. The results are expressed as mg/g FM.

2.2.4. Vitamin B12

The vitamin B12 and pseudovitamin B12 content was determined in fermented batters, doughs and breads by ultra-high performance liquid chromatography (UHPLC), as previously described (Chamlagain et al., 2018, 2015; Wang et al., 2022). Briefly, ca. 2 g of the sample was mixed

with 15 mL of the extraction buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid, pH 4.5) and 100 µL of sodium cyanide (1 % w/v in water), boiled for 30 min with occasional shaking and then cooled on ice. Only in the case of dough or bread samples, further treatment was done before centrifugation: 750 µL of α-amylase solution (20 mg/mL) was added and the samples were incubated at 37 °C for 30 min to digest starch. The supernatant was recovered by centrifugation (6900 × *g*, 10 min). The resulting pellets were washed with another 5 mL of the extraction buffer and after a second centrifugation the supernatants were combined and the final volume was adjusted to 25 mL with the extraction buffer. 10–15 mL of the extract was purified through an immunoaffinity column (Easi-Extract; R-Biopharma, Glasgow, UK), recovered with 300 µL of MilliQ water and filtered (PTFE 0.2 µm; Pall, USA) into UHPLC vials. Vitamin B12 was determined by employing a Waters UHPLC system (Milford, MA, USA) equipped with a photodiode array detector (set at 361 nm) and an Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 µm). The mobile phase consisted of a 0.32 mL/min gradient flow of Milli-Q water and acetonitrile, both containing 0.025 % of trifluoroacetic acid. A multilevel standard curve of cyanocobalamin was used to quantitate the vitamin B12 concentrations (0.4–8 ng/injection). Pseudovitamin B12 was identified based on its absorption spectrum and retention time Chamlagain et al. (2015) and quantified as cyanocobalamin. Results are expressed in ng/g FM.

2.2.5. Phenolic compounds

The analysis of the soluble-free, soluble-conjugated, and insoluble-bound phenolic compounds was performed as outlined by Estivi et al. (2022). Briefly, 1 g of sample was extracted three times with 15 mL of 80 % methanol in water. Pooled supernatants and pellet were obtained after centrifugation at 11,200 × *g*. For free phenolic quantification, the supernatant was evaporated under vacuum at 35 °C for 50 min, dried with nitrogen flux, resuspended in 2 mL of methanol:MilliQ water (8:2 v/v) and filtered through a 0.45 µm PTFE membrane. For soluble-conjugated phenolics, the pooled supernatant was partially concentrated by evaporation, digested with 15 mL of 4 M NaOH under nitrogen for 4 h at room temperature, adjusted to pH 1.5–2 with 6 M HCl and extracted twice with 20 mL of diethyl ether:ethyl acetate (1:1 v/v). The extracts were clarified with anhydrous sodium sulphate, filtered through 110 µm glass fiber (Whatman, Maidstone, England), evaporated under vacuum at 35 °C for 5 min, resuspended in 2 mL of methanol: MilliQ water (1:1 v/v) and filtered through a 0.22 µm PTFE membrane. For the analysis of the insoluble-bound phenolic compounds, the pellet was digested and then extracted as outlined above for the soluble-conjugated phenolics. The HPLC analysis was performed using a SepaChrom C18 250 mm × 4.6 mm column, 5 µm (Sepachrom SRL, Italia) and a precolumn C18 10 mm × 4.6 mm, 5 µm (Sepachrom SRL, Rho, Italy) thermostated at 30 °C with a column oven l-2300 Elite LaChrom (Hitachi, Tokyo, Japan). The system included the following: a Rheodyne injector mounting a 20 µL loop; a l-2130 Elite LaChrom (VWR, Hitachi, Tokyo, Japan) pump; a l-2450 Elite LaChrom (Merck, Hitachi, Tokyo, Japan) Diode Array Detector; and the EZChrom Client/Server v3.1.7 software. Gradient elution was achieved using 1 % formic acid in water and acetonitrile mobile phases at 1 mL/min flow rate. The identity of the compounds was verified by the congruence of retention times and UV/VIS spectra with those of 33 pure authentic standards. The unidentified peaks were pooled according to their absorbance spectrum and their similarity to the standard spectra, designating them as the respective standard, but with the addition of the term "derivative", as proposed by several authors (Brandolini et al., 2022;Campos and Markham, 2007; Dueñas et al., 2009; Magalhães et al., 2017). The calibration curves of the identified phenolics were constructed using external standards. Results are expressed as µg/g DM.

