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Fermentation of Cereal, Pseudo-cereal and Legume Materials with *Propionibacterium freudenreichii* and *Levilactobacillus brevis* for Vitamin B12 Fortification

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ABSTRACT

The present study investigated the *in situ* production of vitamin B12 in eleven cereal, pseudo-cereal and legume materials by fermentation with *Propionibacterium freudenreichii* DSM 20271 and *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) ATCC 14869. *P. freudenreichii* was used as the vitamin producer and *L. brevis* was selected to improve the consistency and microbial safety of the process. The study showed that more than 300 ng/g dw of vitamin B12 (daily requirement: 2.4 µg) were produced during fermentation in most of the studied brans and legumes. The highest vitamin B12 production was observed in the fermentation of the rice bran (ca. 742 ng/g dw), followed by the fermentation of buckwheat bran (ca. 631 ng/g dw). Furthermore, partial least squares (PLS) regression analysis suggested that the production of vitamin B12 was greatly influenced by the nutrient composition of the fermented raw materials. Meanwhile, *L. brevis* was found to effectively inhibit the growth of *Enterobacteriaceae* during fermentation. These results demonstrated that fermentation of cereal, pseudo-cereal and legume materials with *P. freudenreichii* and *L. brevis* is effective in fortifying plant-based food with vitamin B12.

Keywords: *Propionibacterium freudenreichii*; *Levilactobacillus brevis*; Vitamin B12; *In situ* fortification; Brans

1. Introduction

Vitamin B12 (hereafter called B12) is naturally present in foods of animal origin and can be found in plant-based foods only as a consequence of fermentation or chemical fortification (Watanabe, 2007). Humans require B12 as a cofactor in cellular metabolism, and its deficiency may cause various health disorders, such as megaloblastic anemia, ataxia, and cognitive decline (Green & Miller, 2013). Globally, clinical deficiency of B12 is relatively uncommon nowadays, but subclinical deficiency of B12 is common among people of all ages, especially among people with low intake of animal products (Green et al., 2017; Smith, Warren, & Refsum, 2018). On the other hand, the current trend of replacing animal-based products with plant-based materials could result in a considerable global decrease of dietary B12 intake (Marsh, Zeuschner, & Saunders, 2011). Therefore, developing B12-fortified plant-based food products is increasing in importance for future food trends.

In situ fortification of food materials by fermentation with selected starter cultures is an economical and efficient way to provide micronutrients to the consumer. Certain *Lactobacillus* species (e.g. *Lactobacillus reuteri*) have been reported to be able to synthesize B12-like compounds (Taranto et al., 2003; Molina, Médici, de Valdez, & Taranto 2012), which on identification revealed to be mainly pseudo-vitamin B12 (Crofts et al., 2013; Santos et al., 2007), an inactive form for humans (Watanabe, 2007). *Propionibacterium freudenreichii* is a food-grade bacterium with the ability to *de novo* synthesize active form of B12 (Martens, Barg, Warren, & Jahn, 2002). However, the ability of B12 production among different strains of *P. freudenreichii* differs considerably (Hugenschmidt, Schwenninger, Gnehm & Lacroix, 2010;

Chamlagain et al. 2016). In the previous study (Xie et al., 2018), we found that wheat flour and wheat bran were promising substrates for B12 fortification using *P. freudenreichii* DSM 20271. Moreover, *P. freudenreichii* increased B12 production in wheat bran significantly during mixed culture fermentation with *Levilactobacillus brevis* ATCC 14869 (formerly *Lactobacillus brevis*) added to control the growth of indigenous microbes during the fermentation process (Xie et al., 2019).

Although wheat is one of the most widely cultivated crops, a great proportion of people around the world consume other grains as staple foods (Curtis, 2002). Moreover, for people suffering from allergy or intolerance to wheat products, other grain materials should be considered for B12 fortification by fermentation. Sorghum, millet, pseudocereals, and legumes are popular gluten-free alternatives to wheat because they can adapt to harsh environments and contain various health-promoting ingredients, such as dietary fiber and phenolic compounds (Gobbetti, De Angelis, Di Cagno, Polo, & Rizzello, 2019; Taylor, Belton, Beta, & Duodu, 2014). Cereal brans, which are by-products of the cereal milling process, contain high levels of dietary fiber, proteins, and various beneficial compounds, such as polyphenols and phytosterols (Luithui, Nisha, & Meera, 2019). The brans are, however, underutilized for food purposes because of their detrimental effect on the technological and sensory quality of products (Prückler et al., 2014). The development of innovative technologies for upgrading these by-products into the food system is widely suggested for a more resilient food chain.

Therefore, the present study aimed to investigate the *in situ* production of B12 by mixed culture fermentation with *P. freudenreichii* and *L. brevis* in various grain

materials, including cereals (oat bran, rice bran, rye bran, millet flour, and sorghum flours), pseudocereals (buckwheat bran, amaranth flour, and quinoa flour), and legumes (soybean flour, faba bean flour, and lupine flour). Acidification properties and content of acids were also monitored to elucidate the metabolism of starters during fermentation. The content of nutrient composition and cobalt, a limiting factor for the production of B12 by *P. freudenreichii* (Hugenschmidt et al., 2010), were measured and their effects on B12 production in different materials was evaluated. The main compositional factors influencing B12 production in different raw materials were studied using partial least squares regression analysis (PLS).

2. Materials and Methods

2.1 Grain materials, microbial strains, and culture preparation

Eleven kinds of grain materials used in this study were purchased from markets. Brans and grains of sorghum and millet were milled by a rotor mill (Ultra centrifugal mill ZM 200, Retsch GmbH & Co. KG, Haan, Germany) at a speed of 10,000 × g. The particle size of each material is shown in Supplemental Table 1. The nutrient composition of millet flour, sorghum flour, and faba bean flour was analyzed at Eurofins Scientific (Finland). The contents of fat and protein were determined following the methods of NordVal International (NMKL) 160 (1998) and NMKL 6:2003 (2003), respectively. Dietary fiber contents were determined according to the AOAC method 985.29 (2003). The contents of available carbohydrate (monosaccharides, disaccharides, and starch) were determined by subtracting the fiber, fat, ash, and protein content from the total. The nutrient composition of other materials was obtained from their nutrition fact labels. The cobalt contents were

103 determined by the Finnish Environment Institute using a method based on
104 microwave-assisted digestion and inductively coupled plasma mass spectrometry
105 (ICP-MS) quantitation as described by Nóbrega et al. (2012).

106 Both *P. freudenreichii* DSM 20271 and *L. brevis* ATCC 14869 cultures were
107 cryopreserved at –80 °C in 15% glycerol (v/v). Fermentation inocula were prepared as
108 follows: *P. freudenreichii* was propagated in the yeast extract lactate (YEL) medium
109 (Malik, Reinbold, & Vedamuthu, 1968) at 30 °C for 3 d. *L. brevis* was propagated in
110 de Man, Rogosa, and Sharpe (MRS) medium (Lab M, Lancashire, UK) at 37 °C for 1
111 d. The bacterial cells were harvested by centrifugation (3,200 × g, 10 min) and
112 resuspended in MilliQ water prior to inoculation.

