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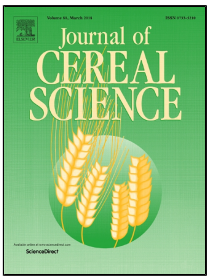

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In situ fortification of vitamin B12 in wheat flour and wheat bran by fermentation with *Propionibacterium freudenreichii*

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Keywords: *Propionibacterium freudenreichii*; *In situ* fortification; Vitamin B12; Wheat bran

[1] **Abbreviation:** AA, Acetic Acid; CFU, Colony Forming Unit; DMBI, 5,6 - dimethylbenzimidazole; dw, dry weight; HPLC, High-Performance Liquid Chromatography; LA, Lactic Acid; LAB, Lactic Acid Bacteria; MRS, de Man, Rogosa and Sharpe; PA, Propionic Acid; PCA, Plate Count Agar; TTA, Total Titratable Acid; VRBGA, Violet Red Bile Glucose Agar; YEL, Yeast Extract Lactate; YM, Yeast Mold.

Abstract

Vitamin B12 is a micronutrient naturally existing in animal products. A growing interest and need to replace animal protein with plant protein sources have resulted in increased attention to developing vitamin B12-fortified plant-based food. Natural fortification by *Propionibacterium freudenreichii* is a promising alternative to chemical fortification, as *P. freudenreichii* can synthesize active vitamin B12. In this work, we studied vitamin B12 production in non-sterile matrices prepared from three raw materials of wheat: durum flour, wholewheat flour and wheat bran. Viable cell counts, pH, total titratable acidity and concentration of acids were determined. After seven days of fermentation, vitamin B12 levels reached 33 ± 4, 87 ± 10 and 155 ± 17 ng/g dry weight in durum flour, wholewheat flour, and wheat bran, respectively. While durum flour supported the growth of *P. freudenreichii* to higher cell densities and more efficient propionic acid production compared with the other two matrices, wholewheat flour and wheat bran were found to be the most promising of the three matrices for *in situ* production of vitamin B12.

1. Introduction

Vitamin B12, also known as cobalamin, has the most complex chemical structure of all vitamins. Humans require it as a cofactor for two enzymes: cytoplasmic methionine synthase, which is involved in nucleotide synthesis together with folate, and for methylmalonyl–CoA mutase, which is important in the metabolism of odd-chain fatty acids and branched amino acids (Nielsen et al., 2012). A vitamin B12 deficiency may result in health problems, such as megaloblastic anemia and various neurological disorders (Nielsen et al., 2012). A vitamin B12 intake of 2.4 μg/day is recommended for adults according to the Institute of Medicine (1998).

Vitamin B12 is known to be synthesized by only a few microorganisms, such as *Pseudomonas denitrificans*, *Bacillus megaterium*, and *Propionibacterium freudenreichii* (Martens et al., 2002). Foods of animal origin are still the main dietary sources of vitamin B12, and without chemical supplementation or fortification due to fermentation, it is not present in appreciable amounts in foods of plant-origin (Watanabe, 2007). Vitamin B12 deficiency, mainly caused by inadequate dietary intake or malabsorption, is reported to be a problem worldwide (Green et al., 2017).

Developing plant products fortified with vitamin B12 is a promising way to ensure sufficient intake by people consuming limited amounts of animal products. As wheat is one of the most produced and important cereals for human diet, wheat products would be good candidates for delivering vitamin B12.

Specific strains of several *Lactobacillus* species have been reported to produce vitamin B12 (Bhushan et al., 2017; Taranto et al., 2003). However, the few studies

64 employing methods capable of distinguishing between the different B12 forms
65 indicate that *Lactobacilli* produce pseudovitamin B12 (Crofts et al., 2013; Santos et
66 al., 2007). Pseudovitamin B12 differs from the active B12 by the presence of adenine
67 in the place of 5, 6-dimethylbenzimidazole (DMBI) as the lower ligand and it is
68 inactive in humans (Stupperich and Nexo, 1991; Watanabe, 2007). The ability of
69 *Lactobacillus* strains to synthesize DMBI has not been addressed in detail yet. *P.*
70 *freudenreichii*, a bacterium with generally recognized as safe status, synthesizes
71 DMBI (Deptula et al., 2015) and produces the active form of vitamin B12 in
72 nutritionally relevant amounts, also under food-like conditions (Chamlagain et al.,
73 2016; Deptula et al., 2017a).

