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In situ fortification of protein-enriched brewer's spent grain with vitamin B12 by fermentation with *Priestia megaterium* and *Propionibacterium freudenreichii*

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A B S T R A C T

Meat is an important protein source in the human diet; however, the consumption of animal proteins needs to be reduced. Plant-based foods naturally lack cobalamin, vitamin B12 (VitB12), an essential vitamin in the human diet. Brewer's spent grain (BSG) is a side stream available in large volumes that was chosen as a substrate for food-grade VitB12-producing microorganisms.

VitB12-producing microorganisms were identified in the scientific literature and selected to study in situ production of VitB12 in a protein-enriched (53%) BSG. A first screening was performed using *Propionibacterium freudenreichii* and *Priestia megaterium* (formerly known as *Bacillus megaterium*) strains. Further optimisations were exploited to increase the BSG content further and resulted in levels of up to 21 μg VitB12/100 g in the BSG at lab scale. Finally, experiments were performed at 12 kg scale fermentation with a stabilized pH and resulted in 7.7 μg VitB12/100 g BSG, indicating the potential of in situ fortification of BSG with VitB12 by fermentation with *P. freudenreichii*. Overall, it was found that the VitB12-fortified BSG could serve as a relatively inexpensive plant-based source of VitB12-fortified protein for food and feed applications. This study shows that fermentation offers opportunities to fortify (protein-enriched) BSG or other BSG derivatives with VitB12.

1. Introduction

Meat is an important protein source in the human diet; however, the current amount of available animal-based proteins is insufficient to satisfy the global demand for meat. Moreover, to reduce the impact on public health and the environment, the intake of meat should be reduced by the consumption of more plant-based protein sources (Searchinger, Waite, Hanson, Ranganathan, & Matthews, 2019). Meat not only provides high-value proteins but is also an important source of several essential micronutrients such as cobalamin (vitamin B12; VitB12). VitB12 has an important role in the functioning of the brain and nervous system as well as in the formation of red blood cells (Hunt, Harrington, & Robinson, 2014). This vitamin is not synthesised by the human body. The usual source for intake is via animal-derived food products such as meat, cheese, eggs, fish and shellfish (Gille & Schmid, 2015; Watanabe, 2007). Hence, the consumption of only plant-based products poses a higher risk of VitB12 deficiency (Hunt et al., 2014).

In situ fortification of plant-based products by fermentation with a bacterium that synthesises biologically active VitB12 and has a GRAS (Generally Regarded As Safe) or QPS (Qualified Presumption of Safety) status could provide a cost-effective technique to improve the availability of this vitamin for populations at risk of VitB12 deficiency. Different microorganisms can produce VitB12, including the food-associated species *Propionibacterium freudenreichii*, several lactobacilli, several *Bacillus* species and *Priestia megaterium* (formerly known as *Bacillus megaterium*) (Assis, Matte, Aschidamini, Rodrigues, & Záchia Ayub, 2020; Shelton et al., 2019). De novo biosynthesis of VitB12 in bacteria is complex and involves over 30 enzymatic steps (Balabanova, Averianova, Marchenok, Son, & Tekutyeva, 2021). In the first stage of VitB12 biosynthesis, the anaerobic biosynthesis pathway, either glutamate or glycine is used to start the synthesis. Cobalt is inserted to promote ring contraction. The precursor cobamide is synthesised using threonine and incorporated. In the second stage of the pathway, another precursor 5, 6-dimethylbenzimidazole (DMBI) is required to form bioactive cobalamin; this part of the pathway requires oxygen (Fang, Kang, & Zhang, 2017).

In situ fortification has been exploited for tempeh by co-culturing *Rhizopus oryzae* and *P. freudenreichii* lupin beans resulting in a VitB12-

Abbreviations: VitB12, Vitamin B12; GRAS, Generally regarded as safe; fw, Fresh weight; dw, Dry weight; DMBI, 5,6-dimethylbenzimidazole; BSG, Brewer's spent grain; SL, Sodium lactate; PPS, Peptone physiological salt; QPS, Qualified presumption of safety; RDI, Recommended dietary intake.

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enriched lupin tempeh of up to 1 μg per 100 g fresh weight (fw) of product (Wolkers-Rooijackers, Endika, & Smid, 2018). In situ fermentation of wheat bran with *P. freudenreichii* subsp. *freudenreichii* DSM 20271 resulted in production of up to 33 μg VitB12 per 100 g dry weight (dw) (Xie et al., 2019). In another study, soya flour was fortified by in situ fermentation using the same strain reaching levels of up to 9.1 μg/100 g fw (Wang et al., 2022). In addition, different examples have been reported for plant-based side streams derived from food production processes that have been fortified with VitB12 by fermentation. For example, sunflower seed milk, a side stream resulting from press cake of sunflower seeds, reached VitB12 levels of up to 17 μg/100 g fw of product by co-culturing *P. freudenreichii* with *Bacillus amyloliquefaciens* (Tangyu et al., 2022). Another study used *P. freudenreichii* to fortify a mixture of potato wastewater and apple pomace with 290 μg VitB12/100 mL fw (Piwowarek, Lipińska, Hać-Szymańczuk, Kolotylo, & Kieliszek, 2022). By using a two-step fermentation process, consisting of an anaerobic and an aerobic phase with *P. freudenreichii,* VitB12 could be produced in rice brain oil to a final level of 2.94 mg/L fw of product (Hedayati, Hosseini, & Najafpour, 2020).

Brewer's spent grain (BSG) represents the major side stream from the brewing industry that is currently largely disposed of as low-cost cattle feed (Bianco et al., 2020). For Europe, about 6–8 million tons of BSG is produced annually as a side stream of beer production (Allegretti et al., 2022). About 70% of the BSG is used as animal feed, and the remainder is used either for biogas production or disposed of as landfill (Parchami, Ferreira, & Taherzadeh, 2021). For a more sustainable use of the side streams of food production processes, it would be desirable to convert low-value side streams into products of higher value (co-products) that can be fed back into the economy as new raw material. Unprocessed BSG contains relatively high protein levels of 26%–30% (Wen, Zhang, Duan, Zhang, & Ma, 2019), while decanted BSG can contain higher levels due to water loss and a wide range of other nutritional constituents (Mitri et al., 2022). There is a growing interest in the possibilities for the use of BSG side streams as a food ingredient such as a meat replacer in the human diet (Bianco et al., 2020), and fortification of this protein-rich product with VitB12 would increase its value. Therefore, this study explored the in situ fortification of a protein-enriched BSG with VitB12 via fermentation.

Based on information from the scientific literature, food-grade bacterial species that are known to produce VitB12 were selected in this study. Three strains of *P. megaterium* and two strains of *P. freudenreichii* were chosen to screen for VitB12 production in BSG based on (1) their commercial availability, (2) the existence of GRAS or QPS status and (3) reported production levels of VitB12 in a plant-based food matrix (Abou-Taleb, Mashoor, Sohair, & Sharaf, 2005; Chamlagain et al., 2016; Moore, Mayer, Biedendieck, Deery, & Warren, 2014). Finally, based on performance, *P. freudenreichii* was used to test VitB12 production at 12 kg scale in a bioreactor under controlled conditions. The resulting product could serve as a relatively inexpensive plant-based source of VitB12-fortified protein for food and feed applications.

2. Material and methods

2.1. Scientific literature search

A Scientific literature search was performed to identify studies reporting on the in situ production of VitB12 in plant-based food matrices using the Scopus database, Web of Science database and Google. After screening based on reference title, keywords and abstracts, relevant full articles were downloaded and analysed. Snowball citation was also applied to gain additional relevant publications.

2.2. Substrates

Decanted BSG cake with a protein content of 53% (w/w) (Fig. 1) and 60% (w/w) corn steep liquor containing lactate and free amino acids

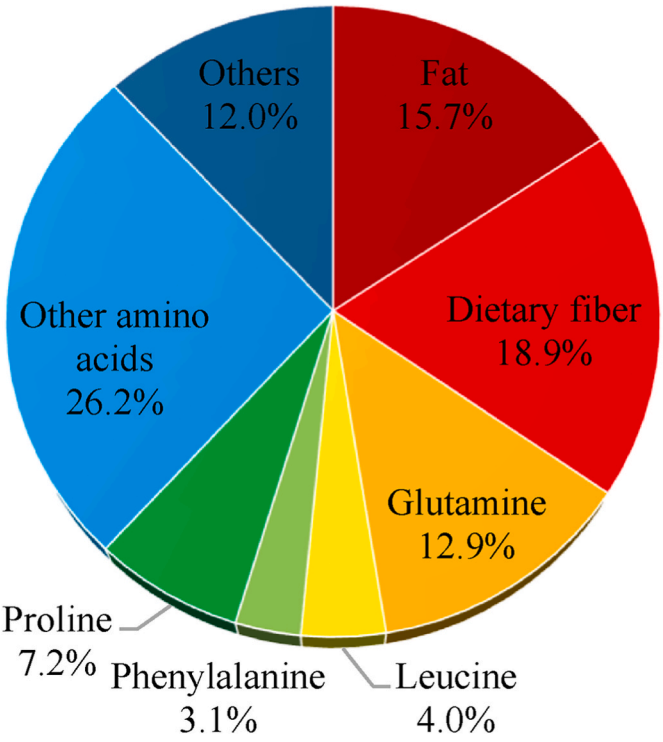

Fig. 1. Composition of decanted BSG. Total protein content is 53% (w/w). All values are related to the dry weight (% w/w).

was provided by a commercial processor of food co-products (Duynie Holding B.V, Alphen aan den Rijn, The Netherlands) and stored at −20 °C until use. The total protein content of the BSG used was 53% (w/w) on the basis of dw (Fig. 1), and this was used as the basis for preparing either the slurry or the paste mentioned below. The sugar content of BSG is known to be low and was 0.5% (w/w) dw in this study, whereas the lactic acid content was 3% (w/w) dw (data not shown). Glutamine is a precursor of glutamate, which is a precursor of VitB12, and the glutamine content in the BSG was 12.9% (w/w) on the basis of dw (Fig. 1). Glycine and threonine, both precursors for VitB12, were present at 1.8% (w/w) and 3.5% (w/w), respectively (data not shown). The content of riboflavin (vitamin B2) and nicotinamide (vitamin B3), precursors of DMBI, in the BSG was 0.04 mg/100 g BSG and 0.35 mg/100 g BSG, respectively, and detected cobalt levels in the BSG were 0.03 mg/kg BSG (data not shown).