2.2.6. Bread colour

The bread colour was evaluated in the CIElab L^* a^* b^* colour space using a Chroma Meter CR-II tristimulus colorimeter (Minolta Camera Co., Osaka, Japan) with a C standard illuminant. Eight measurements were performed.

2.2.7. Bread volume and texture analysis

The bread volume profile was acquired using a Volscan Profiler 300 (Stable Micro Systems, Godalming, UK). Crumb texture analysis was performed after one day with a TA.XT2i texture analyser (Stable Micro Systems) according to Sammalisto et al. (2021). 25 mm-thick slices were taken from the centre of breads removing crusts to obtain 40 × 40 × 25 mm crumb parallelepipeds. The crumb was compressed two times to a 40 % deformation at 5 mm/s speed under a 36 mm diameter cylindrical probe using a 10 kg load cell.

2.3. Statistical analysis

Different lots of batter, dough or bread were analysed in triplicate, except phenolic compounds that were determined in duplicate on the two lots of 20 %-enriched bread as biological replicates. Average values and standard deviations were computed using Microsoft Excel (Microsoft, Redmond, WA, USA). After verifying the normal distribution of data, one-way analysis of variance (ANOVA) was performed with the Statgraphics Centurion 18 statistical software (Statpoint Technologies Inc., Warrenton VA, USA). The power transformation was applied to the colour data for coordinate a^* in the breadcrumb and crust, as well as for coordinate b^* in the crust. When significant differences were found ($p \leq 0.05$), Fisher's lowest significant difference (LSD) at 95 % significance was computed.

3. Results and discussion

3.1. By-product characterization

The pea by-product had a moisture content of 77.9 ± 1.0 g/100 g, while protein (24.3 ± 2.2 g/100 g DM), fat (3.9 ± 0.2 g/100 g DM), raw fibre (33.2 ± 1.0 g/100 g DM), ash (3.4 ± 0.5 g/100 g DM) and residual carbohydrates (35.2 ± 2.0 g/100 g DM) constituted the dry matter. These values were consistent considering the average contents in by-product components. In fact, pea pods are constituted by protein (10.8–13.4 g/100 g DM), fat (1.1–1.3 g/100 g DM), soluble carbohydrates (24.3–26.4 g/100 g; mainly glucose and sucrose), starch (3.7–6.8 g/100 g DM), insoluble (35.6–54.5 g/100 g DM) and soluble (4.2–8.3 g/100 g DM) dietary fiber, and ash (6.6–8.1 g/100 g DM) (Belghith Fendri et al., 2016a; Mateos-Aparicio et al., 2010). Instead, pea cotyledons contain 14–31 g/100 g DM of protein, 1–4 g/100 g DM fat, 5–12 g/100 g DM sugars, 30–49 g/100 g DM starch, 10–20 g/100 g insoluble fiber DM, 2–9 g/100 g DM soluble fiber and 2.3–3.7 g/100 g ash (Hall et al., 2017).

3.2. Preliminary fermentation trials

The results of the preliminary trials are summarized in Table 1. In the first trial, the pH decreased from 5.12 to 3.79 during the fermentation, primarily due to the production of lactic acid (17.2 mg/g FM) by resident lactic acid bacteria both pre- and post-inoculum. PAB exhibited no growth, decreasing from the theoretical initial cell concentration of 8.5 log CFU/g FM to 8.1 after 72 h. This lack of growth explains the absence of propionic acid and the modest vitamin B12 production (12.5 ng/g FM). The already low pH of the matrix, a critical factor limiting *Propionibacterium* growth (Chamlagain et al., 2024; Xie et al., 2019), suggested us to adjust the pH at least to 6.0. This adjustment made the lactic acid available in its neutral form, which is the preferred carbon source of *Propionibacterium*. The beneficial effect of pH control on vitamin synthesis was previously confirmed by Xie et al. (2019) in their co-fermentation of non-sterile wheat bran containing resident or inoculated LAB. In the second trial, the vitamin B12 production increased to 24.1 ng/g FM, but 4.0 ng/g of pseudo vitamin was also observed, likely due to insufficient synthesis of the lower ligand of the active form (i.e., 5,