113 **2.2 Batter preparation and fermentation**

114 2.2.1 Screening of grain materials for *in situ* production of B12

115 Batters were prepared by mixing MilliQ-water and the flours. The ratio of water to
116 flour in each batter (Table 1) was decided according to preliminary experiments to
117 achieve a semi-liquid batter. After mixing, 30 g of batters were transferred into Falcon
118 tubes (50 mL) and inoculated with *P. freudenreichii* (ca. 9.0 log CFU/g) and *L. brevis*
119 (ca. 6.0 log CFU/g). The inoculation levels of *P. freudenreichii* and *L. brevis* were
120 established in the previous studies to provide a meaningful level of B12 production
121 without compromising the microbial safety in non-sterilized wheat bran (Xie et al.,
122 2018; Xie et al., 2019). Triplicate cultures were prepared for each time point (0 d, 1 d
123 and 3 d). Fermentation was carried out in a shaking (200 rpm) incubator at 25 °C for 3
124 d. At each time point (0 d, 1 d and 3 d), three replicate samples of each batter type

were analyzed for pH value, total titratable acids (TTA), as well as, contents of lactic acid, acetic acid, propionic acid, and B12.

2.2.2 Fermentation with different levels of starter cultures

Four materials (buckwheat bran, oat bran, rice bran, and sorghum flour) were chosen for further experiments. Batters were prepared and fermented in the same way as in the screening experiments. In each material, batters were prepared in four ways as follows: spontaneously fermented (no inoculum, Control); inoculated with 9.0 log CFU/g *P. freudenreichii* and 6.0 log CFU/g *L. brevis* (P9/L6); inoculated with 9.0 log CFU/g *P. freudenreichii* and 7.0 log CFU/g *L. brevis* (P9/L7); and inoculated with 9.0 log CFU/g *P. freudenreichii* and 8.0 log CFU/g *L. brevis* (P9/L8). Different levels of *L. brevis* were tested to determine the optimal inoculation level for a meaningful B12 production together with inhibition of *Enterobacteriaceae* growth. Triplicate tubes were prepared for each time point, and at d 0, 1, and 3, samples were taken out for measurement of pH, TTA, viable cell counts, and content of B12.

2.3 Microbial cell counts

Common plate count techniques were used to determine viable cell numbers. Batters (10 g) were serially diluted in sterile sodium chloride solution (8.5 g/l) and plated on respective agar plates for the monitoring of different microbial groups. YEL plates for the determination of *P. freudenreichii* were incubated anaerobically (Anaerogen, Oxoid, Basingstoke, UK) for 4 d and aerobically for 1 d at 30 °C to allow the colonies of *P. freudenreichii* to turn brownish and distinguishable from other bacteria. Lactic acid bacteria (LAB) were counted on MRS agar (Lab M) plates

147 supplemented with 0.1 mg/g cycloheximide (Sigma Chemical Co., USA) and
148 incubated at 30 °C for 48 h. The plate count agar (PCA) plates (Lab M) and violet red
149 bile glucose agar (VRBGA) plates (Lab M) were used for the cell counts of total
150 aerobic bacteria (TAB) and total *Enterobacteriaceae,* respectively. The PCA plates
151 were incubated at 30 °C for 48 h, and the VRBGA plates were incubated at 37 °C for
152 24 h.

153 **2.4 Determination of pH, TTA, and content of acids**

154 A pH meter (Portamess 752 Calimatic, Knick, Berlin, Germany) was used for the
155 measurement of pH value. TTA was determined by a titrator (EasyPlus, Mettler
156 Toledo, Schott, Germany), and the value is expressed as the volume of 0.1 mol/L
157 NaOH solution (ml) required to adjust the pH of 10-g samples in 90 ml Milli-Q water
158 to 8.5.

159 Batter samples (1 g) were diluted (1:1~1:10, w/v) in water and centrifuged (3,200
160 × g, 10 min). The supernatants were filtered (0.45 mm, Pall, USA) into vials. The
161 contents of lactic acid (LA), acetic acid (AA), and propionic acid (PA) were
162 determined using a high-performance liquid chromatography (HPLC) method, as
163 reported earlier (Xie et al., 2018).

164 **2.5 Determination of B12 content in batters**

165 B12 in batters was determined by an ultra-high performance liquid
166 chromatography (UHPLC) method, as described by Chamlagain, Edelmann,
167 Kariluoto, Ollilainen, and Piironen (2015), with minor modifications. Briefly, 3 g of
168 batter samples were mixed with 15 mL of extraction buffer (8.3 mmol/L sodium

hydroxide and 20.7 mmol/L acetic acid, pH 4.5) and 100 µL of sodium cyanide (1g/100g in water). After extraction in boiling water (30 min), cooled mixtures were incubated in a water bath (30 min, 37°C) with the addition of 300 µL α-amylase (50 mg/ml) to allow the breakdown of starch before centrifugation (6,900 × *g*, 10 min). Residues after centrifugation were suspended in 5 mL of extraction buffer and centrifuged again. Both supernatants were combined and adjusted to 25 mL with the extraction buffer. Finally, 10 mL of the extracts were purified using an immunoaffinity column (Easi-Extract; Glasgow, Scotland) and analyzed with a UPLC system (Waters, Milford, MA, USA) equipped with a photodiode array detector (at 361 nm) and an Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 µm). The mobile phase was a gradient flow of MilliQ water and acetonitrile, both with 0.25 mg/g trifluoroacetic acid. The presence of any other corrinoids in the samples, including pseudo-vitamin B12, was followed in the chromatograms based on knowledge of their UV-absorption spectra and retention times from previous studies (Chamlagain et al., 2015; Chamlagain et al., 2018).

2.6 Statistical analysis

Statistical analysis was performed using SPSS 24.0 for Windows (IBM Corporation, NY, USA). One-way analysis of variance (ANOVA) and Tukey's *post hoc* test were used to determine significant differences in all parameters during fermentation at a p-value < 0.05 among the samples. Multivariate data analysis was performed by PLS, using Simca 15.0 (Umetrics AB, Malmö, Sweden).

3. Results

3.1 Nutrient and cobalt content of grain materials

The nutrient composition of the grain materials is shown in Table 1. In general, cereal and pseudocereal flours had the highest content of available carbohydrates (57–68 g/100 g), a moderate protein content (9–14 g/100 g), and low levels of lipids (5 –6 g/100 g) and dietary fiber (6–10 g/100 g). Brans differed greatly in their nutrient content (available carbohydrates 26–49 g/100 g, protein 13–40 g/100 g, dietary fiber 9–39 g/100 g, and lipids 4–20 g/100 g). Legume flours had a high protein content (31–43 g/100 g). Faba bean was rich in available carbohydrates (42 g/100 g), whereas lupine and soya bean had high fat content (12–20 g/100 g). The dietary fiber content in legume flours varied from 9 g/100 g to 28 g/100 g.

