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Valorization of fruit by-products: Vitamin B12 fortification in fruit pomace using a co-culture of propionic and lactic acid bacteria☆

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ABSTRACT

The agri-food industry generates large quantities of by-products, e.g., fruit pomace from juice and wine production, which represent underutilized sources of valuable compounds. The popularity of plant-based foods is steadily increasing due to growing concerns about human health, animal welfare, and environmental sustainability, highlighting the need for vitamin B12 fortification – plants cannot synthesize this essential micronutrient. This challenge is particularly relevant given the growing prevalence of B12 deficiency and its associated health risks. The aim of this study was to enrich pomace from blue honeysuckle berries, strawberries, and grapes with B12 via co-fermentation using *Propionibacterium freudenreichii* DSM 20271 and *Lactiplantibacillus plantarum* ATCC 10241 (selected based on screening tests, including the co-existence test of *P. freudenreichii*-different LAB species), and to optimize the pomace matrix for cobalamin biosynthesis in co-culture conditions. The pomace matrices were characterized with respect to carbon and nitrogen sources, as well as cobalamin biosynthesis precursors, including cobalt, riboflavin, and niacin. Nearly complete utilization of available carbon sources was observed in all matrices. B12 (13.91–39.71 μg/kg) and propionic acid (0.73–4.31 g/kg) were produced in all pomace-based media. Optimization experiments indicated that strawberry and grape pomace were the most suitable substrates for B12 synthesis, whereas propionic acid production was primarily associated with strawberry pomace. These findings demonstrate the feasibility of using co-cultures of propionic acid and lactic acid bacteria to enrich fruit pomace with B12. This approach offers a promising strategy for developing B12-fortified plant foods from fermented and edible fruit pomace, thereby supporting sustainable development and zero-waste principles.

1. Introduction

Vitamin B12 (cobalamin, B12) is a water-soluble B-complex vitamin that plays a crucial role in various physiological and metabolic processes in humans (Nielsen et al., 2012). Cobalamin is produced exclusively by microorganisms, including bacteria such as *Pseudomonas* and *Propionibacterium* (Roth et al., 1996; Martens et al., 2002). On an industrial scale, modified strains of *Pseudomonas denitrificans* are primarily used to produce B12 for supplements and pharmaceuticals, as they are significantly more efficient than propionibacteria (Calvillo et al., 2022). However, *P. denitrificans* does not have GRAS status and therefore cannot be used directly in food production (Martens et al., 2002). In contrast, the species *Propionibacterium freudenreichii* (*Pfr*) is considered safe and also produces the biologically active form of cobalamin (Martens et al., 2002), making it an ideal candidate for fortifying foods with B12 through *in situ* fermentation.

Cobalamin occurs naturally only in animal-derived products. In plant-based foods, B12 is present only through fermentation or chemical fortification (Watanabe, 2007). The popularity of plant-based diets has increased worldwide in recent years (Soares et al., 2023), potentially

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leading to a significant global decrease in B12 intake and increasing the risk of B12 deficiency in humans. It is estimated that approximately 6% of the global population suffers from a deficiency of this compound (Azzini et al., 2021). This trend is driven by various factors, including moral, ethical, religious, animal welfare, and environmental protection concerns, as well as lifestyle and health considerations (Cramer et al., 2017). Limiting or giving up meat consumption is not the only cause of the growing problem of cobalamin deficiency; elderly individuals may also suffer from B12 deficiency (Guéant et al., 2021; Niklewicz et al., 2023; Hoteit et al., 2024). Cobalamin deficiency can lead to serious health problems, including anemia, neurological disorders such as ataxia and lethargy, cognitive decline, depression, and other complications (Green et al., 2017; Soares et al., 2023). Therefore, the development of plant-based food products fortified with B12 is becoming increasingly important, considering future nutritional and societal trends.

Another significant global issue is industrial waste, including by-products generated by the food industry. In line with the principles of sustainable development and zero-waste policies, efforts should be made to maximize the utilization of such raw materials, including fruit pomace. Fruit by-products are perishable, and their eco-friendly disposal poses a significant challenge (Iqbal et al., 2021; Patra et al., 2022). Moreover, fruit pomace is rich in fiber, sugars, amino acids, and vitamins (Pieszka et al., 2015; Oszmiański et al., 2016; Sirohi et al., 2020; Piwowarek et al., 2025). Therefore, such side-streams are worth considering as raw materials for further applications, for example, in the food industry, within the framework of the circular economy (Lau et al., 2021). A cost-effective and efficient method of utilizing pomace may involve fermentation processes, such as those employing propionic acid bacteria (PAB), to enrich these raw materials with cobalamin, thereby improving their quality and value. Fermented pomace could then be used in the production of plant-based foods, as an additive to jams, or as an ingredient in vegan yogurts or plant-based products that mimic animal-origin foods.

A previous study (Piwowarek et al., 2025) demonstrated that a monoculture of *Pfr* DSM 20271 can be utilized to enrich fruit pomace with B12, including pomace derived from blue honeysuckle berries (BP), strawberries (SP), and grapes (GP). All these by-products contain carbon sources (such as glucose and fructose), amino acids important for PAB metabolism, and compounds essential for the synthesis of active cobalamin (including cobalt, riboflavin, and niacin) (Piwowarek et al., 2025). Moreover, the tested pomace samples do not contain pesticide residues or mycotoxins, making them safe for potential industrial applications (Piwowarek et al., 2025). In this study, fruit pomace was fermented in a co-culture of *Pfr* and *Lactiplantibacillus plantarum* (*Lp*). To our knowledge, there is currently no literature available on this specific topic. Until now, co-cultures of PAB and lactic acid bacteria (LAB) have primarily been used to enrich cereal- and legume-based materials with B12 (Xie et al., 2019, 2021). PAB and LAB are industrially employed together in the production of Swiss-type cheeses, representing a typical example of commensalism (Thierry et al., 2011). In addition to lactate synthesis, which, according to literature, can stimulate cobalamin production by PAB (Kruk et al., 2024), LAB can also provide vitamins essential for PAB metabolism, e.g., vitamin B2 (Juarez del Valle et al., 2014), a key precursor in the biosynthesis of active cobalamin by *Pfr* (Chamlagain et al., 2025). Moreover, the enzymatic activities of LAB can increase the bioavailability of certain components for PAB in fermented matrices, such as amino acids, thereby enhancing their metabolism (Canon et al., 2020). Furthermore, LAB fermentation can enhance the stability of pomace matrices by producing antimicrobial metabolites (Smid and Lacroix, 2013; Leyva Salas et al., 2017). Lactic acid fermentation also positively affects the organoleptic properties of fermented substrates, including taste, aroma, and texture (De Vuyst and Neysens, 2005) – a phenomenon successfully utilized on an industrial scale in fermented food products. LAB can also improve the health, nutritional, and sanitary properties of products (Canon et al., 2020). All these factors, combined with the simultaneous fortification of active B12, could significantly enhance the technological applicability of fruit pomace.

This study involved screening various LAB species for their effectiveness in co-culture with *Pfr* DSM 20271. Furthermore, all matrices were characterized by their content of key components required for B12 biosynthesis, such as cobalt, riboflavin, and niacin. Additionally, several important fermentation parameters were assessed, including growth of PAB and LAB, alterations in the amino acid profile, sugar consumption, and, finally, the production of organic acids and vitamin B12. Overall, this work presents a comprehensive approach to utilizing fruit pomace through cobalamin enrichment, with potential industrial applications aligned with sustainable development and zero-waste policies.

2. Materials and methods

2.1. Microorganisms

The study used *Propionibacterium freudenreichii* DSM 20271 (*Pfr*), *Levilactobacillus brevis* DSM 20054 (*Lb*, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany), *Lactobacillus reuteri* ATCC 23272 (*Lre*), *Lactiplantibacillus plantarum* ATCC 10241 (*Lp*), *Lacticaseibacillus casei* ATCC 393 (*Lc*), *Lacticaseibacillus rhamnosus* ATCC 7469 (*Lr*) (American Type Culture Collection, ATCC, Manassas, VA, USA), *Lactobacillus acidophilus* Lac4 (*Lac*) (Ezal, France). All bacterial strains were maintained as frozen glycerol stocks at −80 °C.

2.2. Fruit pomace

The study used fruit by-products in the form of pomace from grapes (from white grape varieties – Sibera, Bianca, Seyval; Mazovia Vineyard, Mazowieckie Voivodeship, Poland), blue honeysuckle berries (Wojtek and Zojka varieties; obtained under laboratory conditions, BUCHER Unipectin AG, Niederweningen, Switzerland, Department of Food Technology and Assessment, Warsaw University of Life Sciences – WULS-SGGW and from DiWine winery, Mazowieckie Voivodeship, Poland), and strawberries (Rumba variety, obtained under laboratory conditions, as above). Fruit side-streams were collected directly from wineries or laboratory sources in a fresh state and frozen at −20 °C at the Department of Food Biotechnology and Microbiology (WULS-SGGW).

2.3. Preparation of fruit pomace-based matrices and fermentation parameters (fortification process)

Fruit pomace-based matrices (1–10, optimized medium) were prepared according to Table 1. Distilled water was then added to the matrices at a 1:1.5 pomace:water (*w*/w) ratio and blended. The pH of the pomace matrices was adjusted to 6.5–7.0 using NaOH (Chempur,

Table 1
Composition of fruit pomace-based matrices.

	Matrix	BP [%]	SP [%]	GP [%]
Optimization process	1	100	0	0
	2	0	100	0
	3	0	0	100
	4	50	50	0
	5	50	0	50
	6	0	50	50
	7	66.4	16.8	16.8
	8	16.8	66.4	16.8
	9	16.8	16.8	66.4
	10	33.3	33.3	33.4
Optimized medium		0	65	35

The share of pomace (blue honeysuckle berry pomace – BP, strawberry pomace – SP, grape pomace – GP) in the matrices (1−10) was selected according to the DoE method; the optimized medium was determined based on the optimization process (Fig. 6, Table S8).

Piekary Śląskie, Poland). After homogenization and pH adjustment, 30 g of each matrix was transferred into 50 mL Falcon tubes and sterilized at 121 °C for 15 min. Prior to inoculation with bacteria, the pH of the matrices was checked under sterile conditions using pH indicator strips. If the pH was below 6.0, the matrices were neutralized with sterile NaOH (2.8 mol/L).