Table 1
Preliminary trials: characterization of the fermented batters (72 h) containing 20 % of dried pea by-product (Trials 1 and 2) and effect of pasteurization on the microbial counts of a batter with 15 % by-product (Trial 3).

Trial 1	
pH	3.79 ± 0.01
Vitamin B12 (ng/g FM)	12.5 ± 1.6
Succinic acid (mg/g FM)	0.32 ± 0.05
Lactic acid (mg/g FM)	17.15 ± 1.35
Acetic acid (mg/g FM)	2.72 ± 0.31
Propionic acid (mg/g FM)	nd
Enterobacteriaceae (log CFU/g)	3.45 ± 0.31
Propionic acid bacteria (log CFU/g)	8.08 ± 0.15
Trial 2 (initial pH *adjusted to 6.00 ± 0.05)*	
pH	4.10 ± 0.01
Pseudovitamin B12 (ng/g FM)	4.0 ± 0.2
Vitamin B12 (ng/g FM)	24.1 ± 1.7
Trial 3 (pasteurization)	
Enterobacteriaceae (raw, log CFU/g)	3.18 ± 1.01
Enterobacteriaceae (pasteurized, log CFU/g)	absent
Lactic acid bacteria (raw, log CFU/g)	5.75 ± 0.16
Lactic acid bacteria (pasteurized, log CFU/g)	3.05 ± 0.74

nd: not detected.

6-dimethylbenzimidazole, DMBI hereafter), attributed to oxygen scarcity (Chamlagain et al., 2018; Deptula et al., 2015). To address this, the amount of powdered by-product was reduced to 15 % w/w to obtain a less viscous batter, thereby improving aeration during shaking in subsequent trials. The heavy presence of contaminants (*Enterobacteriaceae* and LAB) led us to include a pasteurization step to ensure safety and enhance the competitiveness of PAB. In the third preliminary trial, a 20-min pasteurisation at 70 °C effectively eradicated *Enterobacteriaceae* from the initial count of 3.18 ± 1.01 log CFU/g, and further decreased LAB from 5.75 to 3.05 log CFU/g.

3.3. Characterization of fermented batters

Table 2 and Table 3 summarize the results for two independent lots of fermented batter. PAB exhibited nearly a 20-fold growth in horizontally positioned tubes and in the half-filled 1 L bottle, where shaking was most effective. This enhanced growth is consistent with the observed vitamin B12 contents, ranging from 189 to 211 ng/g FM, equivalent to 1374–1535 ng/g DM. These B12 levels notably exceeded those reported by Xie et al. (2021) in various fermented cereal and legume matrices (51–742 ng/g DM; average: 301) and by Chamlagain et al. (2024) in a 30 % w/w wheat bran batter (39–49 ng/mL). Propionic (4.05–4.52 mg/g FM or 29.5–32.7 mg/g DM) and acetic acid (2.81 mg/g FM or 20.4 mg/g DM) levels were comparable to those reported by Chamlagain et al. (2018) in fermented barley malt (4.2–4.7 and 3.2–4.0 mg/g FM respectively), and by Xie et al. (2021) in fermented buckwheat bran (27.9–32.8 and 16.2–18.0 mg/g DM). Conversely, vertically shaken tubes exhibited greater variability in vitamin B12 content (87.2–130.8 ng/g FM), with a significant portion in the inactive pseudo form (64.6–88.1 ng/g FM). This variation highlights the importance of adequate oxygen availability for the synthesis of the lower ligand DMBI. Furthermore, butyric acid (4.99 mg/g FM) and some additional acetic acid were detected only in vertically shaken tubes, likely due to sporulating obligate anaerobes contaminants. In fact, the higher oxygen availability appeared to inhibit them, aligning with the known fermentation patterns of species such as *Clostridium butyricum* and *C. tyrobutyricum*, which are involved in starch, sugar, and lactate fermentation, producing butyrate and acetate (Ljungdahl et al., 1989).