The cobalt content among the grain materials varied, and the highest was found in the faba bean flour (ca. 696 ng/g dw). In the rye bran, oat bran, and lupine flour, a low level of cobalt was observed (17–29 ng/g dw). Among other materials, the cobalt contents ranged from 68 ng/g dw to 183 ng/g dw.

3.2 Acidification of batters in the screening of materials for B12 production

3.2.1 pH and TTA

As shown in Table 2, pH in the batters ranged from 5.7 (lupine flour) to 6.8 (rice bran), and TTA varied from 0.5 mL (oat bran) to 4.8 ml (lupine flour) on d 0. In the batters of millet flour and two pseudocereal flours, pH decreased rapidly to ca. 4.0 already on d 1 and a high TTA (up to 18 mL) was reached after fermentation. A similar rapid pH drop and a high TTA level were detected also in the batters of rye bran and faba bean flour during fermentation. In the buckwheat bran batter, high TTA

(ca. 15 mL on d 1 and ca. 18 mL on d 3) and a pH remaining at 4.9 during fermentation were detected. In the batters of rice bran, oat bran, sorghum flour, soybean flour, and lupine flour, pH levels were higher than 5.5 and TTA increased by only about 1 mL during the first day. On d 3, pH in batters of rice bran (5.0), soybean flour (4.7), and lupine flour (4.9) were significantly ($p < 0.05$) lower than in the batters of oat bran (5.2) and sorghum flour (5.3). Accordingly, TTA in the rice bran, soybean flour, and lupine flour (11-13 mL) was significantly ($p < 0.05$) higher than in the batters of oat bran (6.1 mL) and sorghum flour (4.4 mL).

3.2.2 Production of organic acids

During the fermentation of oat bran, rice bran, sorghum flour, and lupine flour, LA was not detected at the measured time points, whereas a drastic increase of AA and PA contents were detected from d 1 to d 3 (Fig. 1). On d 1, the buckwheat bran batter had the highest level of both AA (ca. 9 mg/g dw) and PA (ca. 13 mg/g dw) and contained ca. 28 mg/g dw of LA. On d 3, the buckwheat bran batter contained the highest amount of PA (ca. 33 mg/g dw) and ca. 19 mg/g dw of AA but no LA was detected. During the fermentation of rye bran and flours of amaranth, quinoa, millet, and faba bean, the contents of LA (40.0–98.1 mg/g dw) were much higher than the contents of AA (1.4–4.8 mg/g dw) and PA (1.6–10.4 mg/g dw).

3.3 B12 synthesis in the screening of materials for B12 production

B12 levels of 19 - 38 ng/g dw were detected in the batters immediately following inoculum with *P. freudenreichii* (Table 3). After one day of fermentation, a significant ($p < 0.05$) increase of B12 content was observed in all batters, and the highest content

was found in the buckwheat bran batter (ca. 335 ng/g dw). From d 1 to d 3, there was no significant ($p < 0.05$) increase of B12 content in the batters of rye bran, millet flour, quinoa flour, and amaranth flour. On d 3, the highest content of B12 was found in the batter of rice bran (ca. 742 ng/g dw), followed by the batters of buckwheat bran (ca. 631 ng/g dw) and soybean flour (ca. 407 ng/g dw). In the batters of oat bran, sorghum flour, faba bean flour, and lupine flour, the B12 content ranged from 265 to 343 ng/g dw on d 3.

3.4 Correlation of nutrient composition and acidification properties with B12 production

A PLS modeling was used to investigate how nutrient composition, cobalt content, and acidification properties (pH, TTA, and production of acids) correlated with B12 production on d 1 and d 3 (Fig. 2). The first and second principal components (PC1 and PC2) together explained 68% (d 1) and 64% (d 3) of the variation. The projections of each variable on the line drawn via B12 production and the origin of the plot describes the correlation of the variables and B12 production. Moreover, the distance of each variable's projection from the origin indicate its influence on B12 production. On both d 1 and d 3, the B12 content was positively correlated with high pH value (> 4.7), the content of protein, fat and dietary fiber, and production of propionic acid and acetic acid. On the other hand, B12 production was negatively correlated with high TTA content (> 17 mL), the content of the available carbohydrate, and lactic acid. The cobalt content only had a limited influence on B12 production, both on d 1 and d 3.

3.5 Batters fermented with different inoculum levels of starter cultures

Buckwheat bran, oat bran, rice bran, and sorghum flour were chosen for further experiments to study the effect of different *L. brevis* inoculum levels on the fermentation of each material.

3.5.1 Microbial counts

Propionic acid bacteria (PAB) were not detected throughout the fermentation in any control batters (Table 4). In the inoculated batters, the cell density of PAB ranged from ca. 8.7 log CFU/g to ca. 8.8 log CFU/g. The cell density of inoculated PAB increased by ca. 0.5 log cycle in the batters of oat bran, rice bran, and buckwheat bran on d 1 and remained stable thereafter. In the sorghum batters, the increase of PAB cell density during fermentation was in the range of 0.2–0.4 log cycle.

In the control batters, LAB were detected only in the buckwheat bran and sorghum flour at a cell density at ca. 2.8 log CFU/g and ca. 2.3 log CFU/g, respectively. During fermentation, cell densities of LAB increased in all batters, and inoculation of *L. brevis* led to a higher cell number of LAB compared to the control batters with no inoculum. On d 3, the cell density of LAB reached 9.0 log CFU/g in all batters with inoculation except in the sorghum batter with an inoculum of P9/L6 (ca. 8.1 log CFU/g). Supplemental Table 2 shows the cell densities of total aerobic bacteria (TAB) in all batters. In general, similar cell densities of LAB and TAB were found during the fermentation of the oat bran and the buckwheat bran.

Before fermentation, *Enterobacteriaceae* were detected only in the buckwheat bran batter and sorghum flour batter at a level of 4.4 log CFU/g and 5.4 log CFU/g,

279 respectively. On d 1, the cell density of *Enterobacteriaceae* increased in all the
280 spontaneously fermented batters and ranged from 7.6 log CFU/g to 9.0 log CFU/g. In
281 the batters of oat bran, rice bran, and buckwheat bran, a significantly ($p < 0.05$) lower
282 cell density of *Enterobacteriaceae* was detected when *L. brevis* was inoculated than in
283 the corresponding control batters. In the batter of sorghum flour, a significantly ($p <$
284 0.05) lower cell density of *Enterobacteriaceae* was detected only when the
285 inoculation level of *L. brevis* was higher than 7 log CFU/g. At the end of fermentation,
286 no *Enterobacteriaceae* were detected in batters of rice bran, buckwheat bran, and
287 sorghum flour when the inoculum size of *L. brevis* was greater than 7 log CFU/g.