2.3. Bacterial strains and preculture conditions

All the bacterial strains were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ; Braunschweig, Germany). Three *P. megaterium* strains (with DSM culture collection numbers: DSM 509, DSM 2894 and DSM 319) and two *P. freudenreichii* strains (*P. freudenreichii* subsp. *freudenreichii* DSM 20271 and *P. freudenreichii* subsp. *shermanii* DSM 4902) were used for initial screening for VitB12 production on BSG. For the culturing of *P. freudenreichii*, 50 mL Erlenmeyer flasks containing 10 mL sodium lactate (SL) broth (5 g/L tryptone [bacteriological, J809, VWR Amresco Life Science, Solon, USA], 10 g/L yeast extract [LP0021, Oxoid, Basingstoke, Hants, UK], 8 g/L sodium acetate 3-hydrate [71188, Sigma-Aldrich St. Louis, USA] and 25 g/L DL lactate syrup 60% [w/w; L1375, Sigma-Aldrich St. Louis, USA], pH 6.5) were individually inoculated with a single colony of *P. freudenreichii* and incubated for 2 days, static at 30 °C. After incubation, the cultures were centrifuged for 5 min at 7000×*g* and the pellets were resuspended in peptone physiological salt (PPS; 1 g/L peptone [enzymatic digest of soybean meal, 70178, Sigma-

Aldrich St. Louis, USA] and 8.5 g/L sodium chloride [S9625, Sigma-Aldrich St. Louis, USA]), resulting in a ten-fold concentrated preculture of ~9 log CFU/mL. *P. megaterium* was cultured in nutrient broth (No. 4, 03856, Sigma-Aldrich St. Louis, USA), 150 rpm at 30 °C and washed as described above for *P. freudenreichii*.

2.4. Fermentation conditions for VitB12 fortification

For all the procedures described in this section, care was taken to carry out fermentation and extraction under minimal exposure to light by, for instance, wrapping in aluminium foil.

2.4.1. Submerged fermentation of BSG in erlenmeyer flasks

Submerged fermentation involved either a 10% or 50% (percentage of BSG cake per total weight) BSG slurry in demineralised water in Erlenmeyer flasks or an 85% (percentage of BSG cake per total weight) BSG paste in plastic pouches, with the pH adjusted to 7–8 using 1M KOH. After autoclaving (15 min, 3 bar, 121 °C), sterile 25% (w/v) glucose (G7021, Sigma-Aldrich St. Louis, USA) was added to a final concentration of 2% (v/w). Precultures of *P. freudenreichii* (1:10) or *P. megaterium* were added, and incubation was performed at 30 °C, unless stated otherwise. *P. megaterium* cell numbers in the BSG fermentation were ~3 log CFU/g at the start. An anaerobic atmosphere was created in jars using Anaerocult A pouches (cat. no. 113829, Merck Millipore; Darmstadt, Germany), according to manufacturer instructions. For microaerophilic fermentations, Anaerocult C pouches (cat. no. 116275, Merck Millipore; Darmstadt, Germany) were used. This created an atmosphere of about 8–10% (v/v) CO_2 and 5–7% (v/v) oxygen. An aerobic atmosphere was created by rotation at 150 rpm (Erlenmeyer flasks with BSG slurry) or by leaving plastic pouches containing BSG paste open in large beaker glasses, loosely covered with sterile aluminium foil to prevent contamination. Uninoculated BSG was included as control samples.

2.4.2. Upscaled submerged fermentation of BSG under controlled conditions

Fermentation with *P. freudenreichii* subsp. *freudenreichii* DSM 20271 was executed in a 20-litre Biotwin bioreactor (Biostream International B. V., Doetinchem, The Netherlands). For this process, 12 kg BSG slurry of 10% (w/v) with 2% (w/w) corn steep liquor (see section 2.1) was adjusted to pH 7.0 using 1M KOH, and the bioreactor with slurry was sterilised while stirring (300 rpm) at 121 °C for 20 min. Probes for measuring pH and dissolved oxygen were calibrated. The inoculum (see section 2.3) was precultured in a shake flask, and 120 ml was dosed to the reactor. Incubation was at 30 °C, pH was controlled at 7.0 by the automatic addition of 10% (w/v) KOH and, if needed, the dissolved oxygen level was adjusted manually by administering air to the bioreactor headspace and/or increasing the stirrer speed.

2.5. Determination of microbial numbers

1 mL fermented BSG slurry or 1 g fermented BSG paste was mixed with 9 mL PPS and vortexed vigorously for 10 s. The *P. freudenreichii* or *P. megaterium* numbers were quantified by plating 100 μL of serially diluted aliquots onto SL agar (SL broth containing 12 g/L technical agar No.2, LP0012, Oxoid, Basingstoke, Hants, UK) plates for 4–7 days (*P. freudenreichii*) or onto nutrient agar plates for 1 day (*P. megaterium*) at 30 °C to determine the colony forming unit (CFU) number.

2.6. Cobalt, vitamin B2, vitamin B3 and metabolite analysis

Cobalt, vitamin B2 and B3 determinations were outsourced to an external laboratory (The Netherlands).

Metabolites consumed or formed during the fermentation in the 20-litre Biotwin bioreactor were measured in time by HPLC (lactic acid, acetic acid, propionic acid, glucose and fructose/xylose). A Waters HPLC system was used, equipped with a refractive index detector (Waters, model 2414, Etten-Leur, The Netherlands) and a KC-811 300 × 8 mm column (Shodex, Tokyo, Japan), at 65 °C, with 3 mM H_2SO_4 as mobile phase and a flow rate of 1 mL/min. As an internal standard, 3 mM valeric acid in 1 M H_2SO_4 was used.

2.7. Vitamin B12 analyses

2.7.1. VitaFast assay

VitB12 determination in BSG (fermented) samples was performed using the VitaFast Vitamin B12 (Cyanocobalamin) microtiter plate test according to manufacturer instructions with minor modifications. In short, a 1 g or mL fermented or control (i.e. uninoculated) sample was placed in a sterile 50 mL centrifuge tube to which 20 mL freshly prepared acetate buffer (pH 4.5 using sodium acetate 3-hydrate [71188, Sigma-Aldrich St. Louis, USA]) was added and vortexed vigorously for 10 s. Subsequently, 250 μL freshly prepared 1% KCN (60178, Sigma-Aldrich St. Louis, USA) solution and 300 mg Taka diastase (6247, Sigma-Aldrich St. Louis, USA) were added and mixed. After incubation for 1 h at 37 °C, sterile MilliQ was added to a volume of 40 mL and heated for 30 min at 95 °C in a water bath. The process of cooling down to approximately 30 °C was performed by placing the tubes in tap water. Serial dilutions in sterile MilliQ and the VitB12 standard curve were prepared in microtiter wells (96 wells) that were covered with foil to prevent evaporation. Optical density at 610–630 nm was measured after incubation for 48 h at 37 °C using a spectrophotometer device (InfiniteF200Pro, Tecan, Männedorf, Switzerland).

2.7.2. HPLC-UV analyses

For determination of the VitB12 content of the BSG samples from the Biotwin bioreactor, an HPLC-UV method was applied. Prior to VitB12 analysis, BSG samples were extracted and purified. A 15 g sample was grinded under liquid nitrogen using a grinder (IKA A11 B S000, IKA-Werke GmbH & Co. KG, Staufen, Germany) and diluted in a sodium acetate buffer (50 mM; pH 4.5). Diastase (0.4 g; 84247, Sigma-Aldrich St. Louis, USA) and lysozyme (0.1 g; 62971, Sigma-Aldrich St. Louis, USA) were used for a first digestion (30 min; 37 °C in a shaking water bath), followed by a second digestion (45 min, 37 °C) with the addition of KCN (0.25 ml; 4% [w/v]; 60178, Sigma-Aldrich St. Louis, USA). Prior to centrifugation (4500 rpm, 10 min, 4 °C) and filtration over paper, the digestion was stopped by incubating for 30 min in a water bath of 100 °C. The filtered digest was purified by immunoaffinity columns (P80B, R-Biopharm Rhône Ltd., Glasgow, United Kingdom) according to the supplier's manual.

The purified VitB12 was separated using a 100 mm × 2.1 mm Zorbax RRHD Eclipse Plus C18 – 1.8 μm column (Agilent, Santa Clara, USA). A 90-5% linear elution gradient of 0.025% (v/v) trifluoroacetic acid (91700, Sigma-Aldrich St. Louis, USA) in water and 0.025% (v/v) trifluoracetic acid in acetonitrile (8010228, Actu-All chemicals, Randmeer, Oss, The Netherlands) was used. The detection was performed on a Waters Acquity Arc with a 2998 PDA detector at 361 nm. Chromatograms were analysed using Chromperfect v. 6.0.18 (Denville, NJ, USA) and the data were analysed in Excel software v.16.0 (Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Studies on in situ production of VitB12 in plant-based food matrices

An overview of the scientific studies reporting on the in situ production of VitB12 in plant-based food matrices is presented in Table 1. *P. freudenreichii* has frequently been used in different plant-based matrices, including studies describing the in situ fortification of grain products; however, none of the studies used BSG as a substrate (Table 1). Other reported food-grade bacteria were *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) and other lactic acid bacteria: *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *Lacticaseibacillus*

Table 1
Vitamin B12 production levels and its producing microorganisms in different plant-based food matrices reported in the scientific literature.