Table 2
Average values (mean ± standard deviation; $n = 3$) of propionic acid bacteria (PAB), lactic acid bacteria (LAB) and vitamin B12 in pea by-product batters before (0 h) and after (72 h) fermentation using different types of shaking.

Batch	Time (h)	Shaking	PAB (log CFU/g)	LAB	pseudo B12 (ng/g FM)	B12
1	0		8.64 ± 0.09	3.13 ± 0.12		
	72	Horizontal (tubes)	9.87^{a} ± 0.11	4.38^{b} ± 0.35	nd	189.4^{a} ± 17.2
	72	Vertical (tubes)	9.57^{b} ± 0.16	3.09^{c} ± 0.35	88.1^{a} ± 4.1	130.8^{b} ± 6.5
	72	Vertical (bottle)	9.93^{a} ± 0.04	4.86^{ab} ± 0.20	2.8^{c} ± 0.3	211.3^{a} ± 22.9
2	0		8.63 ± 0.04	3.76 ± 0.07		
	72	Horizontal (tubes)	9.94^{a} ± 0.05	5.46^{a} ± 0.29	nd	192.8^{a} ± 25.0
	72	Vertical (tubes)	9.67^{b} ± 0.12	3.12^{c} ± 0.16	64.6^{b} ± 4.3	87.2^{c} ± 4.6
	72	Vertical (bottle)	9.98^{a} ± 0.01	5.31^{a} ± 0.51	6.1^{c} ± 0.8	206.5^{a} ± 25.8

nd: not detected. *Enterobacteriaceae* were absent in all samples. Different letters in the same column indicate significant differences between the fermented samples according to the LSD test ($p < 0.05$).

3.4. Characterization of breads and doughs

The vitamin B12 content, presented in Table 4, ranged from 32.0 to 39.7 to 51.1–52.3 ng/g FM in 15 % and 20 %-enriched breads, respectively, with consistent results in the two batches of fermented by-product. The averaged levels of B12 in the doughs ranged from 51.7 to 87.1 ng/g DM and the analysis of variance did not highlight significant differences with the corresponding breads, confirming that no loss of vitamin occurred due to baking. In fact, B12 displays greater stability when produced in situ, rather than when supplemented in a straight-dough breadmaking process (Edelmann et al., 2016). Similar B12 values were previously published for six wheat breads supplemented with rice bran or soybean flour obtained by co-fermentation (29.9–48.7 ng/g FM) (Wang et al., 2022). A lower content of vitamin (14 ng/g FM, but starting from 8.9) was instead reported by Zhang et al. (2023) for a whole meal bread obtained by the sourdough method with a co-colture of L. *lactis* and *P. freudenreichii*. Consuming 40 to 70 g of the breads baked in the present study would provide the most widely used recommended dietary allowance for vitamin B12 (2.4 μg/day for adults) (Institute of Medicine, 1998). This amount would correspond to 70–120 g if the adequate intake proposed by EFSA (2015) (4.0 μg/day) is considered.

Fig. 2 shows the images of control and enriched breads. The color coordinates of the bread samples (Table 5) show small but noticeable variations in crumb and crust lightness (L^*), greenness ($-a^*$), and yellowness ($+b^*$). In the crumb, differences were mainly due to the addition of the pea by-product, which resulted in a darker, greener, and more yellow hue, while the control was the lightest and least green. Regarding the crust, the color seems to be influenced by the fermentation of the added material, as the fermented samples were significantly darker and less yellow than the controls.