288 3.5.2 Acidification of batters

289 During fermentation, the control batters reached the highest pH values and the
290 lowest TTA content and the batters with the highest inoculum ratio of *L. brevis* (P9/L8)
291 reached the lowest pH levels and the highest TTA content (Table 5). In both oat bran
292 and buckwheat bran batters, a significantly ($p < 0.05$) higher TTA value was observed
293 in P9/L6 batters than in the control batters. In the rice bran batter, a significant ($p <$
294 0.05) increase in TTA content and a decrease of pH value was found only when the *L.*
295 *brevis* inoculum was 8.0 log CFU/g. In the sorghum batter, a significant ($p < 0.05$)
296 increase of TTA content (from ca. 4.3 ml to 9.2 ml on d 3) and a decrease of pH value
297 (from ca. 5.3 to ca. 3.9 on d 3) was observed for each increased inoculum level of *L.*
298 *brevis*.

299 3.5.3 Content of B12

During d 1, the B12 production did not differ with different inocula of *L. brevis* in the batters of oat bran and rice bran (Table 5). On the contrary, on d 3, B12 content of oat bran P9/L6 batter (ca. 342 ng/g dw) was significantly ($p < 0.05$) higher than that of the oat bran P9/L7 and P9/L8 batters (298 - 312 ng/g dw). In rice bran, B12 content of P9/L8 batter (ca. 675 ng/g dw) was significantly ($p < 0.05$) lower than in P9/L7 and P9/L6 batter (ca. 720 ng/g dw). In the sorghum batters, B12 content was significantly ($p < 0.05$) lower on both d 1 and d 3 when the inoculation level of *L. brevis* was increased. Notably, only ca. 67 ng/g dw of B12 was produced in P9/L8 batter of sorghum at the end of fermentation.

4. Discussion

Our previous study showed that a significant increase of B12 content was achieved in wheat bran by fermentation with *P. freudenreichii* DSM 20271 and *L. brevis* ATCC 14869 (Xie et al., 2019). The present study aimed to study whether the mixed culture fermentation approach is applicable for the *in situ* fortification of other grains with B12. Furthermore, the correlation of B12 production with nutrient composition, cobalt content, pH, and production of acids during fermentation was investigated by PLS modeling to reveal critical factors affecting vitamin synthesis.

The materials used in this study had varying content of nutrients, which lead to different acidification kinetics and a greatly different production of B12 by *P. freudenreichii* (60 - 740 ng/g dw) during fermentation. In general, batters of cereal and pseudocereal flours had a higher level of LA content and TTA value during fermentation, which was probably due to their higher level of available carbohydrates

compared to other materials (brans and legume flours) supporting the growth of microorganisms. In line with this, the PLS model showed a positive correlation of available carbohydrate content with LA production and TTA (Fig. 2).

Although LA produced by LAB is the preferential carbon source for PAB (Piveteau, 1999), a fast drop in environmental pH caused by accumulating LA can inhibit the growth and metabolism of PAB because they preferably grow at neutral pH and stop producing acids when the pH value drops below 4.5 (Ye, Shijo, Jin, & Shimizu, 1996). B12 is a coenzyme in the metabolic pathway for PA and AA production in *P. freudenreichii* (Thierry et al., 2011). The PLS model revealed a positive correlation of B12 production with the production of PA and AA in the present study (Fig. 2). In accordance, the fermentation conditions in batters with high LA contents and correspondingly with low pH values (<4.5), such as millet, amaranth, and quinoa flours, generally produced low PA, AA, and B12 yields.

Compared to cereal and pseudocereal flours, the content of available carbohydrate in brans and legume flours was generally lower, which was consequently accompanied with higher pH values in batters during fermentation. The high pH values in some batters likely resulted from the low production levels of acids, for example, in oat bran (TTA: 6.4 mL on d 3). Besides, the high pH value may also be an effect from the presence of high buffering capacity compounds, such as proteins (Lević, Prodanović, & Sredanović, 2005) and dietary fiber (Tadesse, 1986), in the materials. For instance, in the buckwheat bran batter, despite a high TTA content observed (ca. 15 mL on d 1), the pH value remained at 4.9 from d 1 to d 3. The PLS

344 model also showed that dietary fiber, protein, and lipid contents were positively
345 correlated with the production of B12.

346 Cobalt, the central metal ion of B12, is reported to be a limiting factor for the
347 production of B12 during the fermentation of *P. freudenreichii* in various substrates,
348 such as whey medium (Hugenschmidt, Schwenninger, & Lacroix, 2011) and aqueous
349 cereal-based matrices (Chamlagain et al., 2017). Moreover, during fermentation in
350 whey medium, the production of B12 by *P. freudenreichii* increased significantly
351 when cobalt was added up to 5 µg/mL (Hugenschmidt et al., 2011). Our previous
352 study also showed that adding cobalt (600 ng/g dw) in durum wheat flour batter was
353 able to increase the production of B12 from ca. 33 ng/g dw to ca. 200 ng/g dw during
354 fermentation with *P. freudenreichii* (Xie et al., 2018). PLS modeling indicates that
355 cobalt content was not a limiting factor for B12 production under the conditions used
356 here. Notably, there was no significant ($p > 0.05$) difference in B12 content (ca. 332
357 ng/g dw vs ca. 298 ng/g dw) at the end of fermentation between materials with the
358 lowest level (17 ng/g dw in oat bran) and the highest level (696 ng/g dw in faba bean
359 flour) of cobalt. On the other hand, rice bran, buckwheat bran, millet, sorghum, and
360 amaranth flours had a similar level of cobalt (157–183 ng/g dw) but with a
361 considerably different level of B12 production (51–742 ng/g dw), and the batter with
362 a higher pH tended to have higher B12 production. Thus, the influence of cobalt
363 content on B12 production seemed to be less significant than that of pH during
364 fermentation when above the level of 17 ng/g dw of cobalt measured in this study.

365 Fermented soybean products, such as tempeh and natto, have been found to

366 contain certain amounts of B12 (0.1−8.0 μg/100 g fresh weight; Watanabe., Yabuta,
367 Bito, & Teng, 2014). Moreover, recent studies showed that the inoculation of *P.*
368 *freudenreichii* as a starter culture could significantly increase B12 production (up to
369 1230 ng/g dw) in lupine tempeh (Signorini et al., 2018; Wolkers – Rooijackers,
370 Endika, & Smid, 2018). In the present study, 300–400 ng/g dw of B12 was obtained
371 after the fermentation of three legume materials, which can provide a new option for
372 developing B12-fortified legume products.

373 To the best of our knowledge, this was the first study to ferment oat, rice, and
374 buckwheat bran with *P. freudenreichii* for *in situ* production of B12 and our study
375 shows that they seem to be very promising materials for this purpose. Moreover, as
376 side streams of the milling process, utilization of these brans for developing
377 B12-fortified food can contribute to a more resilient food chain by reducing grain
378 waste. Furthermore, developing B12-fortified sorghum products can be important
379 because they are widely consumed in Asia and Africa (Nout, 2009). People in these
380 two continents have a higher prevalence of B12 deficiency than people in other parts
381 of the world (Hunt, Harrington, & Robinson, 2014). Therefore, sorghum flour and oat,
382 rice, and buckwheat bran were chosen for further experiments.