Microorganism (species)	Strain	Max VitB12 production[a]	(Optimal) conditions	Remarks[a]	Reference
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	0.97 μg/100 g fresh Lupeh (lupin tempeh).	Co-fermentation. 10^7 CFU/g inoculation, 25 °C for 44 h. Pre-acidified chopped lupin beans were used and fermentation together with *Rhizopus oryzae*.		Wolkers-Rooijackers et al. (2018)
Propionibacterium freudenreichii	184	123 μg/100 g dry lupin tempeh.	Co-fermentation for 72 h at 32 °C. Pre-acidified chopped lupin beans were used and fermented together with a *R. oryzae/Rhizopus oligosporus* mixture.		Signorini et al. (2018)
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	290 μg/100 g fresh potato wastewater with apple pomace (1:1, without glycerine).	120 h at 30 °C with neutralization every 24 h using NaOH.		Piwowarek et al. (2022)
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	3.3 μg/100 g dry durum flour dough, 8.7 μg/100 g dry whole wheat flour dough, and 15.5 μg/100 g dry wheat bran dough.	9 log CFU/g inoculation, 25 °C for 7 days. The tubes were aseptically opened once on day 3 to allow air in and then closed.	Addition of 0.6 mg/g dw of cobalt chloride increased vitB12 from 3.3 to 20.3 μg/100 g dry durum flour dough.	Xie et al. (2018)
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	33.2 μg/100 g dry wheat bran dough.	10^9 CFU/g inoculation, 600 rpm, pH 5.0 at 25 °C for 72 h.	Co-culture with *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) reduced vitB12 production, as well as uncontrolled pH during fermentation.	Xie et al. (2019)
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	74.2 μg/100 g dry rice bran batter; 63.1 μg/100 g dry buckwheat bran batter and 40.7 μg/100 g dry soy bean batter.	10^9 CFU/g inoculation, 200 rpm at 25 °C for 3 days.	Co-culture with *L. brevis* to control spoilage microorganisms. Other fermented cereal, pseudo-cereal and legume brans and flours contained less vitB12.	Xie et al. (2021)
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	4.87 μg/100 g fresh bread from soya flour and 3.81 μg/100 g fresh bread from rice bran.	Co-fermentation with *Weissella confusa*; shaking at 150 rpm at 25 °C for 24 h.	Addition of sucrose (dextran) to the dough did not increase vitB12. VitB12 production in sourdoughs: 9.13 μg vitB12/100 g fresh soya and 7.92 μg vitB12/100 g fresh rice bran.	(Y. Wang et al., 2022)
Propionibacterium freudenreichii subsp. *freudenreichii*	PTCC1674	294 μg/100 mL in fresh media containing rice bran oil (RBO).	3 days anaerobic conditions followed by 3 days aerobic conditions; shaking at 150 rpm at 30 °C. Optimal conditions: RBO concentration of 8.648% (v/v), temperature of 38.3 °C, DMBI concentration of 55.758 mg/L and elemental solution concentration of 2 mg/L.		Hedayati et al. (2020)
Propionibacterium freudenreichii subsp. *freudenreichii*	PTCC 1674	274 μg/100 mL fresh media containing waste frying sunflower oil (WFSO).	130 rpm and 30 °C for 120 h. Optimal conditions: 4% WFSO, 35.56 mg/L DMBI, 14.69 mg/L $CoCl_2{\cdot}6H_2O$, 5.82 mg/L $FeSO_4{\cdot}7H_2O$, and 11.41 mg/L $CaCl_2{\cdot}2H_2O$.		Hajfarajollah et al. (2015)
Propionibacterium freudenreichii subsp. *freudenreichii*	Not mentioned	43 μg/100 g dry baked bread from a straight dough containing malt extract (ME).	An anaerobic fermentation at 30 °C for 72 h was followed by incubation under mild aerobic conditions until 168 h with shaking (150 rpm). ME medium contained lactate 8 g/L, tryptone 5 g/L and $CoCl_2$ 5 mg/L.	In situ-produced vitB12 was almost as stable as added cyanocobalamin during baking.	Edelmann et al. (2016)
Propionibacterium freudenreichii	256, 263, and 266	148 μg/100 g fresh malted barley matrix for strain 256. 8.0 μg/100 g fresh barley flour matrix for strain 256. 25.7 μg/100 g fresh aleurone matrix for strain 266.	8 log CFU/g inoculation, 30 °C for 168 h (72 h anaerobically without shaking and then under the microaerobic condition with shaking [150 rpm]). Cobalt (5 mg/kg medium) was added to the matrix on day 0, whereas DMBI (15 mg/kg medium) was provided after 144 h of incubation.	VitB12 levels in these fresh matrices without any added supplements was 0.9–3.7 μg/100 g.	Chamlagain et al. (2018)
Propionibacterium freudenreichii subsp. *freudenreichii*	DSM 20271	1.18 μg/100 g fresh bread made with spray-dried wheat bran powder and 2.47 μg/100 g fresh cooked extruded wheat bran paste.	Wheat bran was bioprocessed with a commercial starter (incubation at 35 °C for 24 h). Next, pH adjustment to pH 6.4 and addition of *P. freudenreichii* for 3 days at 30 °C under microaerophilic conditions (200 rpm).	Spray-dried fermented bran extract powder contained vitB12 levels of 20.93 μg/100 g matrix (fw).	Chamlagain et al. (2021)
Propionibacterium freudenreichii	NCC 1177	17 μg/100 g fresh sunflower seed milk.	10^7 CFU/g inoculation. Co-fermentation with *Bacillus*	*B. amyloliquefaciens* produced lactate, amino acids and vitB7. The	Tangyu et al. (2022)

(*continued on next page*)

Table 1 (*continued*)

Microorganism (species)	Strain	Max VitB12 production[a]	(Optimal) conditions	Remarks[a]	Reference
			amyloliquefaciens of 6.8% (w/w) ultra-high-temperature pre-treated sunflower seed milk, 48 h of anaerobic fermentation and 48 h aerobic conditions at 30 °C.	addition of cobalt alone did not have an impact on vitB12 synthesis.	
Propionibacterium jensenii	112	7.21 μg/100 g fresh sauerkraut.	Sauerkraut was made in the traditional manner from white cabbage. Fermentation: 22 °C for 14 days, subsequent storage at 8 °C for 64 days.	The addition of strain 112 significantly increased vitB12 content (from 2.01 to 7.21 μg/100 g fresh sauerkraut).	Babuchowski, Laniewska-Moroz, and Warminska-Radyko (1999)
Propionibacterium freudenreichii subsp. *shermanii*	1250	0.1853 μg/100 g dry soybean tempeh.	Co-fermentation at 35 °C for 18–24 h. Pre-acidified sterile soy beans were used and fermentation together with *R. oligosporus*.	Addition of *Lacticaseibacillus casei* (formerly *Lactobacillus casei*) during soaking or fermentation decreased vitB12 due to acidification.	Krusong, Yongsmith, and Sanchez (1991)
Propionibacterium freudenreichii subsp. *shermanii*	ZJ01 ZJ03	560 μg/100 mL fresh soy milk.	Co-fermentation with *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*). To the soy milk, 50 g/L glucose was added. Inoculum: 10^6 CFU/mL L. *reuteri* and 10^6 CFU/mL of *P. shermanii*. Starting pH to 6.5–7.0, incubation at 30 °C; 5-day anaerobic followed by 2-day aerobic conditions.	After co-fermentation, *L. reuteri* and *P. shermanii* cell numbers were up to ~10^10 CFU/mL. The mixed cultures produced 1.6–2.4 fold more cobalamin than the single fermentations.	Shi et al. (2018)
Propionibacterium freudenreichii subsp. *shermanii*	ABM 5378	1.93 μg/100 g fresh rye malt matrix and 1.57 μg/100 g fresh barley malt matrix.	24 h at 28 °C.	After the incubation, the cell counts (CFU/g) had increased by a log factor in both matrices.	Chamlagain, Edelmann, Kariluoto, Ollilainen, and Piironen (2015)
Propionibacterium freudenreichii subsp. *shermanii*	NCFB 853	1110 μg/100 mL fresh hydrolysed tomato pomace.	1.7 x 10^7 CFU/mL inoculation, 1.0 mg/L $(NH_4)_2SO_4$, 25 mg/L $CoCl_2$, 5.0 mg/L sodium lactate, anaerobic conditions and when the bacterial count began to decline, an aerobic phase was introduced by purging with air instead of nitrogen; 100 rpm, 144 h at 30 °C.	The tomato pomace was hydrolysed using *Trichoderma reesei*. *P. freudenreichii* cell density reached 9.1 x 10^13 CFU/mL.	Haddadin et al. (2001)
Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*)	O21	0.36 μg/100 g fresh cereal-based vegan dessert.	Oat flour (4% w/v), millet flour (4% w/v), sucrose (4% w/v), short-chain inulin sucrose (3% w/v) and distilled water were gelatinized and 6% of a 9 log starter culture was added. Incubation was at 37 °C for 20 h.	Refrigerated storage (at 8 °C for 21 days) did not decrease vitB12 content significantly.	Szydłowska et al. (2021)
Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*)	299	0.048 μg/100 g fresh cauliflower, white bean mixture (1:1).	2 g sea salt, 30 °C for 44 h.	Control samples (fermented, but without addition of an LAB strain) contained also vitB12: 0.029 μg/100 g fw.	Thompson et al. (2020)
Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*)	BHM10	0.3951 μg/100 mL in fresh soy milk.	Approximately 10^7 CFU/mL inoculation, pH 6.8, initial anaerobic (16 h) and subsequent microaerophilic (8 h) incubation at 37 °C.	*L. plantarum* cell counts reach 3.0 × 10^8 CFU/mL at the end of fermentation.	Bhushan, Tomar, and Chauhan (2017)
Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*)	DW12	1400 μg/100 mL fresh mature coconut water (MCW).	10^7 CFU/mL inoculation, MCW contained 53 g/L sugar and was supplemented with 0.5% monosodium glutamate, pH 6.0, for 48 h.	*L. plantarum* cell counts reached 8.4 log CFU/mL at the end of fermentation.	Kantachote et al. (2017)
Limosilactobacillus reuteri (formerly *Lactobacillus reuteri*)	F2	15.62 μg/100 mL fresh soy milk.	pH 7, 37 °C; 12h (anaerobic and microaerophilic phase).		Kumari et al. (2021)
Limosilactobacillus reuteri (formerly *Lactobacillus reuteri*)	CRL 1098	2.0 μg/100 mL fresh soy milk.	Soy milk was inoculated with 1% (v/v) *L. reuteri* culture and incubated at 37 °C for 6 h.	Final pH after fermentation: 6.8.	Molina, Medici, de Valdez, and Taranto (2012)
Limosilactobacillus reuteri (formerly *Lactobacillus reuteri*)	ZJ03	18.0 μg/100 mL fresh soy-yoghurt.	10^6 CFU/mL inoculation of soy milk with 2% glucose, 1% glycerol and 0.1% fructose, incubation under anaerobic conditions for 72 h.	Addition of 0.5% fructose (compared to 0.1%) decreased vitB12 production (10.0 μg/100 mL fresh soy-yoghurt).	Gu, Zhang, Song, Li, and Zhu (2015)
Limosilactobacillus reuteri (formerly *Lactobacillus reuteri*)	Starter culture (not further specified)	14.17 μg/100 g fresh furu (fermented tofu).	After tofu cubes were covered with (*Mocus*) mycelia, cubes were soaked in brines containing 8% of salt. 10^6 CFU/g of bacteria starter culture (*L. reuteri*) was added as well as 5% monascus colour, 5% unfiltered rice wine, 10% sugar, and 5% spicy oil and incubated at room temperature for 56 days.	Fermentation of furu consists of one fungal and one bacterial fermentation stage. The control group contained 3.60 μg vitB12/100 g fresh furu.	Bao et al. (2019)