The addition of fermented materials had a minor or negligible impact on bread volume. In fact, as reported in Table 6, significant but limited decreases in specific volume were registered, especially with the highest enrichment percentage, also resulting in superior crumb hardness. The addition of non-fermented by-product caused a decrease in hardness, as previously noted by Belghith Fendri et al. (2016b) with values very similar to ours: from 15.2 N in the control to 12.8–13.8 N in breads enriched with 1 % of fiber extracted from pea or faba bean pods. The specific volumes (2.69–2.94 mL/g) were lower than those described by Wang et al. (2022) (3.32–4.37 mL/g) and Belghith Fendri et al. (2016b) (3.00–3.59 mL/g) but included in the range reported by Kasprzak and Rzedzicki (2010) for pea seed coat-enriched breads (2.47–3.75 mL/g). The baking loss ranged from 12.2 to 13.3 % but did not show a clear pattern. Based on subjective tasting, the addition of fermented materials did not affect the overall taste of the bread but resulted in a barely

Table 3
Average values (mean ± standard deviation; $n = 3$) of pH and organic acid contents (mg/g FM) in the fermented (72 h) pea by-product batters.

Batch	Shaking	pH	Succinic acid	Acetic acid	Propionic acid	Butyric acid	Ratio C_3:C_2
1	Horizontal (tubes)	$4.81^{b} \pm 0.02$	$1.85^{b} \pm 0.25$	$2.81^{b} \pm 0.03$	$4.52^{a} \pm 0.04$	nd	$1.61^{a} \pm 0.03$
	Vertical (tubes)	$4.55^{c} \pm 0.01$	$1.83^{b} \pm 0.15$	$4.69^{a} \pm 0.15$	$3.75^{c} \pm 0.21$	4.97 ± 0.03	$0.80^{c} \pm 0.07$
	Vertical (bottle)	$4.79^{b} \pm 0.08$	$1.67^{b} \pm 0.00$	$2.81^{b} \pm 0.01$	$4.05^{b} \pm 0.01$	nd	$1.44^{b} \pm 0.00$
2	Horizontal (tubes)	$4.83^{b} \pm 0.02$	$2.44^{a} \pm 0.07$	$2.80^{b} \pm 0.01$	$4.50^{a} \pm 0.04$	nd	$1.60^{a} \pm 0.02$
	Vertical (tubes)	$4.57^{c} \pm 0.02$	$0.33^{c} \pm 0.00$	$4.81^{a} \pm 0.15$	$3.68^{c} \pm 0.09$	5.01 ± 0.17	$0.77^{c} \pm 0.04$
	Vertical (bottle)	$4.90^{a} \pm 0.01$	$2.61^{a} \pm 0.01$	$2.78^{b} \pm 0.00$	$4.03^{b} \pm 0.01$	nd	$1.45^{b} \pm 0.00$

nd: not detected. Different letters indicate significant differences between samples in the same column according to the LSD test ($p < 0.05$).

Table 4
Average values (mean ± standard deviation; $n = 3$) of vitamin B12 content in enriched doughs (after proofing) and breads, both in terms of fresh matter (FM) and dry matter (DM).

Sample	Enrichment	Vitamin B12 (ng/g FM)		Vitamin B12 (ng/g DM)	
	(%)	Dough	Bread	Dough	Bread
1a	15	32.8^{b} ± 5.4	39.7^{b} ± 0.5	59.9^{b} ± 10.0	$63.8^{b} \pm 0.7$
2a	15	28.3^{b} ± 1.9	32.0^{b} ± 4.1	$51.7^{b} \pm 3.5$	$51.3^{b} \pm 6.6$
1b	20	36.6^{ab} ± 6.4	52.3^{a} ± 6.3	66.6^{ab} ± 11.6	83.7^{a} ± 10.0
2b	20	$37.3^{a} \pm 9.6$	51.1^{a} ± 3.5	87.1^{a} ± 15.7	$81.2^{a} \pm 5.6$

Different letters indicate significant differences between samples in the same column according to the LSD test ($p < 0.05$).

perceivable sandy texture.