The matrices were inoculated with *Pfr* DSM 20271 (ca. 9.0 log CFU/g) and *Lp* ATCC 10241 (ca. 6.0 log CFU/g); the initial inoculum was selected based on Xie et al. (2019). The inoculation cultures were prepared as follows. *Pfr* and lactobacilli strains were cultivated in VL (Viande-Levure, medium for anaerobes) liquid medium (30 °C for 2–3 days) (Piwowarek et al., 2025) and in MRS broth (30–37 °C for 2–3 days) (Biomaxima, Lublin, Poland), respectively, under static conditions. Bacterial cells were separated from the medium by centrifugation (4427 ×*g*, 10 min) using an MPW-260R centrifuge (MPW Med. Instruments, Poland), and then resuspended in sterile sodium chloride solution (8.5 g/L; Stanlab, Lublin, Poland) directly before inoculation.

The cultures were carried out for 120 h at 30 °C under stationary conditions with pH neutralization (NaOH, 2.8 mol/L) performed every 24 h. Co-cultures were conducted in three biological replicates. All time points (0 h, 72 h, 120 h) were prepared in separate Falcon tubes.

2.4. Preparation of fruit pomace liquid culture media and cultivation parameters (strain screening)

To enable microcultivation of *Pfr* and lactobacilli in pomace-based media, liquid pomace media were prepared. BP-, SP-, and GP-based media were prepared as described by Piwowarek et al. (2021), with some modifications. Fruit pomace was mixed with distilled water in glass bottles (1:1, *w*/w), and the samples were heated (extracted) for 30 min at 75 °C. Following heating, insoluble matter was removed via filtration through gauze and centrifugation (12,857 ×*g*, 4 °C, 10 min; Centrifuge 5804R, Eppendorf, Germany). The supernatants were then sterilized at 121 °C for 15 min. The pH of the media was adjusted to 6.5–7.0 using NaOH before sterilization. The pH was checked after sterilization and, if necessary, neutralized immediately before inoculation, as described above.

The inoculation cultures were prepared as follows: *Pfr* and lactobacilli strains were cultivated as described above. Bacterial cells were separated from the medium by centrifugation (4427 ×*g*, 10 min) using an MPW-260R centrifuge (MPW Med. Instruments, Poland) and subsequently resuspended in sterile pomace-based liquid media (BP, SP, and GP).

Microcultures were performed using an automated Bioscreen C microbial growth analyzer (Yo AB Ltd., Growth Curves, Finland) for 110 h at 30 °C. Microwells were filled with 270 μL of media (BP, SP, and GP) and inoculated with 30 μL of the tested bacterial strains (*Pfr* or lactobacilli). Control media without added bacteria were also used (i.e., pomace media only – 300 μL). Bacterial growth was monitored by automatically measuring changes in optical density (OD) every two hours at a wavelength of 420–580 nm. Based on the results, growth curves were determined, and growth parameters were calculated.

2.5. Coexistence tests, bioscreen analysis of bacterial growth in the presence of culture supernatant – bacterial strains and standard growth conditions (strain screening)

The *Pfr*, lactobacilli and *Lactococcus lactis* IBB 1339 (COLIBB, Central Collection of Strains of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) strains were maintained as frozen stocks at −80 °C in BHI broth (VWR International, Leuven, Belgium), MRS broth (Merck KGaA, Darmstadt, Germany) or M17 broth (Oxoid, Basingstoke, United Kingdom) supplemented with glucose (5 g/L; GM17), respectively; in the presence of glycerol (150 mL/L) as cryoprotective agent. The nisin-producing *Lc. lactis* IBB1339 strain was used as a positive control for antagonistic activity in the coexistence test (Głowacka-Rutkowska et al., 2020).

For preparation of bacterial suspensions for coexistence tests and Bioscreen growth measurements, all strains were cultured on BHI-agar media at 30 °C for *Propionibacterium* and *Lactococcus* strains and at 37 °C for lactobacilli. Plates with PAB and lactobacilli were incubated for 72 h under anaerobic conditions in 2.5-l jars containing GENbox anaer (Cat. No. 96124, bioMérieux, Marcy l'Etoile, France), whereas *Lc. lactis* IBB1339 was grown for 48 h under standard aerobic conditions.

For the preparation of cell-free supernatant samples for growth analyses with Bioscreen, lactobacilli were grown in MRS broth at 37 °C under anaerobic conditions (GENbox anaer) for 18 h (overnight cultures). Next, 5 mL of culture was centrifuged at 12,410 ×*g* for 10 min at 4 °C using a Centrifuge 5804R (Eppendorf, Germany). The pellet was discarded, and the supernatant was filtered using a 0.22-μm membrane filter (PVDF, Millex-GV; Merck KGaA, Darmstadt, Germany). The cell-free supernatants of lactobacilli cultures were subsequently used as supplements to BHI broth in Bioscreen analysis of *Pfr* growth.

2.5.1. Coexistence tests

The antagonistic activity of the tested lactobacilli strains against *Pfr* DSM 20271 was examined using an inhibitory spectrum assay as previously described by Litwinek et al. (2022), with some modifications. To this end, bacteria from freshly-grown colonies on BHI-agar plates were suspended and diluted to an OD600 nm of 1 in NaCl (9 g/L). Then, 100 μL of the DSM 20271 bacterial suspension was used to inoculate 5 mL or 3 mL of preheated (55 °C) soft BHI agar (7.5 g/L agar), respectively. The molten media were immediately poured onto 140-mm and 90-mm BHI agar plates, respectively, and evenly distributed on them. Following agar setting, 5 μL of the tested LAB strain suspension was spotted on a selected location on the plates. The plates were allowed to absorb the drops, then incubated at 30 °C for 72 h under anaerobic conditions. The antagonism against the strain was visualized as an inhibition zone. The presence of an inhibition zone was denoted as "++", " + " or "+/−", depending on the size, whereas the lack of antagonistic activity was denoted as "-". The test was done in triplicate.

2.5.2. Bioscreen analysis of bacterial growth in the presence of culture supernatant

The growth of *Pfr* DSM 20271 in the presence of lactobacilli culture supernatants was evaluated using the Bioscreen C system (Thermo Labsystems, Helsinki, Finland). *Pfr* from freshly-grown colonies was suspended and diluted to an OD600 nm of 1 in BHI broth. Next, 5 μL of DSM 20271 bacterial dilutions and BHI broth (negative control) were added to 200 μL of BHI broth, BHI broth with MRS (200 mL/L), or BHI broth with cell-free supernatant (200 mL/L) from lactobacilli o/n cultures, on honeycomb 100-well Bioscreen microplates. To obtain anaerobic conditions, a droplet of mineral oil (bioMérieux, Marcy l'Etoile, France) was added to the top of each well. Bacterial growth was monitored at 30 °C every 20 min for up to 72 h by measuring OD at 600 nm with the Bioscreen C automated microbiology growth curve analysis system. First, the growth of PAB was compared in BHI medium and in BHI supplemented with MRS (200 mL/L), relative to negative controls consisting of the corresponding non-inoculated media. The effect of lactobacilli on *Pfr* DSM 20271 growth was evaluated by comparing growth curves in BHI medium supplemented with lactobacilli cell-free supernatants with those obtained in BHI medium containing MRS (200 mL/L), using the corresponding media without added PAB as negative controls. The assay was carried out in duplicate for PAB cultures, while lactobacilli supernatants were tested in triplicate.

2.6. Microbial cell count

The plate count method was used to determine the number of viable *Pfr* DSM 20271 and *Lp* ATCC 10241 cells during the B12 fortification process. Samples (matrices at 0 h, 72 h, and 120 h) were serially diluted in sterile sodium chloride solution (8.5 g/L). Selected dilutions were

plated to assess bacterial growth using YEL agar medium (*Pfr*, Yeast Extract Lactate) (Malik et al., 1968) and MRS agar medium (lactobacilli). YEL plates were incubated anaerobically (Anaerogen, Oxoid, Basingstoke, UK) at 30 °C for 4 days, followed by 1 day incubation under aerobic conditions at 30 °C (in these conditions, *Pfr* colonies become brown to be distinguishable from colonies of other bacteria) (Xie et al., 2019), while MRS plates were incubated at 30 °C for 3–4 days under stationary conditions. Results are expressed as log CFU/g.

2.7. Determination of cobalt content

The cobalt content in fruit pomace-based matrices (before inoculation) was determined as described by Piwowarek et al. (2025). Nitric acid and hydrogen peroxide (trace analysis grade, Sigma-Aldrich, USA) and ultrapure water (18 MΩ·cm, Milli-Q Elix 3, Millipore, France) were used. A cobalt standard solution (100 mg/L in 50 mL/L HNO_3, Agilent, USA) served for calibration. Cobalt was determined using an Agilent 8900 ICP-MS/MS (USA) equipped with Pt cones and a 2.5 mm injector. Instrumental parameters (RF power 1550 W, nebulizer gas 0.95 L/min, H_2 collision gas 6.0 mL/min) were optimized daily ($CeO^+ < 0.2\%$) using a 1 μg/L Li, Y, Tl solution. Quantification was performed by external calibration (0–100 μg/L, 10 points) with $^{289}Y^+$ (2 μg/L) as internal standard. Samples were rinsed with 20 mL/L HNO_3 between analyses.

Dried pomace samples were mineralized using microwave digestion (Speedwave® four, Berghof, Germany) with 5 mL HNO_3 and 2 mL H_2O_2. After digestion, solutions were diluted to 10 mL and further diluted to 20 mL/L in HNO_3 prior to analysis. Results are expressed as μg/kg d.m. (dry matrix) (mean of two analytical replicates).

2.8. Determination of riboflavin, niacin, and biotin

Riboflavin (B2, AOAC-RI – Certificate No. 100902), niacin (B3), and biotin (B7, AOAC-RI – Certificate No. 101001) were analyzed using the VitaFast microbiological test kits (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions (tested matrices, before inoculation).

Detection limit for B2: 0.018 mg/kg; limit of quantification: 0.4 mg/kg. Samples (1 g) were mixed with 20 mL of distilled water, adjusted to pH 5–6, shaken, and autoclaved at 121 °C for 30 min. The cooled extracts (tap water) were brought to 40 mL with distilled water, then centrifuged (12,857 ×*g*, 4 °C, 10 min; 5804R, Eppendorf, Germany). Supernatants were filtered through 0.22 μm PES filters (LABSOLUTE, Th. Geyer Ingredients, Germany) and diluted using sterile water from the kit and sterile Eppendorf tubes. Riboflavin was determined using 96-well microtiter plates coated with *Lacticaseibacillus rhamnosus* (provided with the kit). Equal volumes (150 μL) of medium and sample/standard were added to the wells. Plates were sealed with adhesive foil (provided with the kit) and incubated in the dark at 37 °C for 48 h. Optical density was measured at 540 nm using a Multiskan Sky (Thermo Scientific, USA). Concentration was calculated from a standard curve (0–0.24 mg/100 g). Results are expressed as mg B2/kg f.m. (fresh matrix/fresh matter/wet matrix – before/after fermentation) from three analytical replicates.