383 Raw cereal materials usually contain some undesirable microorganisms, such as
384 potential pathogens from the *Enterobacteriaceae* family, which can cause food
385 poisoning by infecting the human gastrointestinal tract or producing various
386 endotoxins (Singh, Sharma, & Nara, 2015). LAB in cereal materials can show
387 antagonistic activity to pathogens by the production of acids and antimicrobial

388 compounds or by competitive exclusion (De Vuyst & Neysens, 2005). However, a
389 high cell density of *Enterobacteriaceae* was found in the batters of brans and sorghum
390 flour during fermentation in the present study. *Enterobacteriaceae* cell density in the
391 buckwheat bran control batter increased by 4 log units during the first day of
392 fermentation and still had a cell density at ca. 5 log CFU/g at the end of fermentation.
393 In the oat bran and rice bran control batters, the cell numbers of *Enterobacteriaceae*
394 increased to more than 7.0 log CFU/g even though no *Enterobacteriaceae* were
395 detected before the fermentation.

396 As expected, the inoculation of *L. brevis* decreased the cell density of
397 *Enterobacteriaceae* by at least 1 log unit compared to control batters, and stronger
398 inhibitions were observed when the initial level of LAB increased during the
399 fermentation. However, an increase of *L. brevis* inoculation also led to a drastic
400 decrease of B12 production in the sorghum flour batter due to the faster pH drop. The
401 production of PA in sorghum flour batter also drastically decreased (5.9 vs 1.4 mg/g
402 dw) when an initial level of *L. brevis* was increased from 6 log CFU/g to 8 log CFU/g
403 (Supplemental Table 3). In the fermentation of rice bran and buckwheat bran,
404 although the initial level of *L. brevis* was increased from 6 log CFU/g to 8 log CFU/g,
405 the PA production levels were not significantly affected (Supplemental Table 3),
406 possibly because of the good buffering capacity (pH > 4.5 on d 3 despite the high
407 TTA) of the materials. Therefore, the B12 content after d 3 was still high in the rice
408 bran (600 ng/g dw) and in the buckwheat bran (500 ng/g dw). Considering that *in situ*
409 synthesized B12 is stable during food processing (Edelmann, Chamlagain, Santin,

Kariluoto, & Piironen 2016), consuming a food product containing 10 g dry matter of rice bran or buckwheat bran fermented with *P. freudenreichii* could provide the daily requirement of B12 (2.4 µg for adults; Institute of Medicine, 1998). On the other hand, by replacing 30% of wheat flour in a bread recipe with fermented sorghum (with ca. 120 ng/g dw of B12), four slices of bread (120 g) could, theoretically, provide more than 2.4 µg of B12.

Further research on the sensory properties of food containing these fermented batters as ingredients (e.g. baked goods and pasta) could be performed to optimize their sensory quality and increase their acceptance. Moreover, a more in-depth study of each raw material with different nutrient composition (e.g. protein, fat, and dietary fiber) and origin would be needed for a more comprehensive understanding of their full potential in B12 production. In addition, maintaining pH at different levels and adding different ingredients (e.g. protein or materials with high availability of cobalt) during fermentation should be applied in future studies to investigate how these factors influence B12 *in situ* production in grain materials.

5. Conclusion

This work demonstrated that a nutritionally relevant amount of B12 was produced in non-sterilized grain materials by fermentation with *P. freudenreichii*. The higher B12 content in grain batters with a higher pH during fermentation suggests that maintaining optimal pH for *P. freudenreichii* can increase the production of B12. The contents of some components, such as protein and dietary fiber, in grain materials were found to be positively correlated with B12 production in PLS analysis probably due to their pH buffering capacity. Meanwhile, the combination of *P. freudenreichii*

with *L. brevis* inoculated at a certain level can effectively inhibit the growth of *Enterobacteriaceae* to ensure the microbial safety of the fermentation. In conclusion, grain materials fermented with *P. freudenreichii* and *L. brevis* can be a promising alternative to produce B12-fortified grain ingredients for a wide variety of plant-based foods.

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Table 1. Nutrient content (g/100 g) and cobalt content (ng/g) of the materials and dry matter in batters (%).

Materials and their origin	Protein	Fat	Fiber	Available Carbohydr-ates	Cobalt	Ratio with water
Rye bran, Finland	15	4	39	26	20	15%
Oat bran, Finland	18	8	14	49	17	15%
Rice bran, USA	13	20	20	33	166	15%
Sorghum flour, Burkina Faso	10	5	10	65	183	30%
Millet flour, Burkina Faso	9	5	7	68	157	30%
Buckwheat bran, Europe	40	9	9	38	183	20%
Quinoa flour, Peru	14	6	6	57	85	25%
Amaranth flour, Germany	14	6	9	66	182	25%
Faba bean flour, Finland	31	2	9	42	696	30%
Soy bean flour, China	38	20	13	18	68	20%
Lupine flour, France	43	12	28	10	29	20%

Table 2. pH values and total titratable acids (TTA; mL) of batters during fermentation in screening of grain materials for vitamin B12 production. The results are expressed as the mean value (n = 3).

	pH			TTA		
Batters	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Rye bran	6.6^{ez}	3.9^{ay}	3.6^{ax}	2.3^{cx}	12.4^{dy}	17.4^{cz}
Oat bran	6.5^{dez}	5.9^{fy}	5.2^{ex}	0.5^{ax}	1.7^{ay}	6.1^{az}
Rice bran	6.8^{fz}	6.5^{gy}	5.0^{dx}	0.9^{ax}	1.3^{ay}	12.5^{by}
Sorghum flour	6.2^{cz}	5.6^{ey}	5.3^{ex}	1.8^{bx}	3.1^{by}	4.4^{az}
Millet flour	6.0^{by}	3.7^{ax}	3.6^{ax}	3.7^{dx}	11.6^{dy}	17.3^{cz}
Buckwheat bran	6.5^{dy}	4.9^{dx}	4.9^{dx}	2.6^{cx}	14.9^{ey}	18.5^{cdz}
Quinoa flour	6.0^{bz}	3.9^{ay}	3.6^{ax}	4.7^{ex}	18.4^{fy}	21.9^{ez}
Amaranth flour	6.3^{cdz}	4.2^{by}	3.7^{ax}	3.3^{dx}	15.5^{ey}	22.1^{ez}
Faba bean flour	6.4^{dz}	4.5^{cy}	4.3^{bx}	4.4^{ex}	14.8^{ey}	19.5^{dz}
Soy bean flour	6.7^{fz}	6.1^{fy}	4.7^{cx}	2.6^{cx}	3.3^{by}	13.5^{bz}
Lupine flour	5.7^{ay}	5.6^{ey}	4.9^{dx}	4.8^{ex}	5.7^{cy}	11.3^{bz}

Values in the same column (a-f) and same row (x-z) bearing different superscripts are significantly different ($p < 0.05$).