3.5. Phenolic compounds

The contents of the phenolic compounds of pea by-product and breads are reported in **Supplementary Table 2**. In the by-product, the soluble-free fraction (105.8 μg/g DM) accounted for 28 % of total phenolics (373.8 μg/g DM), while the insoluble bound and the soluble conjugated phenolics were 37 % and 34 %, respectively. Differently, the main fraction in control bread, without addition of pea by-product batter, was composed of bound phenolics (81 %) with a slightly lower percentage in enriched breads (77 %) because of pea by-product contribution. Higher values for total free phenolics (740 μg/g DM) were previously documented in fresh green peas, with the greatest contribution from catechin (38 %) followed by protocatechuic acid (25 %), gallic acid (13 %), rutin (6 %), kaempferol (6 %) and quercetin (5 %) (Younis et al., 2023). However, the values obtained in this study were in the range observed for six different varieties of green peas by (Magalhães et al., 2017) (96.5–254.5 μg/g DM) with protocatechuic acid (12.1–163.0 μg/g DM) and *p*-hydroxybenzoic acid (45.4–101.7 μg/g DM) as the main compounds, but glucosides of apigenin (nd–45.8 μg/g DM) and of luteolin (1.4–46.1 μg/g DM) were also detected. In two dark hulled peas a significant difference was registered between phenolics in cotyledons (58.0–62.9 μg/g DM) and in the seed coat (683.1–716.5 μg/g DM) (Dueñas et al., 2004): protocatechuic acid and its glycoside prevailed in the former, apigenin glucosides in the latter. The most abundant phenolic compounds found in pea pods are, in decreasing order: 5-caffeoylquinic acid, epicatechin, hesperidin and catechin (Castaldo et al., 2022), or cinnamoyl glucose, *m*-coumaric acid and quercetin (Belghith Fendri et al., 2022).

Fig. 2. Visualization of control and enriched breads.

Table 5
Average values (mean ± standard deviation; $n = 8$) for color coordinates (L^*, lightness; a^*, green-red; b^*, blue-yellow) of the crumb and crust in control and enriched breads.

Sample	Enrichment	Crumb			Crust		
	(%)	L^*	a^*	b^*	L^*	a^*	b^*
Control		$76.2^{a} \pm 2.0$	$-2.7^{a} \pm 0.3$	$16.1^{b} \pm 1.3$	$56.4^{a} \pm 3.2$	$8.4^{a} \pm 0.7$	$31.4^{a} \pm 1.7$
Control-NF	20	$72.5^{bc} \pm 2.3$	$-3.8^{d} \pm 0.2$	$17.9^{a} \pm 0.7$	$55.1^{a} \pm 4.7$	$6.3^{b} \pm 1.6$	$29.7^{ab} \pm 2.1$
1a	15	$72.4^{bc} \pm 1.5$	$-3.2^{b} \pm 0.2$	$17.7^{a} \pm 0.9$	$48.8^{b} \pm 3.5$	$8.4^{a} \pm 0.8$	$26.4^{c} \pm 2.4$
2a	15	$74.1^{b} \pm 2.4$	$-3.6^{cd} \pm 0.2$	$17.5^{a} \pm 1.1$	$50.0^{b} \pm 2.1$	$8.2^{a} \pm 0.4$	$27.9^{bc} \pm 1.5$
1b	20	$71.4^{c} \pm 1.4$	$-3.5^{c} \pm 0.3$	$17.8^{a} \pm 0.7$	$50.2^{b} \pm 2.0$	$7.4^{b} \pm 0.5$	$27.4^{c} \pm 1.2$
2b	20	$73.1^{bc} \pm 1.4$	$-3.6^{cd} \pm 0.2$	$17.6^{a} \pm 0.6$	$48.4^{b} \pm 2.0$	$8.4^{a} \pm 0.4$	$26.8^{c} \pm 1.4$

Different letters indicate significant differences between samples in the same column according to the LSD test ($p < 0.05$).

Table 6
Average values (mean ± standard deviation; $n = 3$) for baking loss, specific volume and texture analysis of the control and enriched breads.