Detection limit for B3: 0.048 mg/kg; limit of quantification: 0.16 mg/kg. Samples (1 g) were mixed with 20 mL of citrate buffer (pH 4.5) and 300 mg of Taka-diastase (Sigma-Aldrich, USA), then shaken and incubated in the dark at 37 °C for 60 min. Next, after adjusting the volume to 40 mL with distilled water, samples were incubated in a water bath with shaking at 95 °C for 30 min. The cooled extracts (tap water) were centrifuged (12,857 ×*g*, 4 °C, 10 min; 5804R, Eppendorf, Germany), filtered (0.22 μm PES), and diluted (as above). Niacin was determined using microtiter plates coated with *Lactiplantibacillus plantarum* (provided with the kit). Equal volumes (150 μL) of medium and sample/standard were added to the wells. Plates were sealed with adhesive foil (provided with the kit) and incubated in the dark at 37 °C for 48 h. Absorbance was measured at 540 nm (as above). Concentration was calculated from a standard curve (0–0.160 mg/100 g) after subtracting reagent blank values. Results from three analytical replicates are expressed as mg B3/kg f.m.

Detection limit for B7: 0.13 μg/kg; limit of quantification: 0.8 μg/kg. Samples (1 g) were mixed with 20 mL of 0.5 mol/L sulfuric acid, shaken, and autoclaved at 121 °C for 30 min. In the next step, the pH of cooled extracts (tap water) was adjusted to 6.0–7.0 with NaOH, and the volume was brought to 40 mL with distilled water. Extracts were centrifuged (12,857 ×*g*, 4 °C, 10 min; 5804R, Eppendorf, Germany), filtered (0.22 μm PES), and diluted (as above). Biotin was determined using microtiter plates coated with *Lactiplantibacillus plantarum* (provided with the kit). Equal volumes (150 μL) of medium and sample/standard were added to the wells. Plates were sealed with adhesive foil (provided with the kit) and incubated in the dark at 37 °C for 48 h. Optical density was measured at 540 nm (as above). Concentration was calculated from a standard curve (0–0.72 μg/100 g). Results from two analytical replicates are expressed as mg B7/kg f.m.

2.9. Determination of total nitrogen and amino acid profile

Total nitrogen of the tested matices (before inoculation) was determined by the Kjeldahl method. Dried samples (200 mg) were mineralized (SpeedDigester K-436, Büchi, Switzerland) with 12 mL of 18.40 mol/L sulfuric acid and a titanium catalyst tablet (Büchi, Switzerland). Following alkalization with 9.45 mol/L NaOH, ammonia was distilled into 0.66 mol/L boric acid (KjelFlex K-360, Büchi) and titrated with 0.1 mol/L HCl to pH 4.3 (TitroLine 5000, SI Analytics, Germany). Results are expressed as g N/kg d.m.

The amino acid profile in fruit pomace-based matrices was determined using an AAA-500 amino acid analyzer (INGOS, Prague, Czech Republic) according to Gientka et al. (2024). Dried matrices (0 h/before inoculation and 120 h) were hydrolyzed in 6 mol/L HCl (60 mL, previously purged with nitrogen) for 23 h at 110 °C, cooled, filtered through Whatman 3 paper, transferred to 100 mL volumetric flasks, and diluted with demineralized water. HCl was removed under reduced pressure (35 bar, 60 °C), and the dry residues were dissolved in citric buffer (pH 2.6) Samples were analyzed by ion-exchange chromatography with post-column ninhydrin derivatization and LED detection at 440 nm (proline) and 570 nm (other amino acids). Separation was performed on a 250 mm analytical column packed with Poly 8 INGOS cation-exchange resin. Buffers of pH 2.6, 3.0, 4.25, and 7.9 were used. The column temperature ranged from 55 to 74 °C, and the reactor temperature was 121 °C. Quantification was performed using external standards and the Clarity software. Results are expressed as g/kg d.m. Analyses were performed in triplicate using pooled samples prepared by combining matrices from each of three biological replicates (1:1:1, *w*/w/w).

2.10. Determination of carbon sources

The sugar content (glucose + fructose) in the matrices (0 h, 72 h, 120 h) was determined using an enzymatic assay (Megazyme, Co. Wicklow, Ireland) according to the manufacturer's instructions (Sucrose/D-Fructose/D-Glucose Assay Kit). Prior to analysis, samples were diluted with distilled water, vortexed for 1 min, and centrifuged (5804R, Eppendorf, Germany) at 12,857 ×g for 10 min (4 °C). Results are expressed as g/kg f.m.

2.11. Determination of organic acids

PA and acetic acid (AA) concentrations (at 0, 72, and 120 h) were determined by GC-FID (Trace 1300, Thermo Fisher Scientific, USA) according to Piwowarek et al. (2025). Samples (1 g) were mixed with 3.5 mL of distilled water and 0.5 mL of 25% sulfuric acid, then vortexed (1 min) and centrifuged (12,857 ×*g* for 10 min; 5804R, Eppendorf, Germany). Supernatants (1 mL) were vigorously shaken with 2 mL of extraction mixture (diethyl ether:hexane, 1:1, *v*/v) and 200 μL of

internal standard (IS; undecanoic acid, Sigma-Aldrich, USA, 1 mg/mL), and transferred to GC vials. Separation was performed on a ZB-WAXplus column (30 m × 0.25 mm × 0.25 μm). Acids were identified using analytical standards (Sigma-Aldrich, USA) and quantified via the IS method with correction factors. Results are expressed as g of PA or AA/kg f.m.

The concentration of lactic acid/lactate (LA) in the matrices was analyzed at 0 h, 72 h, and 120 h using the Enzytec Liquid d−/l-Lactic Acid Assay Kit (R-Biopharm, Darmstadt, Germany, AOAC-OMA 2024.08 „First Action") (Lacorn and Hektor, 2025). The limit of detection is 3 mg/L, whereas the limit of quantification is 10 mg/L. For sample preparation, 1 g of the matrix was weighed into a Falcon tube, diluted with distilled water, and then centrifuged (MPW Med. Instruments, Poland) at 4427 ×g for 10 min (4 °C). Subsequent procedures were carried out according to the manufacturer's instructions. Results are expressed as g/kg f.m.

2.12. Determination of vitamin B12

Vitamin B12 content in the matrices (1–10: 0 h, 72 h, 120 h; optimized medium: 0 h, 120 h) was determined using the VitaFast® Vitamin B12 microbiological assay (MBA, R-Biopharm, Darmstadt, Germany, AOAC-RI – Certificate No. 101002) following the manufacturer's protocol (limit of quantification: 0.30 μg/kg; detection limit: 0.21 μg/kg), as described by Piwowarek et al. (2025). Samples (1 g) were mixed with 20 mL of acetate buffer (pH 4.5), 250 μL of 1% NaCN (Sigma-Aldrich, USA), and 300 mg of Taka-diastase, then shaken and incubated in the dark at 37 °C for 1 h. In the next step, after adjusting the volume to 40 mL with distilled water, the mixture was heated in a water bath at 95 °C for 30 min with constant stirring. The cooled extracts (tap water) were centrifuged (12,857 ×*g*, 4 °C, 10 min; 5804R, Eppendorf, Germany), filtered (0.22 μm PES), and diluted with sterile water from the kit. Vitamin B12 was determined using a *Lactobacillus delbrueckii*-coated microtiter plate assay. Equal volumes (150 μL) of the kit medium and sample/standard were added to the wells. Plates were sealed with adhesive foil (provided with the kit) and incubated in the dark at 37 °C for 48 h. Optical density was measured at 540 (as above). B12 concentration was calculated against a standard curve (0–0.18 μg/100 g) and expressed as μg B12/kg f.m.

The MBA method does not distinguish between B12 in its active form and pseudocobalamin; consequently, the results may be overestimated. Therefore, for matrices 1–10 (120*h*) and the optimized matrix (120 h), B12 was also determined using the LC-MS/MS method (SOP M 3535, 2024) (accreditation number: D-PL-14115-02-03) to quantify only the active form of cobalamin. The analysis was performed by an external laboratory, SGS Poland. Results are expressed as μg/kg f.m. They are based on one analytical replicate of pooled samples from three biological replicates (1:1:1, *w*/w/w).

2.13. Statistical analysis

Data are presented as means ± standard deviations of three biological replicates, unless stated otherwise. Statistical significance ($p \leq 0.05$) was assessed using ANOVA followed by Tukey's HSD test in STATISTICA 13.1 (StatPoint Technologies, USA). Pearson correlations, heatmaps, and graphical representations were generated in R software (v. 4.1.1). The culture matrix was optimized via Design of Experiments (DoE) according to Piwowarek et al. (2025). Model significance was evaluated by ANOVA, and model fit and predictive ability were assessed using the coefficient of determination (R^2). Quadratic models with the highest R^2 values were selected.

3. Results

3.1. LAB screening

The inhibitory effect of LAB on PAB was evaluated in coexistence tests. Six strains representing different lactobacilli species were analyzed for their antagonistic activity against the *Pfr* DSM 20271 strain (Table S1). The antagonistic effect of LAB was strain-dependent. Four strains showed no antagonistic activity. One strain (*Lre*) caused a slight reduction in culture density around the spot inoculation, and one strain (*Lac*) exhibited antagonistic activity comparable to the positive control (the nisin-producing *Lc. lactis* IBB1339 strain), producing a clear inhibition zone, although smaller than that generated by the control strain.