74 Some strains of *P. freudenreichii* have already been used in cereal-based food for
75 various functions, such as producing exopolysaccharides or antifungal compounds
76 (Denkova et al., 2014; Tinzl-Malang et al., 2015). Chamlagain et al. (2017) reported
77 the production of 37 ng/g (fresh weight) of active vitamin B12 by *P. freudenreichii* in
78 a sterilized barley-based matrix (33% w/v) after a 7-day fermentation period.
79 However, whether *P. freudenreichii* can produce physiologically significant amounts
80 of vitamin B12 in unsterilized cereal matrices with autochthonous microbiota is still
81 unknown. Moreover, to the best of our knowledge, there has been no published study
82 on the *in situ* fortification of vitamin B12 in wheat matrices.

83 The aim of this work was to study the influence of three types of wheat raw
84 materials (durum wheat flour, wholewheat flour, and wheat bran) on the *in situ*
85 production of vitamin B12 by *P. freudenreichii.* Vitamin B12 enriched cereal raw

material could be used for several plant based foods such as bread or extruded products. The acidification properties, microbial growth, and amounts of glucose and riboflavin, a precursor of DMBI, were monitored to follow the metabolism of *P. freudenreichii* during fermentation.

2. Materials and methods

2.1 Raw materials

Durum (*Triticum durum*) flour, obtained from Myllyn Paras (Hyvinkää, Finland), contained 14 g protein, 69 g carbohydrates (4 g fibers), 0.7 g ash and 2 g lipids per 100 g. Wholewheat (*Triticum aestivum*) flour was obtained from Helsinki Mylly Oy (Helsinki, Finland), and it contained 12 g protein, 72 g carbohydrates (12 g fibers), 2.1 g ash and 3 g lipids per 100 g. Wheat (*T. aestivum*) bran, supplied by Lantmännen Cerealia AB (Malmö, Sweden), contained 14 g protein, 65 g carbohydrates (54 g fibers), 7.1 g ash and 6 g lipids per 100 g.

2.2 Culture preparation

P. freudenreichii subsp. *freudenreichii* DSM 20271 that was cryopreserved (–70°C) in 15% glycerol was propagated anaerobically for 4 days on a yeast extract lactate (YEL) agar (Malik et al., 1968). Three individual colonies were transferred into 30 ml of YEL medium as three biological replicates and incubated at 30°C. After 72 h, cultures were recovered by centrifugation (3,200 × *g*, 10 min), resuspended in sterile water, and subsequently used for the inoculation of the doughs.

2.3 Dough preparation and fermentation

Three dough types were prepared by mixing MilliQ-water and flour according to

the recipe given in Table 1. The proportions of flours were set so that the visually estimated viscosity in doughs was similar. Doughs (30 g) were weighed in Falcon tubes (50 ml), and triplicate tubes were prepared for each time point (days 0, 1, 3, and 7).

The doughs were inoculated with propagated cultures of *P. freudenreichii*, so that the cell count was approximately 9.0 log colony forming units (CFU)/g of dough. Since cobalt content in white flour is low in comparison to wholegrain or wheat bran (Ekholm *et al.,* 2007) and availability of cobalt is a potential limiting factor during *in situ* production of vitamin B12, a durum dough enriched with 0.6 µg/g dry weight (dw) of cobalt(II) chloride (Sigma-Aldrich, Steinheim, Germany) was also prepared. In addition, control doughs of each dough type without inoculation were included in the study. The tubes were incubated at 25°C in shaking conditions (200 rpm; Certomat) for up to 7 days. The tubes for day 7 were aseptically opened once on day 3 to allow air in and then closed because oxygen is required for the biosynthesis of DMBI (Deptula et al., 2015). At each time point (days 0, 1, 3, and 7), an aliquot of the samples was immediately taken out for the determination of viable cell counts and acidification properties. The rest of the samples were stored (–20°C) for vitamin B12, total titratable acidity (TTA), riboflavin, glucose and acids analyses.

2.4 Microbial counts

Microbial counts were determined according to common plate count techniques and using different substrates for monitoring of different microbial groups as described by Coda et al. (2014) and Malik et al. (1968) with some modifications.

Serial dilutions of doughs (10 g) were carried out in sterile saline solution (8.5 g/L of NaCl) and appropriate dilutions were plated on the appropriate plate. *P. freudenreichii* were determined on YEL plates incubated anaerobically (Anaerogen, Oxoid, Basingstoke, UK) for 4 days at 30°C and aerobically for at least 1 day, which leads the *P. freudenreichii* colonies to turn brownish and distinguishable from colonies of other bacteria. Presumptive LAB were enumerated on de Man, Rogosa and Sharpe (MRS) agar (Lab M, Lancashire, UK) at 30°C for 48 h. Total aerobic bacteria were counted on PCA agar (Lab M) incubated at 30°C for 48 h, and yeasts were cultivated at 30°C for 48 h on YM agar composed of 3 g/l malt extract, 3 g/l yeast extract, 5 g/l peptone, 10 g/l dextrose, and 0.01% chloramphenicol (Oxoid, Basingstoke, UK) to prevent bacterial growth. The cell counts of *Enterobacteriaceae* were determined on VRBGA Agar (Lab M) after incubation at 37°C for 48 h. The results were expressed in CFU/g of dough.