(*continued on next page*)

Table 1 (*continued*)

Microorganism (species)	Strain	Max VitB12 production[a]	(Optimal) conditions	Remarks[a]	Reference
Lacticaseibacillus casei (formerly *Lactobacillus casei*)	L4	1147 μg/100 mL fresh coconut water.	10^8 CFU/mL inoculum, pH 6.5, 48 h fermentation at 35 °C.	VitB12 content was produced extracellular.	Giri, Sukumaran, Sen, and Park (2018)
Priestia megaterium (formerly *Bacillus megaterium*)	ATCC 13639	5.04 μg/100 g dry corn meal.	10^6 CFU/mL inoculum, pH 6.0, 5 days fermentation at 30 °C.	Co-fermentation with *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) increased vitB12 to 5.57 μg/100 g dry corn meal.	Chung and Fields (1986)

[a] VitB12 concentrations were converted to μg/100 g or mL and expressed for dried or wet (i.e., fresh) food matrix.

casei (formerly *Lactobacillus casei*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) and *Lactobacillus johnsonii* (Szydłowska, Siwińska, & Kołożyn-Krajewska, 2021). *P. megaterium* was reported as producing VitB12 in a matrix containing corn meal (Chung & Fields, 1986). The VitB12 production levels described varied greatly, depending on the producing organism, substrate and fermentation conditions, ranging between 0.048 μg/100 g and 1.4 mg/100 g fw matrix (Kantachote, Ratanaburee, Hayisama-ae, Sukhoom, & Nunkaew, 2017; Thompson, Önning, Holmgren, Strandler, & Hultberg, 2020). In general, levels produced by *P. freudenreichii* were among the highest reported, in hydrolysed tomato pomace (Haddadin, Abu-Reesh, Haddadin, & Robinson, 2001), potato wastewater with apple pomace (Piwowarek et al., 2022), media containing waste frying sunflower oil (Hajfarajollah, Mokhtarani, Mortaheb, & Afaghi, 2015) and mature coconut water (Kantachote et al., 2017). Lactobacilli were reported as producing relatively low levels on a cereal-based matrix, with maximum levels of VitB12 of 0.36 μg/100 g fw (Szydłowska et al., 2021), whereas corn meal slurry could be fortified for VitB12 using fermentation by *P. megaterium* to levels of up to 5 μg/100 g dw (Chung & Fields, 1986).

Based on this information, three strains of *P. megaterium* and two strains of *P. freudenreichii* were selected in this study to screen for VitB12 production in BSG.

3.2. Screening strains and conditions for optimal VitB12 production in BSG

A 10% BSG slurry was used for fermentation by the selected *P. megaterium* and *P. freudenreichii* strains. The resulting final cell counts and VitB12 content in the BSG are shown in Fig. 2. For all three *P. megaterium* strains, two different fermentation conditions were tested. Either a single aerobic fermentation or a two-step fermentation (24 h anaerobic followed by 18 h aerobic incubation) were tested, since both strategies have been described elsewhere for production of VitB12 by *P. megaterium* (Abou-Taleb, Mashoor, Sohair, & Sharaf, 2010; Mohammed, Lee, Kang, & Du, 2014). For the two-step fermentation, all three *P. megaterium* strains produced approximately the same amount of VitB12 (between 0.55 and 0.60 μg VitB12/100 g fw BSG), while for the aerobic-aeration fermentation, *P. megaterium* strain 2894 produced the most VitB12 (at least 0.85 μg/100 fw; Fig. 2). For *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii*, a different fermentation regime was used (Chamlagain et al., 2016; Chamlagain et al., 2018; Edelmann, Chamlagain, Santin, Kariluoto, & Piironen, 2016). Here, a 3-day anaerobic or microaerophilic incubation step was applied to allow for cell growth, followed by 4 days of static aerobic incubation to allow for the formation of the cobalamin precursor (DMBI) and eventually cobalamin (vitB12) (Balabanova et al., 2021). This fermentation regime resulted in approximately 0.70 μg VitB12/100 g fw BSG

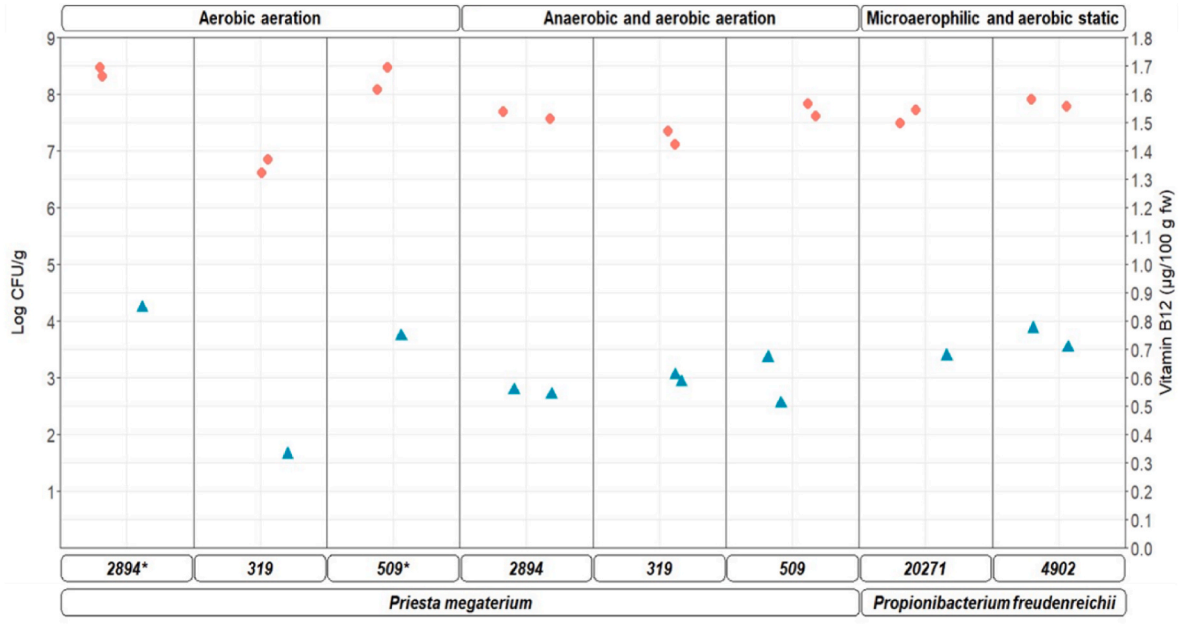

Fig. 2. Screening for VitB12 production levels on BSG. Cell counts of *Priestia megaterium* (three strains) and *Propionibacterium freudenreichii* (two strains) and vitamin B12 levels after submerged fermentation at 30 °C using 10% (w/w) BSG slurry and glucose as substrates. Vitamin B12 concentrations are represented by triangles and expressed per fresh weight (fw); microbial numbers by round dots. *P. megaterium* aerobic fermentation was applied by stirring at 150 rpm; the 2-step fermentation consisted of 24h anaerobically, followed by 18h aerobically at 150 rpm. *P. freudenreichii* was incubated 3 days microaerophilic, followed by 4 days aerobic, static. Asterisk: vitamin B12 levels were near (strain 509) or above (strain 2894) the upper detection limit of the bioassay. The vitamin B12 level in the control BSG were below detection level (data not shown).