To assess the potential stimulatory effect of LAB on PAB, strain *Pfr* DSM 20271 was cultured in the presence of lactobacilli culture supernatants using the Bioscreen C system. Initially, the growth of strain DSM 20271 was evaluated in two different media. A preceding control experiment demonstrated that strain DSM 20271 achieved a higher OD600 nm in BHI supplemented with 20% MRS than in BHI alone, with no difference in growth rate between the two media (Fig. S1). Subsequently, cell-free supernatants (MRS) from six lactobacilli strains were tested for their effects on the growth of strain *Pfr* DSM 20271 in BHI medium, with BHI supplemented with an equivalent volume of MRS used as the reference condition (Fig. S2). The most pronounced decrease in slope of the *Pfr* strain was observed in the presence of supernatants from *Lac* and *Lre* strains. A comparable, though less pronounced, trend was noted for the supernatants of strains *Lc* and *Lr*. In the case of *Lp* and *Lb* supernatants, there was no negative effect on the growth of *Pfr* DSM 20271. Importantly, none of the tested LAB strains showed any capacity to stimulate the growth of strain *Pfr* DSM 20271.

Most of the tested bacteria (*Pfr* and LAB) exhibited growth in all tested pomace types (BP, SP, and GP) (Fig. S3-S5, Table S2-S4), with the exception of *Lc*, *Lre*, and *Lac*. *Lre* did not show growth in BP and SP, whereas *Lc* and *Lac* did not grow in SP. Among LAB, the highest total growth (ΔOD) was observed for *Lp*, reaching 0.369 in BP, 0.490 in SP, and 0.696 in GP. Compared to other LAB strains, *Lp* was characterized by a relatively long adaptation (tlag) phase in all tested fruit by-products, ranging from 20 to 46 h. The shortest adaptation phases, or none at all, were observed for *Lb*. Generation times (g) varied by strain and medium, ranging from 40.76 to 185.59 h. The shortest generation times, particularly among LAB, were observed in SP and GP. Among the tested side-streams, GP supported the highest total growth of the analyzed bacteria. The shortest adaptation phases were observed in BP and SP (0−22*h*), whereas in GP, the adaptation phase varied considerably across strains, ranging from 8 to 88 h.

3.2. Carbon and nitrogen source utilization and bacterial growth

The lowest sugar content (glucose + fructose) was found in matrices 1 and 2, which contained only BP (7.38 ± 0.29 g/kg) and SP (8.15 ± 0.97 g/kg), respectively, whereas the highest sugar content was observed in the matrix composed solely of GP (21.85 ± 1.09 g/kg) (Fig. 1, Table S6). In the remaining matrices, composed of various pomace combinations, the total sugar content ranged from 11.85 to 16.59 g/kg. Glucose, depending on the matrix, accounted for 58–73% of the sugar, with the remaining portion being fructose (27–42%) (Table S5). In most media, sugar utilization (Fig. 1) reached nearly 100% within 72 h. Exceptions were matrices 2, 4, 6, and 9, in which, after 72 h of cultivation, bacterial sugar consumption amounted to 78.7%, 91.7%, 88.2%, and 90.6%, respectively. By 120 h, nearly complete sugar utilization was observed in all tested matrices.

The highest nitrogen content was determined in matrix 1, containing only BP (31.98 ± 1.49), whereas the lowest nitrogen content was observed in matrices 2 (SP) and 3 (GP), amounting to 15.99 ± 1.42 g/kg d.m. and 16.42 ± 2.03 g/kg d.m., respectively (Table S6). In general, higher nitrogen levels were detected in matrices containing BP as one of

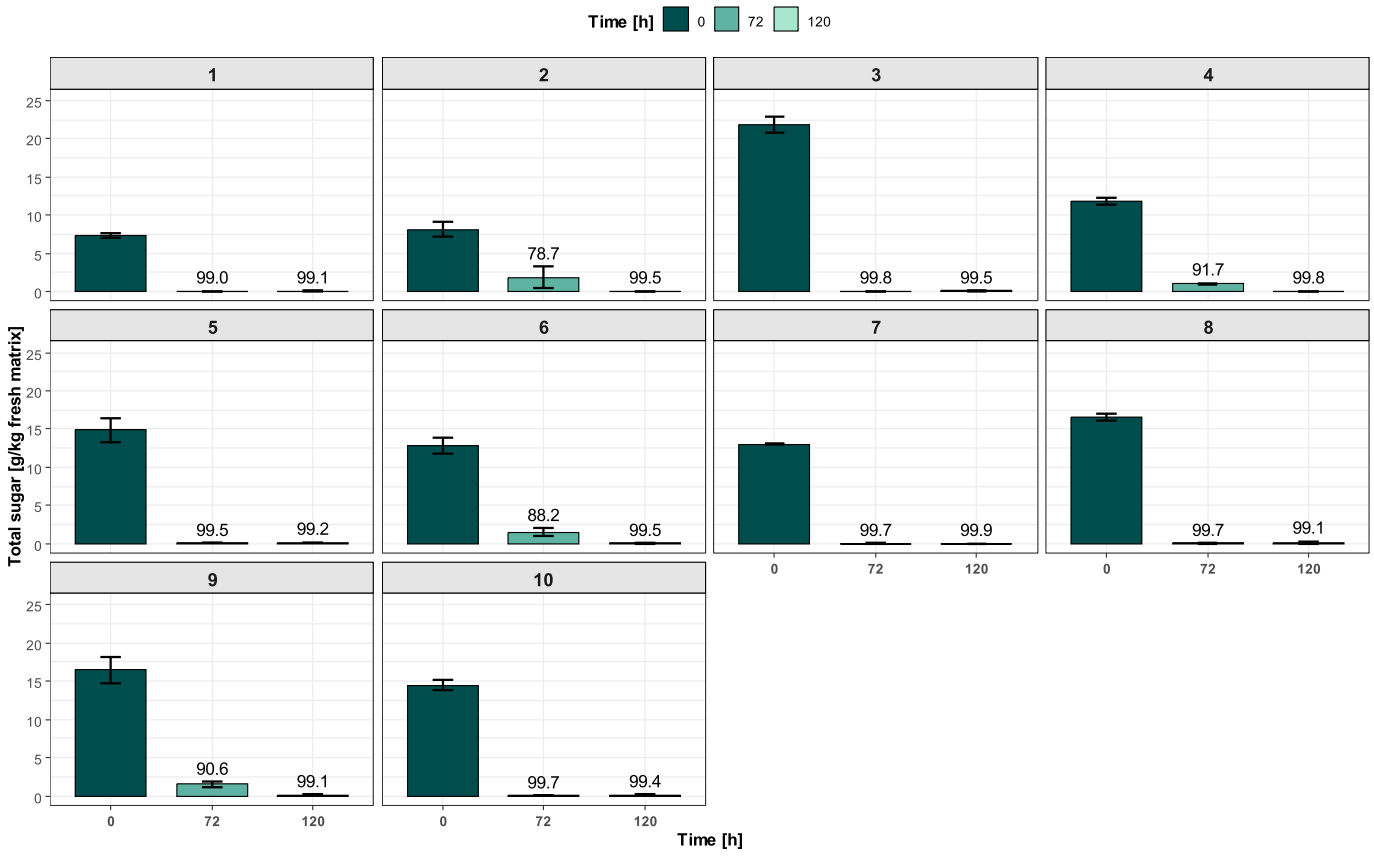

Fig. 1. Total sugar content during fermentation.
1–10 – matrices; numerical values above the bars (72 h and 120 h) indicate the percentage consumption of sugars at the given fermentation time. Bars and error bars represent means±standard deviations of three biological replicates.

the components compared with matrices without BP. The amino acid content was determined in the tested matrices at 0 h and after 120 h of cultivation (Fig. S6), as these compounds serve as nitrogen sources and play an important role in microbial metabolism. The amino acid content in the matrices at 0 h (before inoculation) was consistent with the nitrogen content and ranged from 63.72 ± 1.37 g/kg d.m. (matrix 2) to 201.92 ± 10.60 g/kg d.m. (matrix 1). The highest amino acid content was observed in the matrix containing only BP (matrix 1) and in multicomponent matrices in which BP was included (matrices 4, 5, 7, 8, 9, and 10). Matrices containing only SP and GP contained 63.72 ± 1.37 g/kg d.m. and 77.09 ± 1.21 g/kg d.m., respectively. The most abundant amino acids across all matrices were glutamic acid (Glu) and aspartic acid (Asp). In most matrices, a partial loss of total amino acid content was observed after 120 h of fermentation; amino acid utilization ranged from 11% to 54%, depending on the matrix, although in most cases it was approximately 20–30%. In general, the highest percentage utilization was observed in most matrices for proline (Pro), whereas the highest absolute utilization was observed for Glu and Asp. The only matrix in which an increase in total amino acid content was observed after 120 h, compared to 0 h, was matrix 2, consisting exclusively of SP.

The initial viable cell count of *Pfr* in the matrices was approximately 9.0 log CFU/g (8.64–8.80 log CFU/g) (Fig. 2). In all matrices, a slight increase in *Pfr* was observed up to 72 h of fermentation. Between 72 h and 120 h, no statistically significant differences were detected. The greatest increase in the number of *Pfr* DSM 20271 cells was observed in matrix 2; compared to time 0 h, the bacterial count increased by 0.37 ± 0.05 log CFU/g (Fig. 2). Overall, the addition of SP to the matrices increased the viable cell counts of *Pfr* DSM 20271. The smallest increase of the tested PAB strain was observed in matrices 1 and 3, containing only BP (by 0.19 ± 0.04 log CFU/g) and GP (by 0.16 ± 0.03 log CFU/g). *Lp* ATCC 10241 exhibited substantially more pronounced growth in the tested matrices compared to *Pfr* DSM 20271 (Fig. 2). The initial viable cell count of the LAB strain was approximately 6.0 log CFU/g (5.87–5.96 log CFU/g). Similar to *Pfr*, *Lp* ATCC 10241 showed growth up to 72 h, after which the number of viable cells remained stable across all matrices. Relative to time 0 h, the viable cell count of *Lp* ATCC 10241 increased by approximately 2.55–2.68 log CFU/g (Fig. 2).

3.3. Organic acid production

At the beginning (0 h), neither PA nor AA was detected in any of the analyzed matrices. Production of both acids continued until 120 h of cultivation (Fig. 3). The most intense PA synthesis was observed between 72 and 120 h of fermentation. The highest levels of PA and AA were observed in matrix 2, reaching 4.31 ± 0.13 g PA and 1.83 ± 0.08 g AA/kg f.m. In the remaining matrices, the synthesis of both acids was significantly lower, ranging from 0.73 to 2.88 g PA/kg and 0.30 to 1.29 g AA/kg. The weight ratio of PA to AA (P/A) after 120 h of cultivation in all matrices remained around 2:1 (1.56–2.51) (Table S7). The Pareto chart indicates that SP had the greatest effect on PA synthesis. Optimization of PA synthesis suggests that the highest PA production can be achieved using SP as the sole compound (Fig. 4, Table S8). The simultaneous use of SP with BP and/or GP, regardless of their ratio, should result in lower PA production.