Table 3. Vitamin B12 content (ng/g, dw) of batters during fermentation in screening of grain materials for vitamin B12 production. The results are expressed as the mean ± standard deviation (n = 3).

Batters	Day 0	Day 1	Day 3
Rye bran	38 ± 5^{cx}	103 ± 7^{cy}	104 ± 29^{by}
Oat bran	36 ± 4^{cx}	128 ± 3^{dy}	332 ± 24^{dz}
Rice bran	37 ± 5^{cx}	133 ± 16^{dy}	742 ± 18^{gz}
Sorghum flour	20 ± 3^{ax}	149 ± 9^{dey}	265 ± 13^{cz}
Millet flour	19 ± 3^{ax}	58 ± 1^{ay}	51 ± 6^{ay}
Buckwheat bran	29 ± 4^{bx}	335 ± 76^{gy}	631 ± 61^{fz}
Quinoa flour	32 ± 4^{bx}	60 ± 1^{ay}	65 ± 5^{ay}
Amaranth flour	31 ± 4^{bx}	81 ± 13^{by}	78 ± 4^{ay}
Faba bean flour	20 ± 5^{cx}	155 ± 23^{ey}	298 ± 51^{cdz}
Soy bean flour	29 ± 6^{bx}	154 ± 2^{ey}	407 ± 5^{ez}
Lupine flour	28 ± 4^{bx}	246 ± 15^{fy}	343 ± 71^{dz}

Values in the same column (a-g) and same row (x-z) bearing different superscripts are significantly different ($p < 0.05$).

Table 4. Cell counts of propionic acid bacteria (PAB), lactic acid bacteria (LAB) and *Enterobacteriaceae* during fermentation with different level of starter cultures (log CFU/g). The results are expressed as the mean ± standard deviation (n = 3).

	PAB			LAB			*Enterobacteriaceae*		
	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Oat bran									
Control	-*	-	-	-*	7.1 ± 0.1^{ax}	9.1 ± 0.1^{ay}	-*	7.6 ± 0.1^{dy}	4.7 ± 0.2^{bx}
P9/L6	8.7 ± 0.1^{ax}	9.3 ± 0.1^{ay}	9.4 ± 0.3^{ay}	5.7 ± 0.1^{ax}	8.4 ± 0.1^{by}	9.2 ± 0.1^{az}	-	6.4 ± 0.2^{cy}	4.4 ± 0.2^{bx}
P9/L7	8.8 ± 0.1^{ax}	9.3 ± 0.1^{ay}	9.3 ± 0.1^{ay}	6.9 ± 0.1^{bx}	8.3 ± 0.1^{by}	9.3 ± 0.1^{az}	-	4.8 ± 0.2^{by}	3.9 ± 0.1^{ax}
P9/L8	8.7 ± 0.1^{ax}	9.2 ± 0.1^{ay}	9.3 ± 0.2^{ay}	8.1 ± 0.1^{cx}	9.4 ± 0.1^{cy}	9.3 ± 0.1^{ay}	-	4.3 ± 0.1^{a}	-
Rice bran									
Control	-	-	-	-	7.4 ± 0.1^{ax}	8.1 ± 0.2^{ay}	-	7.8 ± 0.1^{cy}	8.7 ± 0.2^{bx}
P9/L6	8.8 ± 0.0^{ax}	9.4 ± 0.0^{ay}	9.5 ± 0.1^{ay}	5.8 ± 0.1^{ax}	8.1 ± 0.1^{by}	8.9 ± 0.2^{bz}	-	5.4 ± 0.2^{by}	3.5 ± 0.4^{ax}
P9/L7	8.7 ± 0.2^{ax}	9.2 ± 0.2^{ay}	9.3 ± 0.0^{ay}	7.0 ± 0.0^{bx}	8.0 ± 0.2^{by}	9.3 ± 0.2^{cz}	-	4.2 ± 0.2^{a}	-
P9/L8	8.8 ± 0.1^{ax}	9.3 ± 0.1^{ay}	9.3 ± 0.2^{ay}	8.0 ± 0.1^{cx}	9.1 ± 0.1^{cy}	9.5 ± 0.2^{cz}	-	4.3 ± 0.1^{a}	-
Buckwheat bran									
Control	-	-	-	2.8 ± 0.2^{ax}	8.4 ± 0.0^{ay}	9.4 ± 0.1^{az}	4.4 ± 0.2^{ax}	8.6 ± 0.1^{dz}	5.4 ± 0.2^{by}
P9/L6	8.8 ± 0.1^{ax}	9.4 ± 0.1^{by}	9.4 ± 0.1^{by}	5.7 ± 0.2^{bx}	9.1 ± 0.1^{by}	9.3 ± 0.0^{az}	4.3 ± 0.2^{ax}	6.5 ± 0.2^{cz}	5.1 ± 0.0^{ay}
P9/L7	8.7 ± 0.2^{ax}	9.3 ± 0.1^{aby}	9.3 ± 0.1^{by}	7.2 ± 0.1^{cx}	9.2 ± 0.1^{by}	9.3 ± 0.1^{ay}	4.4 ± 0.1^{ax}	5.5 ± 0.1^{by}	-
P9/L8	8.7 ± 0.1^{ax}	9.1 ± 0.2^{ay}	9.0 ± 0.2^{ay}	8.1 ± 0.2^{dx}	9.1 ± 0.1^{by}	9.4 ± 0.2^{ay}	4.3 ± 0.1^{ax}	5.1 ± 0.2^{ay}	-
Sorghum flour									
Control	-	-	-	2.3 ± 0.3^{ax}	6.6 ± 0.1^{ay}	7.9 ± 0.1^{az}	5.4 ± 0.1^{ax}	9.0 ± 0.1^{cy}	8.9 ± 0.1^{ay}
P9/L6	8.7 ± 0.2^{ax}	8.8 ± 0.1^{ax}	9.1 ± 0.2^{by}	5.9 ± 0.0^{bx}	8.1 ± 0.1^{by}	8.1 ± 0.1^{ay}	5.5 ± 0.2^{ax}	8.8 ± 0.1^{cy}	9.2 ± 0.2^{ay}
P9/L7	8.8 ± 0.1^{ax}	9.0 ± 0.1^{ax}	8.9 ± 0.1^{ax}	6.8 ± 0.2^{cx}	8.9 ± 0.1^{by}	9.1 ± 0.1^{by}	5.4 ± 0.0^{ax}	7.9 ± 0.1^{by}	-
P9/L8	8.7 ± 0.1^{ax}	8.9 ± 0.1^{ax}	8.9 ± 0.2^{ax}	7.9 ± 0.1^{dx}	8.8 ± 0.1^{by}	9.0 ± 0.1^{by}	5.5 ± 0.1^{ax}	5.4 ± 0.1^{ax}	-

* Not detected

Control, spontaneously fermented batter; P9/L6, P9/L7 and P9/L8 mean the batters with 9.0 log CFU/g of *P. freudenreichii* and 6.0 log CFU/g, 7.0 log CFU/ and 8.0 log CFU/g of *L. brevis*, respectively.