(Fig. 2). This indicates that both species can grow on BSG and produce VitB12. The single aerobic fermentation was selected for *P. megaterium* strain 2894 over the two-step fermentation for further experiments on VitB12 production.

VitB12 production was tested in 85% BSG paste, which is closer to a solid-state type of fermentation than the 10% BSG slurry. For *P. megaterium* 2894, fermentation of the 85% BSG paste resulted in 2.6 μg VitB12/100 g fw in the single aerobic fermentation. To further increase the VitB12 production of *P. megaterium* 2894 on BSG, a higher incubation temperature was applied (37 °C instead of 30 °C) in combination with an extended incubation time (5 days instead of 3 days). After a 3-day incubation at 37 °C, VitB12 levels (2.5 μg/100 g fw) were comparable to fermentation at 30 °C (2.6 μg/100 g fw); however, extending the incubation time by 2 days at 37 °C increased the VitB12 content to 6.2 μg/100 g fw (Table 2). For *P. freudenreichii* subsp. *freudenreichii* DSM 20271, using the 2-step fermentation, levels as high as 16 μg VitB12/100 g fw (Table 2) were reached.

The timing of the switch from anaerobic/microaerophilic to aerobic conditions could influence the VitB12 production levels. Hence, the influence of oxygen availability on VitB12 production at preculture stage and subsequent fermentation was investigated in a BSG slurry for *P. freudenreichii*. Three different environments for the first-stage fermentation (4 days' duration) were tested, namely aerobic, anaerobic or microaerophilic, followed by a 3-day aerobic fermentation step for all conditions. To allow stirring of the BSG for the aerobic first stage, the percentage of the BSG was reduced to 50% (w/w) in this experiment. For the first fermentation stage under aerobic conditions, 6.0 μg VitB12/100 g fw was reached, whereas levels of 21 and 20 μg VitB12/100 g fw were reached under anaerobic or microaerophilic conditions (Table 3), indicating that aerobic conditions inhibit VitB12 production.

3.3. BSG fermentation under stabilized pH conditions at up-scale level

As a next step, the VitB12 fortification of BSG was tested on a larger scale using *P. freudenreichii* under controlled conditions for pH in a bioreactor. Therefore, 12 kg BSG slurry (standardised to 10% [w/w] to allow stirring in the bioreactor) with corn steep liquor was inoculated with *P. freudenreichii* and incubated anaerobically at 30 °C. Results showed that 9 h after the addition of the *P. freudenreichii* culture to the BSG slurry, the pH decreased and the addition of KOH was needed to stabilise the pH to a value of 7.0, indicating growth of *P. freudenreichii*. Acidification followed an exponential curve until incubation reached 26 h, then the KOH demand decreased, indicating that acidification halted (Fig. 1 in the Supplemented materials). From this timepoint, a slower acidification rate was observed. During the first 4 days of fermentation, low levels of dissolved oxygen were detected (Fig. 2 in the Supplemented materials). After 4 days of incubation, oxygen was actively introduced by aeration of the headspace, resulting in a dissolved oxygen level of 2–4% until the end of fermentation (Fig. 2 in the Supplemented materials). An increase in pH from 7.0 to 7.8 was detected at this stage.

At regular timepoints during the fermentation, samples were taken to measure sugar and organic acid levels. Lactic acid, glucose and fructose/xylose were largely consumed within the first 4 days of the fermentation (Table 4). Propionic acid remained stable at levels between 0.45 and 0.60 g/L, while acetic acid increased over time, resulting in levels of up to 3.9 g/L after 7 days (Table 4).

VitB12 levels were measured at different timepoints during the fermentation (Fig. 3). At the start of the fermentation, VitB12 levels were below the detection limit, after 4 days (end of the first stage of the fermentation) levels as high as 6.6 μg/100 g fw were produced and increased to 7.7 μg/100 g fw during the second stage of the fermentation (days 6 and 7; Fig. 3). This is more than 7 times higher compared to uncontrolled fermentation in 10% BSG (Fig. 2). *P. freudenreichii* viable cell counts reached their highest levels after the first fermentation stage (day 4; 3.0×10^9 CFU/g) and decreased to 1.1×10^8 CFU/g during the second stage (Fig. 3). The maximum detected VitB12 levels were reached at days 6 and 7.

Table 2
Levels of *Priestia megaterium* 2894, *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 and vitamin B12 after improved submerged aerobic fermentation (static; *P. megaterium*) or 2-step fermentation (4 days anerobic, 3 days aerobic); *P. freudenreichii* using 85% (w/w) BSG paste and glucose as substrates.

Species	Fermentation duration and temperature	Microbial level (log CFU/g)	VitB12 (μg/100 g fw)
P. megaterium	3 days at 30 °C	1.8×10^7	2.6
P. megaterium	3 days at 37 °C	1.7×10^7	2.5
P. megaterium	4 days at 37 °C	8.9×10^6	4.5
P. megaterium	5 days at 37 °C	9.1×10^6	6.2
P. freudenreichii	7 days at 30 °C	1.4×10^9	16.3

Table 3
Vitamin B12 production by *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 for different conditions for oxygen availability during the first stage (4 days), namely aerobic, anaerobic and microaerophilic condition. Fermentations were performed at 30 °C with using 50% (w/w) BSG paste and glucose as substrates. The second stage was similar for all fermentations: aerobic atmosphere, static, 30 °C, 3 days.

Conditions for the first stage of the fermentation	Microbial level (log CFU/g)	VitB12 (μg/100 g fw)
Aerobic, 150 rpm	4.8×10^9	6.0
Anaerobic, static	3.3×10^9	21
Microaerophilic, static	2.5×10^9	20

4. Discussion

4.1. BSG fermentation and in situ VitB12 production

BSG is the main side stream of the brewing industry, which makes up to 85% of the brewing waste (Mussatto, 2014). BSG is currently mainly used for feed applications, and only a small amount (5–10%) of BSG finds an application in food products (Parchami et al., 2021). Microbial fermentation offers an attractive method to produce compounds of higher value (Mitri et al., 2022). For example, co-fermentation with *Bacillus velezensis* and *L. brevis* was used in BSG to increase the levels of amino acids, in particular glutamic acids and γ-aminobutyric acid (GABA) (Zeng et al., 2022). Another example is BSG with increased polyphenolic compounds resulting from a fermentation step with *L. plantarum* (Gupta et al., 2013). In the current study, we used fermentation to fortify BSG with VitB12. The resulting product may find application as a VitB12-fortified ingredient in plant-based food and feed formulations (Calvillo, Pellicer, Carnicer, & Planas, 2022).

The chemical synthesis of VitB12 takes up to 70 steps and is costly due to its complexity. Therefore, industrial production of the vitamin is via fermentation. The large-scale microbial production of biologically active VitB12 typically uses *P. freudenreichii* or *Pseudomonas denitrificans* (Marie Sych, Lacroix, & Stevens, 2016; Martens, Barg, Warren, & Jahn, 2002). Propionibacteria have the advantage that they are food-grade

Table 4
Levels (g/L) of metabolites produced at 12 kg scale during *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 submerged fermentation with stabilized pH using 10% (w/w) BSG and corn steep liquor as substrates.

Metabolite	Start fermentation	4 days	6 days	7 days
Lactic acid	3.1	0.00	0.00	0.00
Acetic acid	0.00	2.5	3.9	3.9
Propionic acid	0.50	0.45	0.49	0.62
Glucose	1.9	0.00	0.11	0.00
Fructose/xylose[a]	1.4	0.38	0.38	0.40

[a] Fructose and xylose cannot be discriminated from each other.

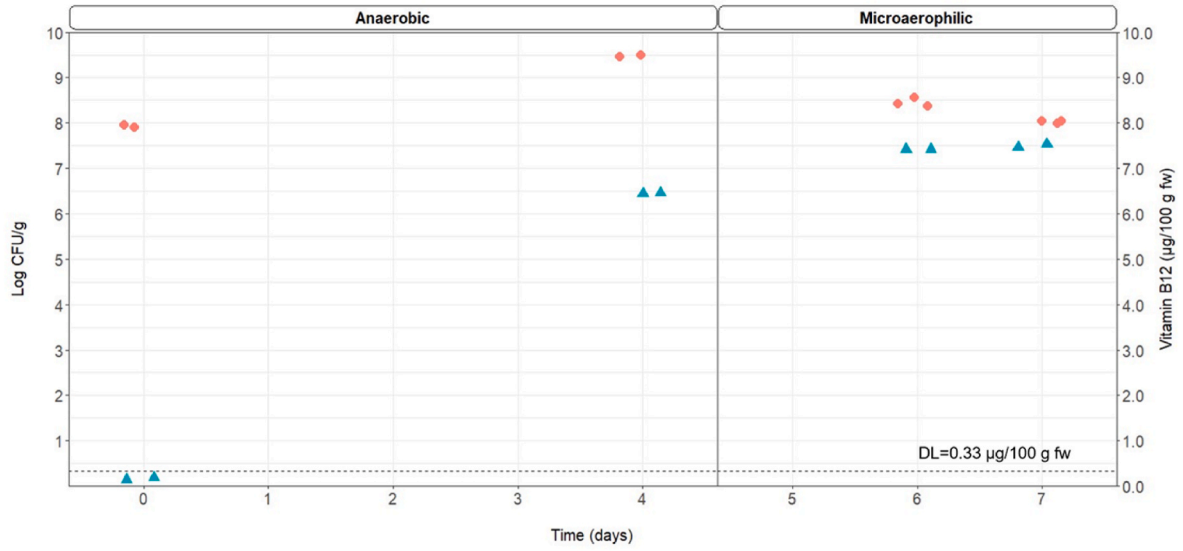

Fig. 3. *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 cell counts (orange dots) and vitamin B12 concentration (green triangles) during 12 kg scale, submerged fermentation at 30 °C with stabilized pH, and using 10% (w/w) BSG, glucose and corn steep liquor as substrates. Switch from anaerobic growth conditions to microaerophilic conditions was on day 4. At day 0, vitamin B12 levels were below the detection level (DL: 0.33 µg VitB12/100 g fresh weight [fw]). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and can thus be used for food applications. *P. megaterium* was one of the first VitB12-producing bacteria used biotechnologically (Vary et al., 2007); it also later served as a genetically accessible model organism to elucidate and engineer the VitB12 biosynthesis pathway. Both species, *P. freudenreichii* and *P. megaterium*, have QPS status, facilitating their use in food applications.