No LA was detected in the analyzed matrices at 0 h. The maximum production of this acid in all matrices was observed at 72 h (Fig. 3), ranging from 4.71 to 7.80 g LA/kg f.m., depending on the variant. Between 72 and 120 h, LA concentrations were significantly lower in all pomace matrices, ranging from 1.07 to 6.53 g LA/kg f.m.

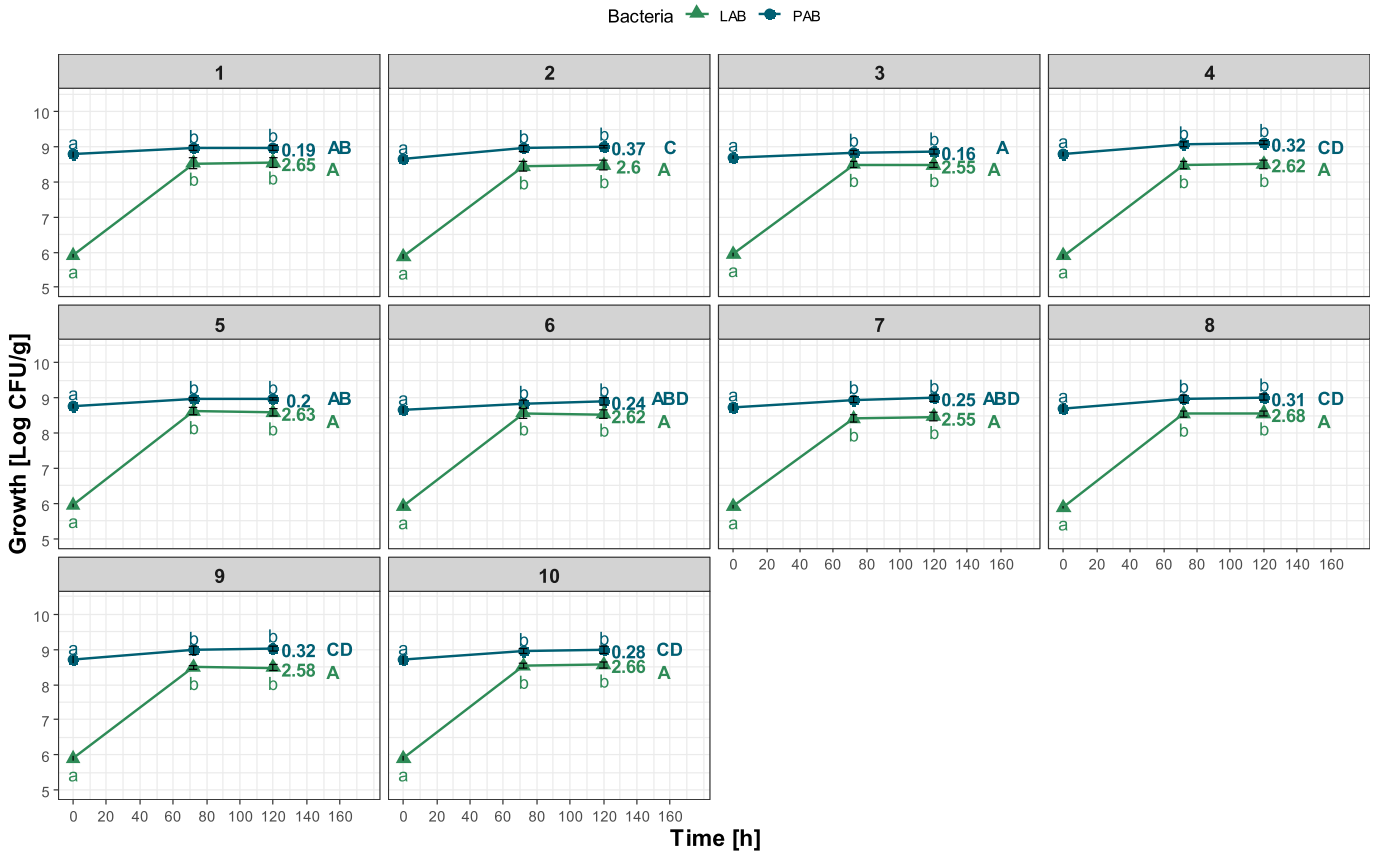

Fig. 2. Cell count of *P. freudenreichii* DSM 20271 and *L. plantarum* ATCC 10241.
1–10 – matrices; numerical values at 120 h indicate the net growth of bacteria (net growth = number of bacteria at 120 h − number of bacteria at 0 h). a-b – homogeneous groups showing the effect of fermentation time on cell counts of *Pfr* and *Lp* (one-way ANOVA, separate analysis for each matrix); A-D – homogeneous groups showing the effect of matrix type on net growth of *Pfr* and *Lp* (120 h) (one-way ANOVA, common analysis for all matrices). Values represent means±standard deviations of three biological replicates. Different letters indicate statistically significant differences ($p \leq 0.05$).

3.4. Vitamin B12 production

The B12 content (Fig. 5) at time 0 h in the analyzed matrices ranged from 3.61 ± 1.21 to 8.79 ± 1.25 μg/kg f.m., resulting from the inoculation of the media with *Pfr* DSM 20271. In most matrices, cobalamin synthesis occurred up to 120 h of cultivation; exceptions were matrices 2, 3, and 10, in which no statistically significant differences in cobalamin content were observed between 72 and 120 h. Vitamin B12 was determined using two methods: the microbiological assay (MBA) at 0 h, 72 h, and 120 h, and LC-MS/MS analysis (120 h). Because the microbiological method cannot distinguish between active B12 and pseudocobalamin, samples collected at 120 h were analyzed by LC-MS/MS to accurately quantify the active form of B12. The MBA results represent total B12 (pseudocobalamin + active form), whereas the LC-MS/MS results reflect only the active form. The highest total B12 yields (Fig. 5) were obtained in matrices 2 (44.98 μg/kg f.m.), 6 (49.81 μg/kg f.m.), and 9 (47.17 μg/kg f.m.); these media mainly contained SP and/or GP. In contrast, the lowest concentrations were observed in matrices 1 (30.71 μg/kg f.m.), 3 (23.92 μg/kg f.m.), and 5 (24.39 μg/kg f.m.), which were composed of BP, GP, and a BP-GP mixture at a 1:1 ratio, respectively. LC-MS/MS analysis revealed that the active cobalamin content was lower in most matrices (compared to the total B12) (Fig. 5), although the overall trend remained the same. The highest concentrations of active B12 were detected in matrices 2 (31.4 μg/kg f.m., 69.9% of total B12), 6 (35.6 μg/kg f.m., 71.5%), and 9 (38.2 μg/kg f.m., 81.0%), whereas the lowest values were found in matrices 1 (26.0 μg/kg f.m.), 3 (26.3 μg/kg f.m.), and 5 (17.0 μg/kg f.m.). The optimization process demonstrated that SP, BP, GP, and SP combined with GP significantly affected B12 synthesis by *Pfr* DSM 20271. The optimization model also showed that B12 production could be enhanced by using a matrix composed of 65% SP and 35% GP (Fig. 6, Table 8S). According to the quadratic model, such a proportion of pomace in the matrix should yield vitamin B12 levels of 43.12–60.04 μg/kg f.m. (predicted values) (Table 2, Table 8S). In the optimized matrix, co-culture of *Pfr* DSM 20271 and *Lp* ATCC 10241 resulted in 40.47 ± 2.05 μg/kg f.m. of total B12 and 36.8 μg/kg f.m. of the active form, corresponding to 90.9% of total B12 (Table 2). The initial cobalamin content in the optimized medium, after inoculation, was 5.40 ± 0.50 μg/kg f.m.

Vitamin B12 production was subjected to correlation analysis in relation to cobalt, riboflavin, niacin, total sugar and nitrogen contents, LA consumption, as well as molar and mass carbon-to-nitrogen (C/N) ratios (Table S6, Fig. S7). It was demonstrated that total B12 production depended on cobalt content and LA consumption, whereas synthesis of the active form was correlated exclusively with cobalt content (Fig. S7).

4. Discussion

4.1. LAB screening

Although the co-culture of *Pfr* and LAB is used industrially in cheese production (Thierry et al., 2011), indicating that LAB generally do not limit *Pfr* metabolism, it is important to recognize that microbial interactions are often species- or even strain-specific. Therefore, various LAB species were screened for their potential to fortify cobalamin in co-culture with *Pfr* DSM 20271. Of the six LAB species tested, only *Lac* exhibited an antagonistic effect against *Pfr* DSM 20271. Analysis of LAB