2.5 Acidification properties

The pH values were determined using a pH meter (Portamess 752 Calimatic, Knick, Berlin, Germany). TTA was determined with a Mettler Toledo EasyPlus Titrator (Schott, Germany). Ten grams of dough mixed in 90 ml of distilled water were titrated against 0.1 M NaOH to a final pH of 8.5. TTA was expressed as the volume of 0.1 M NaOH used (ml).

2.6 Determination of α-amylase, glucose and acids

The activity of α-amylase in each flour was determined using an α-amylase kit (Ceralpha, Megazyme Co., Ltd., Wicklow, Ireland), and the results were expressed in Ceralpha units on a dry matter basis (Table 1). One unit of activity is defined as the amount of enzyme required to release 1 μmol of glucose reducing-sugar equivalents in 1 minute under defined conditions.

Doughs (1 g) were diluted 1:10 (w/v) in water and centrifuged (13,000 × *g*, 10 min). The supernatants were filtered (0.45 μm, Pall, USA) into vials. Glucose, lactic acid (LA), acetic acid (AA), and propionic acid (PA) were determined using a high-performance liquid chromatography (HPLC) method described in Chamlagain et al. (2016), with following modifications. The analysis was performed on a Hi-Plex H column (Agilent, CA, USA; 300 × 6.5 mm), with a Hi-Plex H guard column (Agilent, CA, USA; 50 × 7.7 mm). The HPLC system was equipped with a Waters 515 pump, autosampler, ultraviolet (UV) detector (Waters 717), and refractive index detector (HP 1047A, HP, USA). The mobile phase was 10 mM H_2SO_4 and the flow rate was set at 0.6 ml/min with the column temperature maintained at 65°C.

2.7 Determination of riboflavin

Content of riboflavin in doughs was determined with an ultra-HPLC (UHPLC) method according to Chamlagain et al. (2016). In brief, doughs (1–2 g) were mixed with 15 ml of 0.1 M hydrochloric acid and subjected to a boiling water extraction for 60 min. After cooling on ice, the pH of the extract was adjusted to 4.5 with 2.5 M sodium acetate. Then, the extract was incubated (at 37°C) with Taka-Diastase (50 mg; Pfaltz and Bauer, CT, USA) and β-amylase (5 mg; Sigma-Aldrich) for 24 h. The

extract was filtered (0.2 µm, Pall, USA) and analyzed on a Waters UPLC system with an Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm) and a Waters fluorescence detector using aqueous methanol (30% v/v) containing 20 mM ammonium acetate as an eluent (0.2 ml/min).

2.8 Determination of vitamin B12

Vitamin B12 was determined as cyanocobalamin in dough samples after extraction and purification as presented by Chamlagain, Edelmann, Kariluoto, Ollilainen and Piironen (2015) with minor modifications. In addition, the presence of other corrinoids, especially pseudovitamin B12, was followed in chromatograms based on the knowledge of their retention times and absorption spectra obtained in our previous studies (Chamlagain et al., 2015; Chamlagain et al., 2017; Deptula et al., 2017a; Deptula et al., 2015). Approximately 3 g of fermented doughs were mixed with 15 ml of extraction buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid, pH 4.5) containing 100 µl of sodium cyanide (1% w/v in water) and extracted in boiling water for 30 min. After cooling, 300 µl of α-amylase (50 mg/ml; St Louis, MO, USA) was added, and the samples were incubated in a water bath (30 min, 37°C) 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. After combining the supernatants, the final volume was adjusted to 25 ml with the extraction buffer.

After filtration (0.2 µm, Pall, USA), 10 ml of the extracts was purified through an immunoaffinity column (Easi-Extract; R-Biopharma; Glasgow, Scotland) and

194 analyzed with a Waters UHPLC system (Milford, MA, USA) equipped with a
195 photodiode array detector (at 361 nm) and an Acquity HSS T3 C18 column (2.1 × 100
196 mm, 1.8 μm). The mobile phase was a gradient flow of acetonitrile and MilliQ water
197 with 0.025% trifluoroacetic acid (0.32 ml/min) as explained by Chamlagain et al.
198 (2015). Each sample was injected twice (15 μl).