4.2. VitB12 production levels

Both *P. freudenreichii* strains and all three *P. megaterium* strains used in the current study produced VitB12 on a 10% BSG slurry in the initial screening experiments. When concentrated BSG was used as a substrate, up to 6.2 µg/100 g fw VitB12 was reached for *P. megaterium*, whereas *P. freudenreichii* produced up to 16 µg/100 fw. By using an anaerobic or microaerophilic first stage of fermentation, VitB12 levels of 20–21 µg/100 g fw were detected on 50% BSG. In addition, we showed that at a 12 kg bioreactor scale low levels of oxygen (2–4%) during the second phase of a controlled fermentation could induce high production levels of VitB12 (7.7 µg/100 g fw) using 10% BSG in the matrix. This shows that a microaerophilic environment allows for substantial VitB12 levels and there is no need to completely remove oxygen, which may facilitate industrial implementation.

Overall, *P. freudenreichii* produced higher levels of VitB12 on BSG in comparison to *P. megaterium*. Therefore, *P. freudenreichii* is the preferred production organism of the strains tested in this study for in situ fortification of BSG. Several cereals and pseudocereals were used as a substrate for VitB12 fortification via fermentation with *P. freudenreichii*, and levels of 33.2 µg/100 g (dw) wheat bran dough (Xie et al., 2019), 74.2 µg/100 g (dw) rice bran batter, 63.1 µg/100 g (dw) buckwheat bran batter, 33.2 µg/100 g (dw) oat bran batter and 26.5 µg/100 g (dw) sorghum batter (Xie et al., 2021) were reported. When processed to make a bread, VitB12 contents of 43 µg/100 g (dw) and 3.81 µg/100 g (fw) are reported for breads prepared from a fermented malt extract and from fermented rice bran, respectively (Edelmann et al., 2016; Y. Wang et al., 2022). BSG could be used in the production of bread, cake, cookies and egg pasta (Parchami et al., 2021); these products could contribute significantly to the recommended dietary intake (RDI) of VitB12 when fortified with VitB12 using fermentation. The VitB12 RDI for adults (aged 19 to 64) varies between 1.4 µg/d and 4 µg/d (EFSA Panel on Dietetic Products and Allergies, 2015; NHS; Services; Strohle et al., 2019; Voedingscentrum; Voedingsinformatiecentrum). With the fermented BSG of this study containing 7.7 µg VitB12/100 g, the daily RDI seems within reach, even for consumers on a full plant-based diet.

4.3. Composition of BSG

Cobalt is an ion in the core of the cobalamin molecule, and a cobalt limitation in the substrate could potentially limit VitB12 production levels. Previously, supplementing a rich medium with cobalt (250 µM) showed a positive effect on cobalamin production by *P. megaterium* (Biedendieck et al., 2010) in the medium as well as in fermented durum flour dough or barley flour dough by *P. freudenreichii* (Chamlagain et al., 2018; Xie et al., 2018). Supplementation of the MRS medium with 50 µM of $CoCl_2$ enhanced VitB12 levels produced by *P. freudenreichii* to almost 150 µg/100 g (Tangyu et al., 2022). However, in the same study involving sunflower seed milk, supplementation with cobalt alone did not enhance the VitB12 levels, indicating that the cobalt level naturally present (8 µg/kg) in the product was sufficient. The BSG used in the present study contains 30 µg cobalt/kg (dw), which may not limit the fortification of VitB12 via fermentation under the tested conditions.

DMBI supplementation can also stimulate VitB12 production as shown for *P. freudenreichii* during the fermentation of media containing rice bran oil (Hedayati et al., 2020), media containing waste frying sunflower oil (Hajfarajollah et al., 2015) or a barley dough (Chamlagain et al., 2018). Since DMBI is not allowed as a food ingredient or additive, increased VitB12 levels in BSG by *P. freudenreichii* might be achieved by supplementing with riboflavin and nicotinamide instead as precursors of DMBI (Chamlagain et al., 2018).

4.4. Potential to further increase VitB12 production in a BSG matrix

Besides supplementation of VitB12 precursors, other factors can influence VitB12 production by *P. freudenreichii*. To a certain extent at least, the *P. freudenreichii* yield should be sufficient and, therefore, growth should be stimulated. This can be achieved by optimising the carbon source, fermentation conditions such as pH and dissolved oxygen. Lactate and glucose appeared to be good carbon sources for *P. freudenreichii* growth and subsequent VitB12 production in sunflower seed milk (Tangyu et al., 2022). In this study, at a 12 kg scale, corn steep liquor containing lactate and free amino acid was added to the BSG

(10%). After fermentation for 26 h, the KOH consumption was decreased drastically, indicating a depletion of the carbon source. Therefore, combining increased levels of the carbon source (via the addition of glucose and/or corn steep liquor) and increased BSG content (from 10% to 50%, for example) might increase VitB12 production. A pH of 6–7 is considered optimal for VitB12 production by *P. freudenreichii* because the ratio between undissociated/dissociated acetic and propionic acids then favours the dissociated form (Fang et al., 2017; Hsu & Yang, 1991; Marie Sych et al., 2016; Martens et al., 2002). The study by Wang, Zhang, Jiao, Liu, and Wang (2015) showed that for VitB12 fermentation by *P. freudenreichii* propionic acid levels should be below 10–20 g/L in the first fermentation stage and below 20–30 g/L in the second stage for optimal VitB12 biosynthesis (Wang et al., 2015). In this study, VitB12 levels produced by *P. freudenreichii* were higher in the bioreactor with a regulated pH of 7.0 compared to fermentation under an uncontrolled pH, indicating that pH stabilisation promotes VitB12 production. In addition, microaerophilic conditions, i.e. oxygen availability, seem crucial for VitB12 production (Gray & Escalante-Semerena, 2007; Martens et al., 2002), so the presence of low levels of dissolved oxygen may explain the already relatively high VitB12 concentration detected in this study at day 4 of fermentation. Increased oxygen availability in the second stage of fermentation further increased VitB12 concentration.

Another strategy to influence VitB12 fortification in BSG might be co-fermentation with another microorganism that has features enhancing *P. freudenreichii* growth and VitB12 production. For instance, co-fermentation of BSG with *P. freudenreichii* and *Bacillus amyloliquefaciens* could potentially increase VitB12 levels. *B. amyloliquefaciens* can produce vitamins B2 and B3 as precursors for DMBI production for VitB12 by *P. freudenreichii* and also lactate, amino acids and vitamin B7 to stimulate the growth of *P. freudenreichii* (Tangyu et al., 2022). Co-fermentation with *L. reuteri*, as the producer of lactic acid, also revealed increased VitB12 production by *P. freudenreichii,* especially under adjusted fermentation conditions concerning the initial pH and length of the aerobic phase that stimulated a synergistic effect (Shi et al., 2018).

As discussed above, increased VitB12 concentrations are desired. To keep the production costs low, shortening the incubation period could be a way to realise this. In this study, in the bioreactor at a 12 kg scale, the VitB12 concentration between days 6 and 7 did not increase further. VitB12 levels by co-fermentation of *P. freudenreichii* and *B. amyloliquefaciens* in sunflower seed were equal for 48 and 72 h of anaerobic fermentation of the first stage (Tangyu et al., 2022). Therefore, decreasing the first fermentation stage may potentially save time without any loss of VitB12. It remains to be determined what impact post-processing the fortified BSG has on the VitB12 concentration. In addition, further studies should address the food safety and shelf life of the final product. Overall, the VitB12-fortified BSG might serve as a relatively inexpensive plant-based source of VitB12-fortified protein for food and feed applications.

5. Conclusion

It can be concluded that plant-based, protein-enriched BSG can be fortified with VitB12 by fermentation using food-grade microorganisms, specifically *P. freudenreichii* and *P. megaterium*. This study showed that (protein-enriched) BSG products could serve as a relatively inexpensive plant-based source of VitB12-fortified protein for food and feed applications. Future work for industrial application should focus on further upscaling, food safety, cost reduction and shelf life.

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CRediT authorship contribution statement

Hermien van Bokhorst-van de Veen: Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Lucienne Berendsen:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Mariette Helmond:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Masja Nierop Groot:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116520.