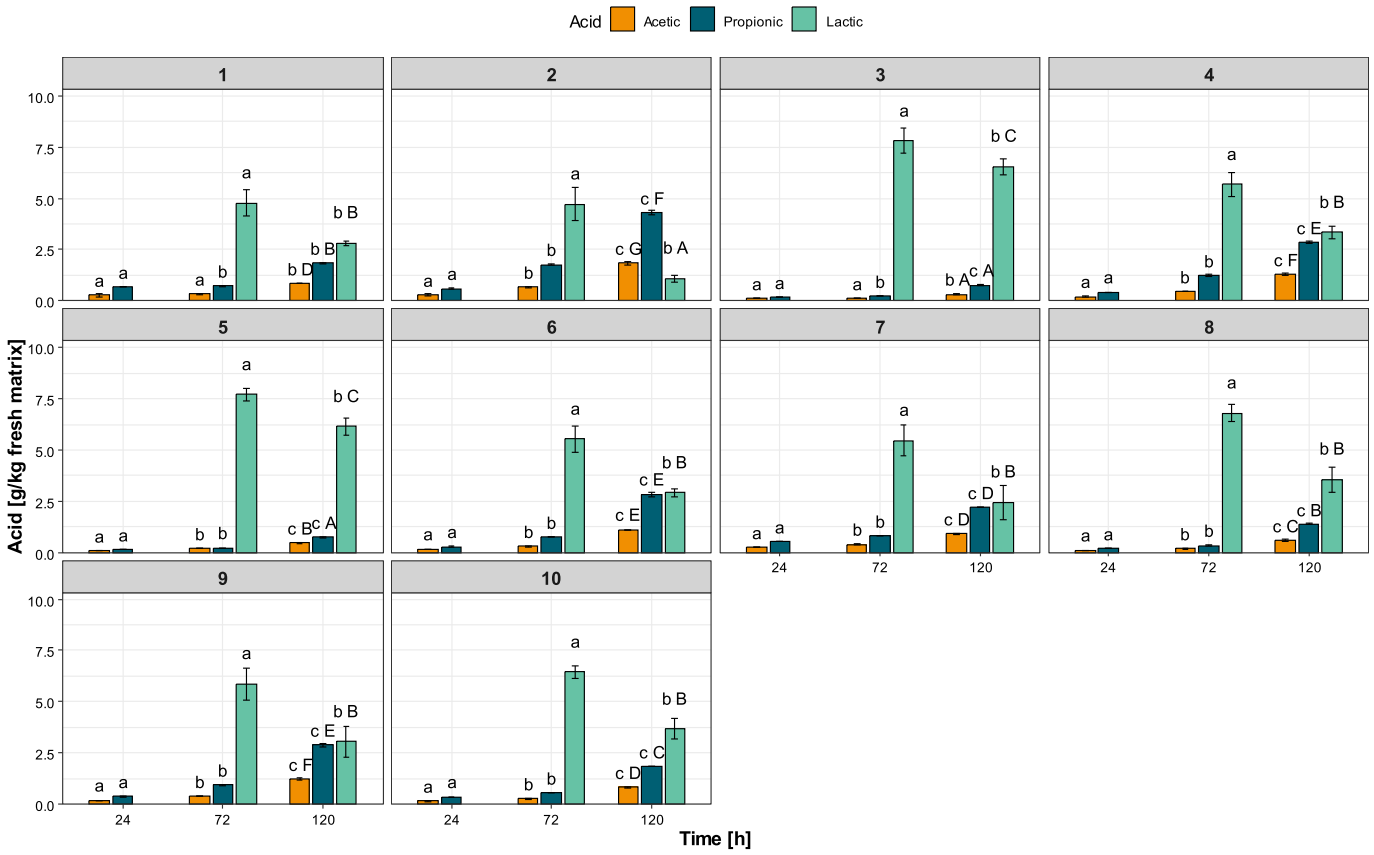

Fig. 3. Organic acid production during co-culture of *P. freudenreichii* DSM 20271 and L. *plantarum* ATCC 10241.
1–10 – matrices. a-c – homogeneous groups showing the effect of fermentation time on acetic/propionic/lactic acid production (one-way ANOVA, separate analysis for each matrix and acid); A-C – homogeneous groups showing the effect of matrix type on acetic/propionic/lactic acid production (120 h) (one-way ANOVA, common analysis for all matrices, but performed separately for each acid). Bars and error bars represent means±standard deviations of three biological replicates. Different letters indicate statistically significant differences ($p \leq 0.05$).

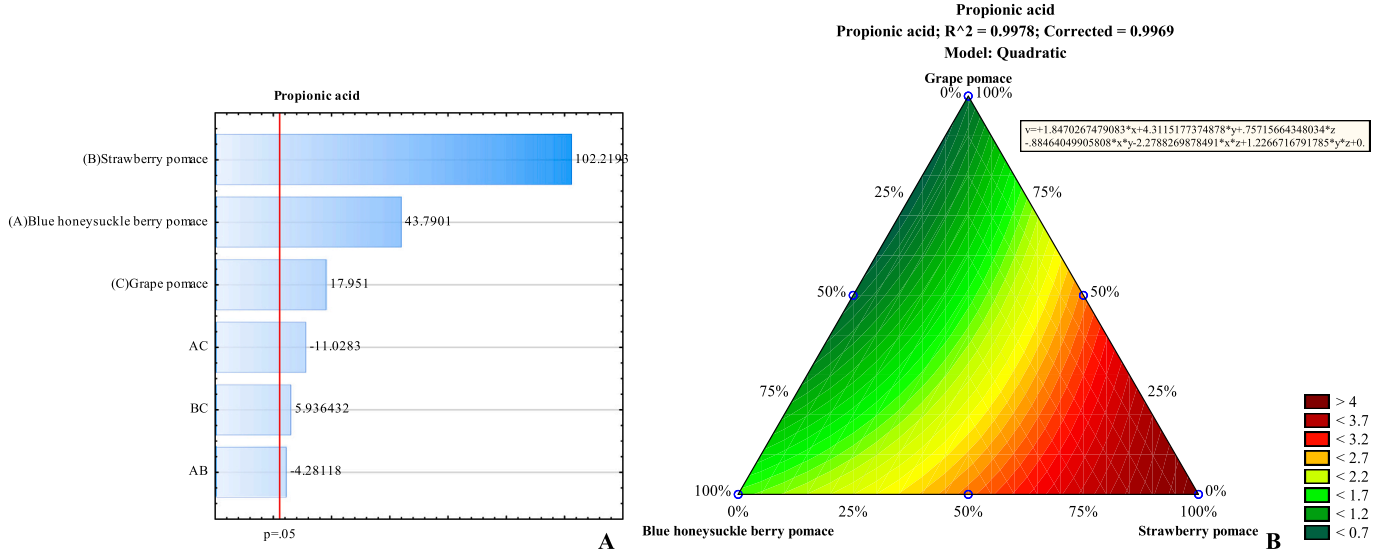

Fig. 4. Pareto chart (A) and triangle plot (B) – influence of the analyzed factors on propionic acid production during co-culture of *P. freudenreichii* DSM 20271 and *L. plantarum* ATCC 10241 (120 h).
Factors outside the red line – significant for the model (Pareto chart); the darker the shade of red on the triangle plot, the higher the predicted propionic acid production (g/kg f.m.).

culture supernatants showed that only *Lb* and *Lp* had no negative impact on *Pfr* growth, whereas the remaining supernatants inhibited *Pfr* development. Supernatants from lactobacilli strains (*Lre*, *Lac*, *Lr*, *Lc*) likely reflected partial depletion of MRS-derived nutrients that could

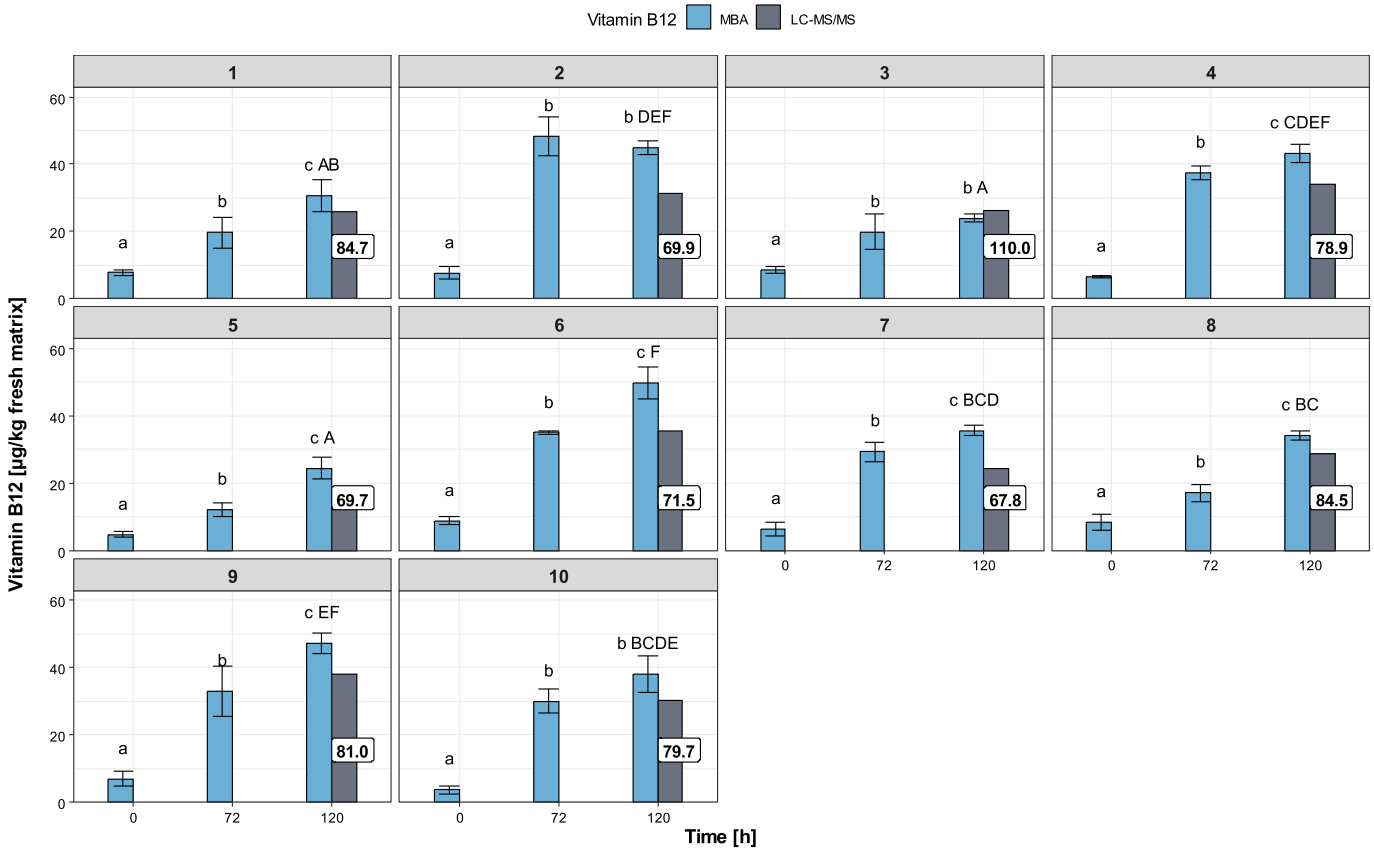

Fig. 5. Vitamin B12 production during co-culture of *P. freudenreichii* DSM 20271 and *L. plantarum* ATCC 10241.
1–10 – matrices; MBA – total B12, LC-MS/MS – active B12. a-c – homogeneous groups showing the effect of fermentation time on B12 production during the optimization process (one-way ANOVA, separate analysis for each matrix); A-F – homogeneous groups showing the effect of matrix type on B12 production during the optimization process (120 h) (one-way ANOVA, common analysis for all matrices). Bars and error bars represent means±standard deviations of three biological replicates (MBA). LC-MS/MS – one analytical replicate from mixed samples from 3 different cultures. Numerical values (brackets) on the LC-MS/MS bars refer to the percentage of active cobalamin in relation to total B12. Different letters indicate statistically significant differences ($p \leq 0.05$).