199 **2.9 Reporting of results and statistical analysis**

200 Contents of glucose, acids, riboflavin, and vitamin B12 were reported on a dry
201 matter basis. Statistical analysis was performed using SPSS 21.0 for Windows (IBM
202 Corporation, NY, USA). One-way analysis of variance (ANOVA) and Tukey's *post*
203 *hoc* test were employed to determine significant differences among the samples. The
204 results were calculated based on three replicates, and the level of statistical
205 significance was defined at a p-value < 0.05.

206 **3. Results**

207 **3.1 Acidification and microbial counts**

208 Before fermentation, the pH value in the doughs ranged from 6.24 to 6.72 (Fig.
209 1A). In the first 3 days, the pH in all doughs decreased sharply. Generally, the pH in
210 the inoculated doughs were higher than those in the control doughs. On day 1, the pH
211 of the wholewheat flour dough was the highest (5.86 ± 0.02), while it was the lowest
212 in the durum flour control dough (4.63 ± 0.06). On day 3, only in wheat bran doughs
213 the pH was higher than 4.0. At the end of the fermentation, the pH of all doughs was
214 lower than 4.0; the lowest value of 3.5 was reached in the durum doughs.

215 The initial TTAs in durum flour, wholewheat flour, and wheat bran doughs were

1.5 ± 0.2 ml, 2.7 ± 0.2 ml, and 2.9 ± 0.5 ml, respectively (Fig. 1B). During the fermentation, the TTA increased, and the inoculated doughs had higher TTA levels than the corresponding control doughs. The wheat bran dough had the highest TTA levels, which reached 24.8 ± 0.2 ml in the inoculated dough and 23.4 ± 0.2 ml in the control dough on day 7. In contrast, the durum flour control dough had the lowest TTA (17.3 ± 0.1 ml) on day 7.

At the beginning of fermentation, the number of *P. freudenreichii* colony forming units in the inoculated doughs ranged from 8.7 log CFU/g to 9.0 log CFU/g (Table 2). After one day of fermentation the cell number of *P. freudenreichii* reached 9.6 ± 0.1 log and 9.4 ± 0.1 log CFU/g in durum flour dough and in wheat bran dough, respectively, and remained approximately at the level until the end of fermentation (day 7). In the wholewheat flour dough, the number of *P. freudenreichii* CFU remained stable during the fermentation. The low initial number of LAB (< 3 log CFU/g) increased to 8.6 ± 0.1 log CFU/g in wholewheat flour doughs and 9.2 log CFU/g in other doughs after one day of fermentation. Similar LAB cell numbers were counted in doughs with and without *P. freudenreichii*. In doughs of durum flour and wheat bran the cell number of LAB remained stable after day 1. In wholewheat flour dough with *P. freudenreichii*, the cell density of LAB further increased to 9.0 ± 0.1 log CFU/g on day 3 and remained stable after that.

The inoculation of *P. freudenreichii* had no influence on the cell numbers of total aerobic bacteria, total *Enterobacteriaceae*, or yeasts. The cell density of the total aerobic bacteria in all doughs increased from approximately 4 log CFU/g to 9 log

CFU/g on the first day of fermentation and remained stable after that. The cell density of total *Enterobacteriaceae* ranged from 4.5 ± 0.1 log CFU/g to 4.9 ± 0.1 log CFU/g in all doughs before fermentation, and further, it grew 100-fold from days 0 to 1. After day 1, the cell count of total *Enterobacteriaceae* decreased in all doughs, and *Enterobacteriaceae* were not found on day 7. The initial cell density of yeasts was approximately 3.8 log CFU/g in durum flour dough and less than 2 log CFU/g in the other doughs. The cell count of yeasts in all doughs increased to approximately 5.0 on day 1, but decreased after that and became undetectable on day 7.

3.2 Glucose, α-amylase activity and organic acids

The α-amylase activity in durum flour, wholewheat flour and wheat bran were 0.12 units/g, 0.19 units/g and 0.15 units/g, respectively.

On day 0, the glucose contents in durum flour, wholewheat flour, and wheat bran doughs were 8.5 ± 0.4 mg/g, 18.4 ± 1.2 mg/g, and 26.8 ± 1.1 mg/g dw, respectively (Fig. 2A). In the bran doughs, the glucose concentrations decreased during the first 24 h and almost totally depleted by day 3. In contrast, the glucose level in the other doughs increased considerably during fermentation. The wholewheat flour doughs had a higher glucose concentration than the durum flour doughs. After fermentation, 67.7 ± 3.1 mg/g and 63.7 ± 3.5 mg/g dw of glucose were found in wholewheat inoculated dough and wholewheat control dough, respectively.