	Enrichment (%)	Baking loss (%)	Specific volume (mL/g)	Hardness (N)	Cohesiveness	Gumminess (N)
Control	0	$13.3^{a} \pm 0.5$	$2.94^{a} \pm 0.03$	$14.1^{bc} \pm 1.1$	$0.76^{a} \pm 0.01$	$10.6^{bc} \pm 0.6$
Control-NF	20	$12.2^{d} \pm 0.7$	$2.89^{b} \pm 0.03$	$11.9^{c} \pm 0.8$	$0.72^{c} \pm 0.01$	$8.5^{d} \pm 0.5$
1a	15	$12.3^{cd} \pm 0.3$	$2.89^{b} \pm 0.03$	$16.2^{ab} \pm 1.6$	$0.75^{a} \pm 0.01$	$12.1^{ab} \pm 1.3$
2a	15	$13.0^{ab} \pm 0.2$	$2.82^{c} \pm 0.02$	$14.1^{bc} \pm 1.1$	$0.74^{ab} \pm 0.00$	$10.4^{c} \pm 0.8$
1b	20	$12.5^{bcd} \pm 0.2$	$2.73^{d} \pm 0.03$	$17.8^{a} \pm 2.0$	$0.71^{c} \pm 0.02$	$12.7^{a} \pm 1.2$
2b	20	$13.0^{abc} \pm 0.2$	$2.69^{d} \pm 0.01$	$16.3^{ab} \pm 0.6$	$0.72^{bc} \pm 0.01$	$11.8^{abc} \pm 0.6$

Different letters indicate significant differences between samples in the same column according to the LSD test ($p < 0.05$).

The ferulic acid found in the insoluble-bound fraction in enriched breads was due to its predominant presence in wheat (Hidalgo et al., 2016; Yilmaz et al., 2015). The addition of pea by-product in breads, estimated to be 4.3 % on dry weight, led only to a slight increase in bound *p*-coumaric acid. Previously, Niño-Medina et al. (2019) reported a 5-fold increase in bread total phenolics after a 2 % enrichment with chickpea or soybean fiber.

The propionic fermentation did not seem to modify the phenolic profile, differently from lactic fermentation, which is known to release phenolic aglycones from bound and conjugated fractions, improving their bioaccessibility, through the activities of glycosyl hydrolase, phenolic acid decarboxylase and reductase, and esterase activities (Gobbetti et al., 2020).

4. Conclusions

The by-product from industrial canned green pea processing was successfully fermented with *Propionibacterium freudenreichii* DSM 20271 to produce a vitamin B12-rich batter (1374–1535 ng/g DM). The best results were obtained by fermenting a 15 % w/v pea by-product batter, pasteurized at 70 °C for 20 min, neutralized to pH 6, and inoculated at 10 %, under microaerophilic conditions. Such a high vitamin content led to significant levels of vitamin B12 in the fortified breads (32.0–52.3 ng/g FM) with relatively modest additions and minimal impact on bread volume and texture. A portion of 40 to 70 g of the enriched bread can supply the recommended dietary allowance of vitamin B12, effectively addressing the nutritional deficiencies common in strictly plant-based diets. This study emphasizes the importance of upcycling by-products to reduce food waste while enhancing the nutritional profile of foods. Due to the high levels of microbial contaminants, particularly lactic acid bacteria, a pasteurization step was necessary, underscoring the need to treat by-products as food-grade materials. *Propionibacterium freudenreichii* DSM 20271 showed consistently high levels of vitamin B12 under the tested conditions but comparative assessments with multiple strains may be considered in further studies. Future investigations should mainly include objective sensory evaluations to assess consumer acceptability, as unpleasant flavours and/or texture often result from the supplementation of food with fermented materials.

Our findings support further exploration of legume by-products, as the demand for sustainable and nutritious foods grows, and strategies that add value to waste materials gain interest from both industries and consumers.

Ethical statement

This study does not involve human participants or animals.

CRediT authorship contribution statement

Lorenzo Estivi: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Bhawani Chamlagain:** Writing – review & editing, Methodology, Investigation. **Minnamari Edelmann:** Writing – review & editing, Resources, Methodology, Investigation. **Pekka Varmanen:** Writing – review & editing, Resources, Methodology, Investigation. **Claudio Gardana:** Resources, Investigation. **Alyssa Hidalgo:** Writing – review & editing, Conceptualization. **Vieno Piironen:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100755.

Data availability

Data will be made available on request.

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