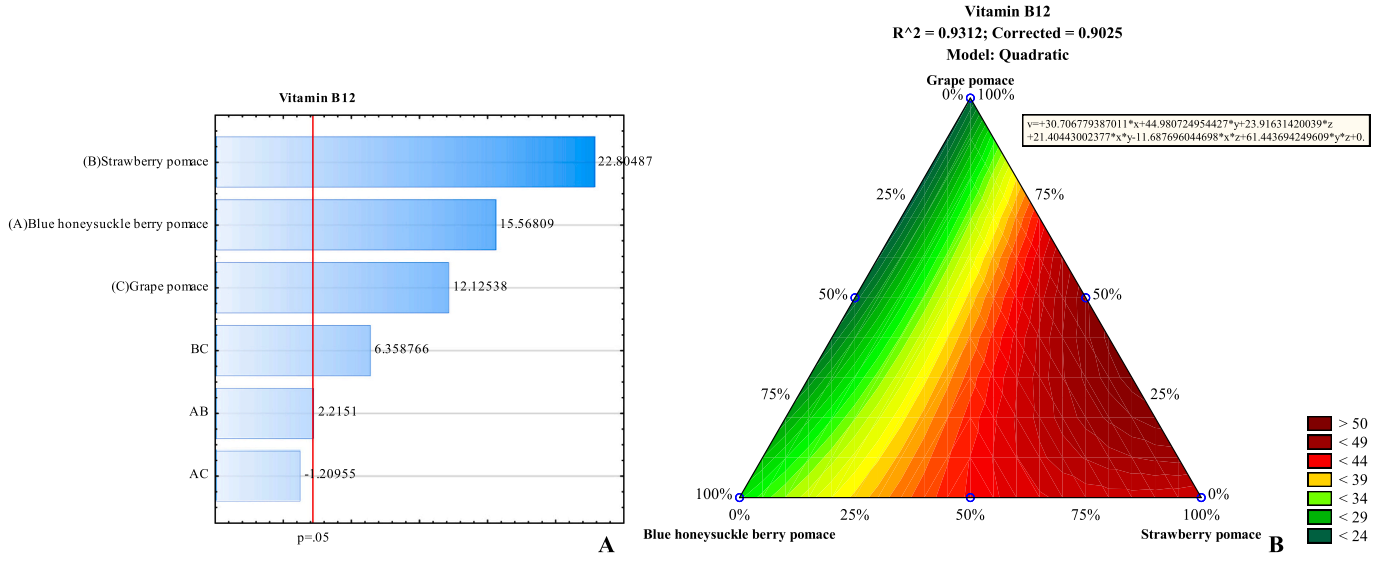

Fig. 6. Pareto chart (A) and triangle plot (B) – influence of the analyzed factors on vitamin B12 production during co-culture of *P. freudenreichii* DSM 20271 and *L. plantarum* ATCC 10241 (120 h).
Factors outside the red line – significant for the model (Pareto chart); the darker the shade of red on the triangle plot, the higher the predicted B12 production (µg/kg f.m.).

also be utilized by strain *Pfr* DSM 20271. This suggests that in some co-cultures, competition for nutrients from the matrices could limit *Pfr*

Table 2
Validation of the optimization model for B12 fortification.

Parameter	Predicted value	95% Confidence interval	Observed value (MAB)*	Observed value (LC-MS/MS)*
B12 [μg/kg fresh matrix]	51.59	43.12–60.04	40.41 ± 2.05	36.8

Based on the optimization model (Fig. 6, Table S8).
* Values obtained experimentally.

metabolism. LAB are also known to possess a broad antimicrobial spectrum due to the production of metabolites such as bacteriocins, organic acids, hydrogen peroxide, diacetyl, or exopolysaccharides (Ahansaz et al., 2023). It is likely that the supernatants of *Lre*, *Lac*, *Lc*, and *Lr* contained metabolites that suppressed *Pfr* growth.

Microculture experiments in pomace-based media demonstrated that most LAB species grew in the tested substrates. Special attention was given to *Lb* and *Lp*, which did not negatively affect *Pfr* metabolism. Overall, *Lp* exhibited the highest total growth across all fruit pomace matrices, whereas *Lb* showed shorter lag phases and faster generation times. Considering that the selected LAB were intended for co-culture with *Pfr*, *Lp* ATCC 10241 was chosen for the B12 fortification stage. This selection was based primarily on its lack of antagonistic activity toward *Pfr*. Another factor was its extended lag phase and longer generation time. These traits were expected to allow *Pfr* to adapt more effectively in pomace matrices and access nutrients more efficiently. In contrast, Lb adapts and multiplies rapidly. Consequently, Lb could dominate the environment through nutrient competition, thereby limiting *Pfr* activity.

The *Lp* strain was also tested for its potential to produce vitamin B12 (total B12, MBA) in MRS medium; no cobalamin synthesis was detected. Therefore, it can be concluded that the results regarding cobalamin fortification are not disturbed by the synthesis of, e.g., pseudocobalamin by the tested LAB strain.

4.2. Carbon and nitrogen source utilization and bacterial growth

Successful bacterial metabolism, encompassing both cell growth and metabolite synthesis, relies on microorganisms' ability to adapt to the conditions of the cultivation environment. This is a time-dependent process that is strongly influenced by the availability of utilizable nutrients, particularly carbon. Nitrogen sources are also important factors influencing bacterial metabolism and should be readily digestible, as they serve as building blocks for structural and enzymatic proteins (Piwowarek et al., 2023). Because nutrient uptake reflects the intensity of microbial metabolic activity, sugar and nitrogen consumption were evaluated during fermentation.

The initial sugar content (glucose and fructose) in the matrices varied, reflecting the different proportions of individual pomace types. Matrices containing GP, a rich carbon source (Piwowarek et al., 2025), exhibited higher sugar levels compared to matrices 1 and 2, which consisted exclusively of BP or SP. It is consistent with previously reported data (Oszmiański et al., 2016; Jin et al., 2019; Piwowarek et al., 2025). Under co-culture conditions (this study), carbon sources, including glucose and fructose, were almost completely consumed in all tested matrices. In contrast, Piwowarek et al. (2025) reported that monoculture of *Pfr* DSM 20271, cultivated under identical conditions and in the same fruit pomace-based matrices, utilized only 25–56% of the available sugars, depending on the matrix composition. The results obtained indicate that the matrices provided a favorable environment for bacterial metabolism, likely conferring benefits to *Lp* ATCC 10241. This may explain the substantially higher carbon utilization observed in co-cultures compared with *Pfr* DSM 20271 monocultures (Piwowarek et al., 2025), especially considering the enhanced *Lp* growth observed in the present study.

The nitrogen content and amino acid content of the tested matrices at 0 h (before inoculation), including the predominance of Glu and Asp, are consistent with literature data for fruit pomace and fruits (Palíková et al., 2008; Pieszka et al., 2015; Bordiga et al., 2019; Piwowarek et al., 2025). The approximately threefold higher total amino acid content observed in matrix 1, as well as the generally higher amino acid levels in BP-containing matrices, may result from the origin of BP from red fruit wine production. In this process, fermentation occurs in the pulp, and separation of the wine from the must typically takes place after fermentation, i.e., after yeast activity. Consequently, the elevated nitrogen and amino acid content may partially originate from yeast biomass generated during fermentation. The results showed a decrease in amino acid content in most matrices after 120 h of fermentation, consistent with the literature on LAB fermentation (Moore et al., 2022). The highest quantitative utilization of amino acids was observed for Glu and Asp. Glu serves as a precursor for cobalamin synthesis, whereas Asp can be converted into fumarate or succinate, thereby generating energy and acting as an intermediate energy substrate for *Pfr* (Thierry et al., 2011). A decrease in alanine and serine levels was also observed in the analyzed matrices. Both amino acids can be converted into pyruvate, an important carbon source in microbial metabolism, including PAB (Kruk et al., 2024). The highest percentage utilization was observed for Pro, which *Pfr* uses to protect against osmotic stress (Gagnaire et al., 2015).

In the tested fruit pomace-based matrices, the *Lp* strain exhibited significantly better growth across all media variants. In contrast, *Pfr* showed only a slight increase in cell numbers during cultivation. However, it remained viable in the pomace-based media throughout the entire cultivation period, regardless of matrix composition. The relatively abundant growth of *Lp* ATCC 10241 may indicate that this strain dominated the tested matrices, potentially suggesting a competitive limitation of *Pfr* growth. However, screening studies demonstrated that *Lp* ATCC 10241 should not inhibit the growth of the tested PAB strain. In the study by Piwowarek et al. (2025), *Pfr* DSM 20271 cultivated under monoculture conditions in the same matrices also exhibited only a slight increase in cell numbers, nearly identical to that observed in the present study. This finding indicates that the LAB strain used in co-culture did not inhibit the growth of *Pfr* DSM 20271, consistent with the results from the screening stage.

Fruit and fruit pomace do not constitute environments conducive to microbial growth. They contain numerous compounds that limit bacterial proliferation, including phenolic acids and flavonoids, which exhibit antibacterial properties (Palíková et al., 2008; Oliveira et al., 2013; Tumbas Šaponjac et al., 2015). LAB, such as *Lp*, naturally occur on plants, including fruits like grapes and berries (Cong et al., 2024; Vargas-Luna et al., 2025). Therefore, they are likely better adapted to such environments. This adaptation may explain *Lp* more efficient growth in pomace matrices than *Pfr*. In contrast, PAB, which naturally occur mainly in the rumen of ruminants and in their feces, are likely more susceptible to the biologically active compounds present in fruit pomace. Indeed, several studies have reported that polyphenols and flavonoids inhibit PAB growth (Wang et al., 2013; Kruk et al., 2024). The limited growth of *Pfr* DSM 20271 in fruit pomace-based matrices may also be attributed to the potentially low availability and bioaccessibility of certain vitamins, such as pantothenic acid (B5) (Falentin et al., 2010; Chamlagain et al., 2020).

Nevertheless, despite the limited increase of *Pfr* in the tested matrices, the number of viable cells did not decrease during cultivation. This observation indicates high resistance and stress tolerance of *Pfr* (Loivamaa et al., 2025), which may be attributed, among other factors, to its ability to accumulate various storage compounds (e.g., inorganic polyphosphate, trehalose, and glycogen) (Thierry et al., 2011).