Values in the same column and batter type bearing different superscripts (a-c) are significantly different ($p < 0.05$). Values in the same row bearing different superscripts (x-z) are significantly different ($p < 0.05$).

Table 5. pH, total titratable acids (TTA; mL) and vitamin B12 content (ng/g, dw) during fermentation with different level of starter cultures. The results of pH and TTA are expressed as the mean. The results of vitamin B12 content are expressed as the mean ± standard deviation (n = 3).

	pH			TTA			Vitamin B12		
	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3	Day 0	Day 1	Day 3
Oat bran									
Control	6.5 ^{az}	6.2 ^{cy}	5.8 ^{cx}	0.5 ^{ax}	1.0 ^{ay}	2.1 ^{az}	-*	-	-
P9/L6	6.5 ^{az}	5.8 ^{by}	5.2 ^{bx}	0.5 ^{ax}	1.6 ^{by}	5.4 ^{bz}	38±5^{ax}	119±7 ^{ay}	342±14 ^{bz}
P9/L7	6.5 ^{az}	5.4 ^{ay}	4.5 ^{ax}	0.5 ^{ax}	4.8 ^{cy}	6.1 ^{bz}	38±5^{ax}	104±18^{ay}	312±3 ^{az}
P9/L8	6.5 ^{az}	5.4 ^{ay}	4.4 ^{ax}	0.5 ^{ax}	4.7 ^{cy}	6.3 ^{bz}	38±5^{ax}	128±12^{ay}	298±10 ^{az}
Rice bran									
Control	6.8 ^{az}	6.4 ^{by}	5.3 ^{bx}	0.9 ^{ax}	1.3 ^{ay}	11.2 ^{az}	-	-	-
P9/L6	6.8 ^{az}	6.5 ^{by}	5.1 ^{abx}	0.9 ^{ax}	1.1 ^{ay}	11.9 ^{bz}	38±5^{ax}	112±16 ^{ay}	722±21 ^{bz}
P9/L7	6.8 ^{az}	6.4 ^{by}	5.0 ^{ax}	0.9 ^{ax}	1.8 ^{ay}	13.1 ^{bz}	38±5^{ax}	119±24 ^{ay}	717±10 ^{bz}
P9/L8	6.8 ^{az}	5.9 ^{ay}	4.9 ^{ax}	0.9 ^{ax}	3.9 ^{by}	16.4 ^{cz}	38±5^{ax}	122±3 ^{ay}	675±30 ^{az}
Buckwheat bran									
Control	6.5 ^{az}	5.3 ^{cy}	4.9 ^{bx}	2.6 ^{ax}	12.4 ^{ay}	17.0 ^{az}	-	-	-
P9/L6	6.5 ^{az}	4.9 ^{bx}	4.9 ^{bx}	2.6 ^{ax}	14.3 ^{by}	17.7 ^{bz}	29±4^{ax}	312±76^{by}	604±41 ^{bz}
P9/L7	6.5 ^{ay}	4.7 ^{ax}	4.6 ^{ax}	2.6 ^{ax}	19.1 ^{cy}	20.0 ^{cz}	29±4^{ax}	237±38^{ay}	572±58^{abz}
P9/L8	6.5 ^{ay}	4.6 ^{ax}	4.6 ^{ax}	2.6 ^{ax}	19.8 ^{cy}	20.7 ^{cz}	29±4^{ax}	256±28^{ay}	508±26 ^{az}
Sorghum flour									
Control	6.2 ^{az}	5.7 ^{by}	5.4 ^{cx}	1.8 ^{ax}	2.4 ^{ay}	3.7 ^{az}	-*	-	-
P9/L6	6.2 ^{az}	5.6 ^{by}	5.3 ^{cx}	1.8 ^{ax}	3.2 ^{ay}	4.3 ^{az}	19±3^{ax}	151±16^{cy}	249±11 ^{cz}
P9/L7	6.2 ^{az}	4.7 ^{ay}	4.5 ^{bx}	1.8 ^{ax}	4.8 ^{by}	7.8 ^{bz}	19±3^{ax}	88±9 ^{by}	125±27 ^{bz}
P9/L8	6.2 ^{az}	4.6 ^{ay}	3.9 ^{ax}	1.8 ^{ax}	5.5 ^{cy}	9.2 ^{cz}	19±3^{ax}	68±6 ^{ay}	67±14 ^{ay}

* Not detected

Control, spontaneously fermented batter; P9/L6, P9/L7 and P9/L8 mean the batters with 9.0 log CFU/g of *P. freudenreichii* and 6.0 log CFU/g, 7.0 log CFU/ and 8.0 log CFU/g of *L. brevis*, respectively.

Values in the same column and batter type bearing different superscripts (a-c) are significantly different ($p < 0.05$). Values in the same row bearing different superscripts (x-z) are significantly different ($p < 0.05$).

Figure legends

Figure 1. Contents (mg/g dw) of lactic acid (), acetic acid () and propionic acid () during fermentation in screening of grain materials for vitamin B12 production. The results are expressed as the mean value of three replicates and error bars indicate standard deviation of total acid content.

Figure 2. Partial least square regression (PLS) loading plots showing the distribution of x variables and y variables along the first and second principal components (PC) on day 1 (A) and day 3 (B). In each plot, the x variables are pH, total titratable acid (TTA) and content of protein, fat, fiber, available carbohydrates, cobalt, lactic acid (LA), acetic acid (AA) and propionic acid (PA). The y variable is production of vitamin B12. The projection of each x variable on the line drawn via the y variable and the origin of the plot describes the correlation of the x variables and the y variable; distance of each x variable's projection from the origin indicate its influence on the y variable.

Figure 1.