References

Abou-Taleb, K., Mashoor, W. A., Sohair, A. N., & Sharaf, M. S. (2005). Production of vitamin B12 by *Propionibacterium freudenreichii* and *Bacillus megaterium*. *Journal of Agricultural Chemistry and Biotechnology, 30*(7), 4149–4162. https://doi.org/10.21608/jacb.2005.235832

Abou-Taleb, K., Mashoor, W. A., Sohair, A. N., & Sharaf, M. S. (2010). Effect of environmental factors on vitamin B12 production by *Propionibacterium freudenreichii* and *Bacillus megaterium*. *Journal of Biological Chemistry and Environmental Sciences, 5*(4), 13.

Allegretti, C., Bellinetto, E., D'Arrigo, P., Griffini, G., Marzorati, S., Rossato, L. A. M., … Turri, S. (2022). Towards a complete exploitation of brewers' spent grain from a circular economy perspective. *Fermentation, 8*(4). https://doi.org/10.3390/fermentation8040151

Assis, D. A.d., Matte, C., Aschidamini, B., Rodrigues, E., & Záchia Ayub, M. A. (2020). Biosynthesis of vitamin B12 by *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673 using liquid acid protein residue of soybean as culture medium. *Biotechnology Progress, 36*(5), Article e3011. https://doi.org/10.1002/btpr.3011

Babuchowski, A., Laniewska-Moroz, L., & Warminska-Radyko, I. (1999). *Propionibacteria* in fermented vegetables. *Le Lait, 79*(1), 113–124. https://doi.org/10.1051/lait:199919. Retrieved from.

Balabanova, L., Averianova, L., Marchenok, M., Son, O., & Tekutyeva, L. (2021). Microbial and genetic resources for cobalamin (vitamin B12) biosynthesis: From ecosystems to industrial biotechnology. *International Journal of Molecular Sciences, 22*(9). https://doi.org/10.3390/ijms22094522

Bao, X., Xiang, S. S., Chen, J., Shi, Y. G., Chen, Y. W., Wang, H. H., et al. (2019). Effect of *Lactobacillus reuteri* on vitamin B12 content and microbiota composition of furu fermentation. *LWT-Food Science & Technology, 100*, 138–143. https://doi.org/10.1016/j.lwt.2018.10.045

Bhushan, B., Tomar, S. K., & Chauhan, A. (2017). Techno-functional differentiation of two vitamin B12 producing *Lactobacillus plantarum* strains: An elucidation for diverse future use. *Applied Microbiology and Biotechnology, 101*(2), 697–709. https://doi.org/10.1007/s00253-016-7903-z

Bianco, A., Budroni, M., Zara, S., Mannazzu, I., Fancello, F., & Zara, G. (2020). The role of microorganisms on biotransformation of brewers' spent grain. *Applied Microbiology and Biotechnology, 104*(20), 8661–8678. https://doi.org/10.1007/s00253-020-10843-1

Biedendieck, R., Malten, M., Barg, H., Bunk, B., Martens, J. H., Deery, E., … Jahn, D. (2010). Metabolic engineering of cobalamin (vitamin B12) production in *Bacillus megaterium*. *Microbial Biotechnology, 3*(1), 24–37. https://doi.org/10.1111/j.1751-7915.2009.00125.x

Calvillo, A., Pellicer, T., Carnicer, M., & Planas, A. (2022). Bioprocess strategies for vitamin B12 production by microbial fermentation and its market applications. *Bioengineering-Basel, 9*(8). https://doi.org/10.3390/bioengineering9080365

Chamlagain, B., Deptula, P., Edelmann, M., Kariluoto, S., Grattepanche, F., Lacroix, C., … Piironen, V. (2016). Effect of the lower ligand precursors on vitamin B12 production by food-grade *Propionibacteria*. *LWT - Food Science and Technology, 72*, 117–124. https://doi.org/10.1016/j.lwt.2016.04.023

Chamlagain, B., Edelmann, M., Kariluoto, S., Ollilainen, V., & Piironen, V. (2015). Ultra-high performance liquid chromatographic and mass spectrometric analysis of active vitamin B12 in cells of *Propionibacterium* and fermented cereal matrices. *Food Chemistry, 166*, 630–638. https://doi.org/10.1016/j.foodchem.2014.06.068

Chamlagain, B., Peltonen, L., Edelmann, M., Ramos-Diaz, J. M., Kemppinen, A., Jouppila, K., … Piironen, V. (2021). Bioaccessibility of vitamin B12 synthesized by *Propionibacterium freudenreichii* and from products made with fermented wheat bran extract. *Current Research in Food Science, 4*, 499–502. https://doi.org/10.1016/j.crfs.2021.07.009

Chamlagain, B., Sugito, T. A., Deptula, P., Edelmann, M., Kariluoto, S., Varmanen, P., et al. (2018). *In situ* production of active vitamin B12 in cereal matrices using *Propionibacterium freudenreichii*. *Food Science and Nutrition, 6*(1), 67–76. https://doi.org/10.1002/fsn3.528

Chung, H. J., & Fields, M. L. (1986). Production of riboflavin and vitamin B12 by *Bacillus megaterium* ATCC 13639 and *Enterobacter aerogenes* in corn meal. *Journal of Food Science, 51*(6), 1514–1517. https://doi.org/10.1111/j.1365-2621.1986.tb13848.x

Edelmann, M., Chamlagain, B., Santin, M., Kariluoto, S., & Piironen, V. (2016). Stability of added and *in situ*-produced vitamin B12 in breadmaking. *Food Chemistry, 204*, 21–28. https://doi.org/10.1016/j.foodchem.2016.02.071

EFSA Panel on Dietetic Products, & Allergies, N. (2015). Scientific opinion on dietary reference values for cobalamin (vitamin B12). *EFSA Journal, 13*(7), 4150. https://doi.org/10.2903/j.efsa.2015.4150

Fang, H., Kang, J., & Zhang, D. (2017). Microbial production of vitamin B12: A review and future perspectives. *Microbial Cell Factories, 16*(1), 15. https://doi.org/10.1186/s12934-017-0631-y

Gille, D., & Schmid, A. (2015). Vitamin B12 in meat and dairy products. *Nutrition Reviews, 73*(2), 106–115. https://doi.org/10.1093/nutrit/nuu011

Giri, S. S., Sukumaran, V., Sen, S. S., & Park, S. C. (2018). Use of a potential probiotic, *Lactobacillus casei* L4, in the preparation of fermented coconut water beverage. *Frontiers in Microbiology, 9*. https://doi.org/10.3389/fmicb.2018.01976

Gray, M. J., & Escalante-Semerena, J. C. (2007). Single-enzyme conversion of FMNH2 to 5,6-dimethylbenzimidazole, the lower ligand of B12. *Proc Natl Acad Sci U S A, 104*(8), 2921–2926. https://doi.org/10.1073/pnas.0609270104

Gu, Q., Zhang, C., Song, D., Li, P., & Zhu, X. (2015). Enhancing vitamin B12 content in soy-yogurt by *Lactobacillus reuteri*. *International Journal of Food Microbiology, 206*, 56–59. https://doi.org/10.1016/j.ijfoodmicro.2015.04.033

Gupta, S., Jaiswal, A. K., & Abu-Ghannam, N. (2013). Optimization of fermentation conditions for the utilization of brewing waste to develop a nutraceutical rich liquid product. *Industrial Crops and Products, 44*, 272–282. https://doi.org/10.1016/j.indcrop.2012.11.015

Haddadin, M. S. Y., Abu-Reesh, I. M., Haddadin, F. A. S., & Robinson, R. K. (2001). Utilisation of tomato pomace as a substrate for the production of vitamin B12 – a preliminary appraisal. *Bioresource Technology, 78*(3), 225–230. https://doi.org/10.1016/S0960-8524(01)00018-9

Hajfarajollah, H., Mokhtarani, B., Mortaheb, H., & Afaghi, A. (2015). Vitamin B12 biosynthesis over waste frying sunflower oil as a cost effective and renewable substrate. *Journal of Food Science and Technology, 52*(6), 3273–3282. https://doi.org/10.1007/s13197-014-1383-x

Hedayati, R., Hosseini, M., & Najafpour, G. D. (2020). Optimization of semi-anaerobic vitamin B12 (cyanocobalamin) production from rice bran oil using *Propionibacterium freudenreichii* PTCC1674. *Biocatalysis and Agricultural Biotechnology, 23*. https://doi.org/10.1016/j.bcab.2019.101444

Hsu, S. T., & Yang, S. T. (1991). Propionic acid fermentation of lactose by *Propionibacterium acidipropionici* - effects of pH. *Biotechnology and Bioengineering, 38*(6), 571–578. https://doi.org/10.1002/bit.260380603

Hunt, A., Harrington, D., & Robinson, S. (2014). Vitamin B12 deficiency. *BMJ British Medical Journal, 349*, Article g5226. https://doi.org/10.1136/bmj.g5226

Kantachote, D., Ratanaburee, A., Hayisama-ae, W., Sukhoom, A., & Nunkaew, T. (2017). The use of potential probiotic *Lactobacillus plantarum* DW12 for producing a novel functional beverage from mature coconut water. *Journal of Functional Foods, 32*, 401–408. https://doi.org/10.1016/j.jff.2017.03.018

Krusong, W., Yongsmith, B., & Sanchez, P. C. (1991). Influence of *Lactobacillus casei* in production of high vitamin B12-tempeh. *Agriculture and Natural Resources, 25*(4), 458–462. Retrieved from https://li01.tci-thaijo.org/index.php/anres/article/view/241999.