4.3. Organic acid production

In the present study, PA production was detected in all analyzed matrices. The most pronounced increase in PA concentrations occurred between 72 and 120 h of cultivation, a period in which the sugar level was already low. This suggests that *Pfr* utilized alternative carbon sources during this time, most likely LA produced by *Lp*. LA concentration decreased significantly between 72 and 120 h in all matrices. The literature indicates that lactate can serve as a carbon source for PAB (Lee et al., 1974; Dank et al., 2021). The PA concentrations obtained in the present study were higher than those in *Pfr* monocultures in the same matrices (Piwowarek et al., 2025), demonstrating that co-culturing *Pfr* with LAB (*Lp*) can enhance PAB metabolism by enriching the medium with essential substrates, in this case, lactate. DoE statistical analysis confirmed that SP was the most influential factor in PA synthesis, and the addition of other side-streams reduced PA production. These findings are consistent with results from *Pfr* monocultures in the same pomace matrices (Piwowarek et al., 2025).

Biotin is an important factor in PA synthesis by *Pfr* (Falentin et al., 2010). This bacterium produces PA via the Wood-Werkman cycle, which begins with the biotin-dependent conversion of pyruvate to oxaloacetate. Importantly, *Pfr* does not produce biotin – it must be available in the environment. Interestingly, the highest concentration of B7 was found (Table S6) in matrices dominated by BP and GP, i.e., in media with relatively low PA synthesis. In contrast, the matrix composed solely of SP contained the lowest biotin level among all matrices, yet it yielded the highest PA concentration. It is likely that the biotin concentration was sufficient and that other factors played a more important role in PA production.

It should be noted that PA produced in pomace matrices, along with AA and LA, can function as natural preservatives, which could potentially extend the shelf life of fermented pomace. This may be particularly important in practical applications. LA has a long-standing tradition of preserving plant materials. PA is also commonly used in food production (EFSA ANS Panel, 2014) to prevent mold growth (the primary spoilage agent in fruits), which can produce harmful mycotoxins (Fernández-Cruz et al., 2010).

4.4. Vitamin B12 production

Plants do not naturally contain cobalamin. Therefore, the presence of B12 in the pomace matrices at 0 h resulted from the introduction of *Pfr* DSM 20271, a cobalamin-producing strain. The increase in cobalamin concentration observed at subsequent time points was solely attributable to the metabolic activity of *Pfr* DSM 20271. These results are consistent with those of Piwowarek et al. (2025). In *Pfr* DSM 20271 monocultures, the highest cobalamin concentrations, although lower than those observed under co-culture conditions (this study), were obtained in matrices 2 and 6, reaching 39.20 and 39.71 μg/kg f.m., respectively, without distinguishing between pseudocobalamin and the active form (Piwowarek et al., 2025). In the *Pfr-Lp* co-cultures, strain DSM 20271 had access to lactate as a carbon source, which may explain the higher cobalamin production observed in the present study compared to monocultures (Piwowarek et al., 2025). Correlation analysis revealed that LA consumption was associated with more efficient total B12 synthesis. No correlation was observed between lactate consumption and the production of the active form of B12. Kruk et al. (2024) also demonstrated that lactate metabolism enhances cobalamin synthesis by *Pfr*. The most efficient cobalamin synthesis observed in matrices 2, 6, and 9 likely resulted from their higher cobalt contents – 26.34, 37.23, and 26.93 μg/kg f.m., respectively (Table S6). This was confirmed by a correlation analysis of cobalt content with cobalamin production, both total and in its active form (Fig. S7). The literature indicates that the yield of vitamin B12 from *P. freudenreichii* depends on cobalt availability, and that cobalt limitation in the medium can restrict its production (Chamlagain et al., 2018). Conversely, Tangyu et al. (2022) reported that the cobalt supplementation of sunflower seed milk did not increase B12 production, suggesting that the natural level of cobalt in the plant milk (8 μg/kg) was sufficient. This demonstrates that the effectiveness of cobalt supplementation depends heavily on the base medium. Supplementing pomace with cobalt could enhance vitamin B12 production by *P. freudenreichii*; however, this hypothesis requires experimental confirmation. Although the direct addition of cobalt is not permitted in food-grade fermentations, pomace could be supplemented with raw materials that are naturally rich sources of this microelement, e.g., rice bran (Xie et al., 2021) or yeast extract (Kruk et al., 2024).

The literature indicates that *Pfr* can convert pseudocobalamin to the active form of B12 during prolonged cultivation (Deptula et al., 2017). In most matrices inoculated with *Pfr* DSM 20271, both active cobalamin and pseudocobalamin were produced; however, a proportion of pseudocobalamin was not converted to the active form for some reason. Active cobalamin contains 5,6-dimethylbenzimidazole as the lower ligand, which is synthesized by *Pfr* from riboflavin. Vitamin B3 is also believed to play a crucial role in the synthesis of active cobalamin. B3 is likely involved in the activation of DMBI to α-ribazole, thereby enabling its incorporation into the B12 structure (Deptula et al., 2015, 2017). *Pfr* can synthesize both B2 and B3; however, the literature indicates that their presence in the growth medium enhances cobalamin synthesis (Chamlagain et al., 2025). Perhaps the higher content and bioavailability of vitamins B2 and B3 (Chamlagain et al., 2016, 2020) in the pomace matrices could result in increased synthesis of active cobalamin. This requires further experimental validation.

Although LA produced by LAB is the preferred carbon source for PAB, the rapid decrease in pH caused by LA accumulation could have limited PAB growth, as these bacteria prefer neutral pH conditions, and their metabolism is limited when the pH drops below 4.5 (Ye et al., 1996). In the tested matrices, whose initial pH was approximately 6.3, the pH decreased to values even below 4.0, depending on the variant and cultivation time (Table S9). Nevertheless, PAB demonstrated metabolic activity by synthesizing PA and B12. This was primarily due to the medium being neutralized every 24 h to approximately 7.0, which temporarily ensured optimal pH conditions for PAB. More precise pH control, such as in a bioreactor cultivation, could enhance PAB metabolism, potentially leading to more efficient propioniac acid and cobalamin production.

Based on the optimization process, the most efficient cobalamin production can be achieved with a mixture of SP (65%) and GP (35%). This result is fully consistent with the optimization performed using a *Pfr* monoculture (Piwowarek et al., 2025). According to the optimization model, the predicted total B12 production (pseudocobalamin + active form) at these SP and GP proportions should range from 43.12 to 60.05 μg/kg f.m. As a result of the *Pfr–Lp* co-culture in the optimized matrix, the total B12 concentration of 40.41 ± 2.05 μg/kg f.m. was obtained, which is almost consistent with the model prediction. Differences in absolute B12 concentrations were observed between the optimization process and the optimized matrix, with the total cobalamin concentration being lower in the latter. This variability likely reflects batch-to-batch differences, which are typical of small-scale cultivations in complex media under microaerobic and co-culture conditions. Perhaps using a bioreactor culture on a larger scale, with more precise control of fermentation parameters, such as pH and dissolved oxygen, would allow greater process repeatability. On the other hand, the production of the active form of B12 in the optimized matrix reached 36.8 μg/kg f.m., accounting for 90.9% of total B12. In contrast, in matrices 2, 6, and 9, the proportion of active B12 during the optimization process ranged from ca. 70 to 80%, depending on the matrix. Thus, the optimized matrix composition enabled a higher relative share of active cobalamin. This may have resulted from a more favorable vitamin of the matrix. Riboflavin is among the components that could have contributed to this effect.

Culture systems based on co-cultivation of *Pfr* with other microorganisms, including LAB, for cobalamin fortification have already been

investigated using various raw materials, such as cereal-based matrices (wheat bran, oat bran, rice bran, buckwheat bran, and sorghum flour) (Xie et al., 2019, 2021), faba bean flour (Kantanen et al., 2024), or faba bean, pea, and lentil (Kahala et al., 2024). The results of the present study, combined with existing literature, clearly demonstrate the potential for effective cobalamin fortification of plant-based materials using co-cultures of *Pfr* with LAB without compromising B12 production. This is particularly important because LAB can extend shelf life, improve microbiological quality, enrich fermented raw materials with bioactive metabolites, and positively influence sensory attributes (taste, aroma, texture). Moreover, some LAB, including *Lp*, are considered probiotic microorganisms. Their presence in the final product may enhance its functional and health-promoting value (Echegaray et al., 2023).

Recommended daily intake values for B12 vary across countries. The Recommended Dietary Allowance (RDA) for B12 is 2.4 μg/day for adults in the United States and Canada. The European Food Safety Authority (EFSA) recommends an adequate intake (AI) of 4 μg/day (Zhou et al., 2025). Considering these recommendations and the active B12 content of the optimized matrix, the amount of fermented matrix required to meet the daily B12 requirement would be approximately 65–109 g. This value varies depending on the country or region. Fermented pomace could be used in food production, for example, as an ingredient in fruit jams and yogurts (including plant-based yogurts), or as a component in the manufacture of plant-based foods designed to mimic animal-derived products. The literature indicates that B12 produced *in situ* by *Pfr* exhibits high stability and resistance to degradation (caused by light, heat, and pH), even after processing (Edelmann et al., 2016; Loivamaa et al., 2025). The fortified B12 is also characterized by good bioavailability (Chamlagain et al., 2021). These findings support the potential industrial application of fermented pomace matrices, particularly in the food sector, in alignment with the circular economy principle and zero-waste policy.

5. Conclusions

The research presented in this paper demonstrates that the co-culture of *Pfr* DSM 20271 and *Lp* ATCC 10241 offers a promising approach for valorizing fruit by-products, with potential environmental, economic, and consumer benefits. Further studies are needed to assess the stability of the vitamin produced during fermentation, its resistance to processing, and its bioavailability. Future research should also focus on assessing B12 production in systems with more precisely controlled pH and oxygen conditions, for example, through bioreactor-based cultivation.

CRediT authorship contribution statement

Kamil Piwowarek: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zuzanna Makowska:** Investigation, Formal analysis. **Magdalena Kowalczyk:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Izabela Kern-Zdanowicz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Lena Ruzik:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Alicja Synowiec:** Visualization, Methodology, Formal analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT and Grammarly in order to check and improve the language of the manuscript. After using these tools/services, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the published article. The text was not generated by AI. All research data were obtained experimentally.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2026.111910.

Data availability

The experimental data are available in an open repository at the following link: doi:10.18150/JW40ND

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