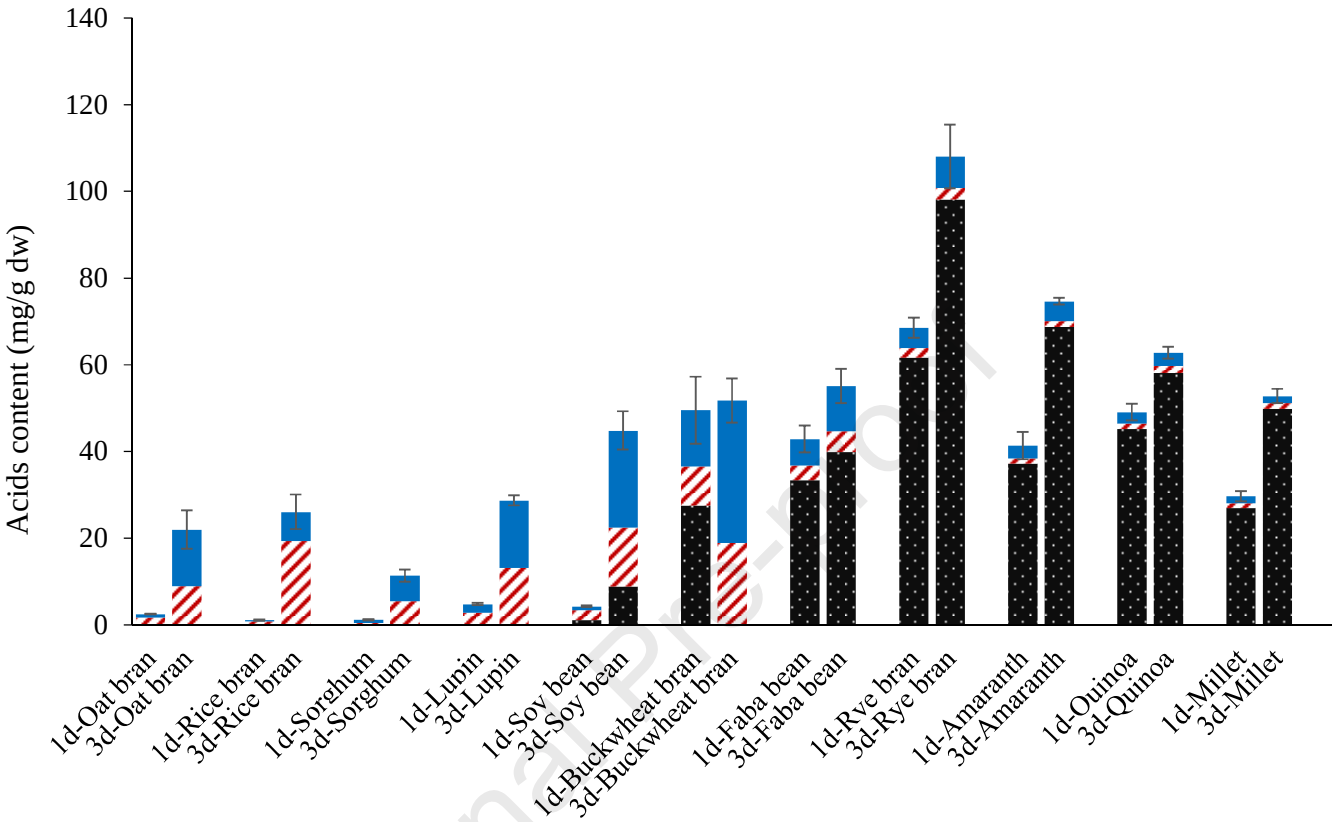
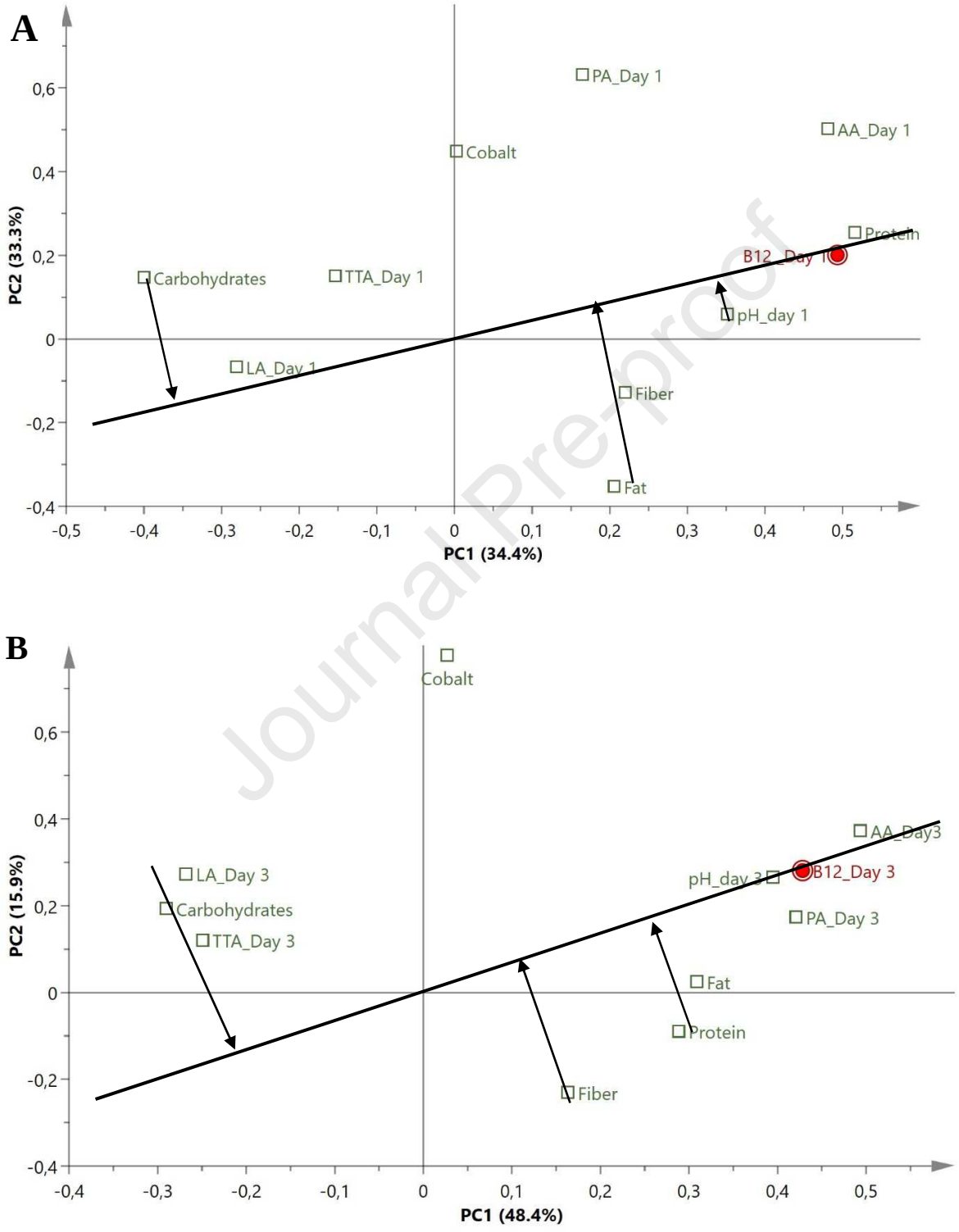


Figure 2.



1. More than 300 ng/g of vitamin B12 was produced in various materials

2. The highest vitamin B12 production was found in the rice bran fermentation

3. Nutrient composition of materials significantly influenced vitamin B12 production

4. *L. brevis* can inhibit the growth of *Enterobacteriaceae* during fermentation

The authors declared that no conflict of interest exists in the submission of this manuscript for publication.