Kumari, M., Bhushan, B., Kokkiligadda, A., Kumar, V., Behare, P., & Tomar, S. K. (2021). Vitamin B12 biofortification of soymilk through optimized fermentation with extracellular B12 producing *Lactobacillus* isolates of human fecal origin. *Current Research in Food Science, 4*, 646–654. https://doi.org/10.1016/j.crfs.2021.09.003

Marie Sych, J., Lacroix, C., & Stevens, M. J. A. (2016). Vitamin B12 – physiology, production and application. In *Industrial biotechnology of vitamins, biopigments, and antioxidants* (pp. 129–159).

Martens, J. H., Barg, H., Warren, M., & Jahn, D. (2002). Microbial production of vitamin B12. *Applied Microbiology and Biotechnology, 58*(3), 275–285. https://doi.org/10.1007/s00253-001-0902-7

Mitri, S., Salameh, S.-J., Khelfa, A., Leonard, E., Maroun, R. G., Louka, N., et al. (2022). Valorization of brewers' spent grains: Pretreatments and fermentation, a review. *Fermentation, 8*(2), 50. Retrieved from https://www.mdpi.com/2311-5637/8/2/50.

Mohammed, Y., Lee, B., Kang, Z., & Du, G. (2014). Development of a two-step cultivation strategy for the production of vitamin B12 by *Bacillus megaterium*. *Microbial Cell Factories, 13*(1), 102. https://doi.org/10.1186/s12934-014-0102-7

Molina, V., Medici, M., de Valdez, G. F., & Taranto, M. P. (2012). Soybean-based functional food with vitamin B12-producing lactic acid bacteria. *Journal of Functional Foods, 4*(4), 831–836. https://doi.org/10.1016/j.jff.2012.05.011

Moore, S. J., Mayer, M. J., Biedendieck, R., Deery, E., & Warren, M. J. (2014). Towards a cell factory for vitamin B12 production in *Bacillus megaterium*: Bypassing of the cobalamin riboswitch control elements. *New Biotech, 31*(6), 553–561. https://doi.org/10.1016/j.nbt.2014.03.003

Mussatto, S. I. (2014). Brewer's spent grain: A valuable feedstock for industrial applications. *Journal of the Science of Food and Agriculture, 94*(7), 1264–1275. https://doi.org/10.1002/jsfa.6486

NHS. B vitamins and folic acid. Retrieved from https://www.nhs.uk/conditions/vitamins-and-minerals/vitamin-b/#:~:text=How%20much%20vitamin%20B12%20do,vitamin%20B12%20from%20your%20diet.

Parchami, M., Ferreira, J. A., & Taherzadeh, M. J. (2021). Brewing process development by integration of edible filamentous fungi to upgrade the quality of brewer's spent grain (BSG). *Bioresources, 16*(1), 1686–1701. https://doi.org/10.15376/biores.16.1.1686-1701

Piwowarek, K., Lipińska, E., Hać-Szymańczuk, E., Kolotylo, V., & Kieliszek, M. (2022). Use of apple pomace, glycerine, and potato wastewater for the production of propionic acid and vitamin B12. *Applied Microbiology and Biotechnology, 106*(17), 5433–5448. https://doi.org/10.1007/s00253-022-12076-w

Searchinger, T., Waite, R., Hanson, C., Ranganathan, J., & Matthews, E. (2019). Creating a sustainable food future: Synthesis report. A Menu of Solutions to Feed Nearly 10 Billion People by 2050. Retrieved from https://www.wri.org/research/creating-sustainable-food-future.

Services, U. D. o. H. H. Vitamin B12. Retrieved from https://ods.od.nih.gov/factsheets/VitaminB12-HealthProfessional/.

Shelton, A. N., Seth, E. C., Mok, K. C., Han, A. W., Jackson, S. N., Haft, D. R., et al. (2019). Uneven distribution of cobamide biosynthesis and dependence in bacteria predicted by comparative genomics. *Isme Journal, 13*(3), 789–804. https://doi.org/10.1038/s41396-018-0304-9

Shi, L., Xu, Y., Zhan, L., Xiang, S., Zhu, X., Wang, X., et al. (2018). Enhancing vitamin B12 content in co-fermented soy-milk via a Lotka Volterra model. *Turkish Journal of Biochemistry, 43*(6), 671–678. https://doi.org/10.1515/tjb-2017-0365

Signorini, C., Carpen, A., Coletto, L., Borgonovo, G., Galanti, E., Capraro, J., … Scarafoni, A. (2018). Enhanced vitamin B12 production in an innovative lupin tempeh is due to synergic effects of *Rhizopus* and *Propionibacterium* in cofermentation. *International Journal of Food Sciences & Nutrition, 69*(4), 451–457. https://doi.org/10.1080/09637486.2017.1386627

Strohle, A., Richter, M., Gonzalez-Gross, M., Neuhauser-Berthold, M., Wagner, K. H., Leschik-Bonnet, E., et al. (2019). The revised D-A-CH-reference values for the intake of vitamin B12: Prevention of deficiency and beyond. *Molecular Nutrition & Food Research, 63*(6), Article 1801178. https://doi.org/10.1002/mnfr.201801178

Szydłowska, A., Siwińska, J., & Kołożyn-Krajewska, D. (2021). Cereal-based vegan desserts as container of potentially probiotic bacteria isolated from fermented plant-origin food. *CyTA - Journal of Food, 19*(1), 691–700. https://doi.org/10.1080/19476337.2021.1963320

Tangyu, M., Fritz, M., Ye, L., Aragao Borner, R., Morin-Rivron, D., Campos-Gimenez, E., … Wittmann, C. (2022). Co-cultures of *Propionibacterium freudenreichii* and *Bacillus amyloliquefaciens* cooperatively upgrade sunflower seed milk to high levels of vitamin B12 and multiple co-benefits. *Microbial Cell Factories, 21*(1), 48. https://doi.org/10.1186/s12934-022-01773-w

Thompson, H. O., Önning, G., Holmgren, K., Strandler, H. S., & Hultberg, M. (2020). Fermentation of cauliflower and white beans with *Lactobacillus plantarum* – impact on levels of riboflavin, folate, vitamin B12, and amino acid composition. *Plant Foods for Human Nutrition, 75*(2), 236–242. https://doi.org/10.1007/s11130-020-00806-2

Vary, P. S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W. D., et al. (2007). *Bacillus megaterium*–from simple soil bacterium to industrial protein production host. *Applied Microbiology and Biotechnology, 76*(5), 957–967. https://doi.org/10.1007/s00253-007-1089-3

Voedingscentrum. Vitamine B12 (cobalamine). Retrieved from https://www.voedingscentrum.nl/encyclopedie/vitamine-b12.aspx.

Voedingsinformatiecentrum. Vitamine B12 - Onmisbaar voor jong en oud. Retrieved from https://www.nice-info.be/nutrinews/vitamine-b12-onmisbaar-voor-jong-en-oud.

Wang, Y., Xie, C., Pulkkinen, M., Edelmann, M., Chamlagain, B., Coda, R., & Katina, K. (2022). *In situ* production of vitamin B12 and dextran in soya flour and rice bran: A tool to improve flavour and texture of B12-fortified bread. *Lwt, 161*. https://doi.org/10.1016/j.lwt.2022.113407

Wang, Zhang, Z., Jiao, Y., Liu, S., & Wang, Y. (2015). Improved propionic acid and 5,6-dimethylbenzimidazole control strategy for vitamin B12 fermentation by *Propionibacterium freudenreichii*. *Journal of Biotechnology, 193*, 123–129. https://doi.org/10.1016/j.jbiotec.2014.11.019

Watanabe, F. (2007). Vitamin B12 sources and bioavailability. *Experimental Biology and Medicine, 232*(10), 1266–1274. https://doi.org/10.3181/0703-MR-67

Wen, C., Zhang, J., Duan, Y., Zhang, H., & Ma, H. (2019). A mini-review on brewer's spent grain protein: Isolation, physicochemical properties, application of protein, and functional properties of hydrolysates. *Journal of Food Science, 84*(12), 3330–3340. https://doi.org/10.1111/1750-3841.14906

Wolkers-Rooijackers, J. C. M., Endika, M. F., & Smid, E. J. (2018). Enhancing vitamin B-12 in lupin tempeh by *in situ* fortification. *LWT-Food Science & Technology, 96*, 513–518. https://doi.org/10.1016/j.lwt.2018.05.062

Xie, C., Coda, R., Chamlagain, B., Edelmann, M., Deptula, P., Varmanen, P., … Katina, K. (2018). *In situ* fortification of vitamin B12 in wheat flour and wheat bran by fermentation with *Propionibacterium freudenreichii*. *Journal of Cereal Science, 81*, 133–139. https://doi.org/10.1016/j.jcs.2018.05.002

Xie, C., Coda, R., Chamlagain, B., Edelmann, M., Varmanen, P., Piironen, V., et al. (2021). Fermentation of cereal, pseudo-cereal and legume materials with *Propionibacterium freudenreichii* and *Levilactobacillus brevis* for vitamin B12 fortification. *Lwt, 137*, Article 110431. https://doi.org/10.1016/j.lwt.2020.110431

Xie, C., Coda, R., Chamlagain, B., Varmanen, P., Piironen, V., & Katina, K. (2019). Co-Fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in wheat bran for *in situ* production of vitamin B12. *Frontiers in Microbiology, 10*, 1541. https://doi.org/10.3389/fmicb.2019.01541

Zeng, J., Sheng, F., Hu, X., Huang, Z., Tian, X., & Wu, Z. (2022). Nutrition promotion of brewer's spent grain by symbiotic fermentation adding *Bacillus velezensis* and *Levilactobacillus brevis*. *Food Bioscience, 49*. https://doi.org/10.1016/j.fbio.2022.101941