Before the fermentation, no detectable levels of organic acids were found in any of the doughs (Fig. 2B–D). On day 1, 11.2 ± 1.2 mg/g and 10.3 ± 0.3 mg/g dw of LA were already measured in the durum and wholewheat flour control doughs,

respectively. Notably, in the matching doughs inoculated with *P. freudenreichii*, the LA level was < 0.5 mg/g dw. After day 1, the LA contents in these doughs increased, ultimately reaching 62.5 ± 3.7 mg/g dw in durum flour and 62.9 ± 6.8 mg/g dw in wholewheat flour, respectively. In the wheat bran doughs, the amounts of LA were 14.7 ± 1.7 mg/g and 23.8 ± 5.5 mg/g dw after 1 day, and on day 7, the levels reached 59.5 ± 1.2 mg/g and 78.3 ± 9.4 mg/g dw in inoculated dough and control dough, respectively.

In the inoculated doughs, PA was detected on day 1 and increased to 8.3 ± 1.1 mg/g and 9.3 ± 0.9 mg/g dw in durum flour and wheat bran, respectively, by the end of fermentation. In the inoculated wholewheat flour dough, PA was not detected on day 1, but a level of 1.3 ± 0.2 mg/g dw was measured on day 7. AA was detected from day 1 and further increased during the fermentation. Wheat bran doughs had the highest concentrations of AA during fermentation, reaching up to 16.4 ± 1.8 mg/g dw in the inoculated dough and 7.0 ± 1.6 mg/g dw in the control dough on day 7.

3.3 Riboflavin

At the start of fermentation, the amounts of riboflavin in the doughs of durum flour and wholewheat flour were 0.18 ± 0.03 μg/g dw and 0.45 ± 0.05 μg/g dw, respectively (Fig. 3). After 7-day fermentation, riboflavin concentration increased to 0.42 ± 0.02 μg/g dw in durum flour dough and 0.73 ± 0.03 μg/g dw in wholewheat flour dough. In contrast, the riboflavin in the wheat bran dough was 1.93 ± 0.11 μg/g dw at the beginning and decreased to 1.78 ± 0.07 μg/g dw on day 7.

3.4 Production of vitamin B12

The UHPLC chromatograms showed that all the vitamin B12 detected in this study was active B12, and no detectable level of pseudovitamin B12 was observed. In the control doughs, no vitamin B12 was detected during fermentation (Table 3). In the inoculated doughs, 17 ± 1 ng/g to 47 ± 6 ng/g dw of vitamin B12 was found on day 0, contributed by the *P. freudenreichii* inoculum. By day 1, the vitamin B12 contents significantly increased ($p < 0.05$) in the doughs inoculated with *P. freudenreichii*. From day 1 to day 7, the vitamin B12 content in durum flour inoculated dough did not increase, with a final level of 33 ± 4 ng/g dw. In inoculated durum flour dough with cobalt chloride (0.6 μg/g dw) supplementation, 5.2-fold vitamin B12 (203 ± 24 ng/g dw) was produced by the end of the 7-day fermentation period. On day 3, vitamin B12 concentration in the inoculated wholewheat flour dough was significantly ($p < 0.05$) higher than in the inoculated durum flour dough, but without a significant ($p > 0.05$) increase afterwards (87 ± 10 ng/g dw on day 7). In inoculated wheat bran dough, the concentration of vitamin B12 significantly ($p < 0.05$) increased from days 3 to 7 and reached 155 ± 17 ng/g dw at the end of fermentation.

4. Discussion

P. freudenreichii DSM 20271 was used as a starter in wheat flours and bran for a 7-day fermentation period to study the possibility of *in situ* fortification of vitamin B12. The non-sterile cereal materials used in this study had various endogenous microorganisms and enzymes activated during fermentation, which is required to obtain full technological functionality of wheat-based raw materials but also leads to rapid acidification by lactic acid bacteria. The differences in nutritional composition,

304 endogenous microflora and enzyme activity among the three materials have led to
305 significantly different glucose content, microbiota, acidity, and vitamin B12 content
306 during fermentation.

307 The increase of glucose content in durum flour and wholewheat flour doughs in
308 the first 3 days was likely due to starch hydrolysis by endogenous amylases. Activities
309 of α-amylase in three matrices was measured to explain the difference of glucose
310 content among them during fermentation. A higher content of glucose was found in
311 wholewheat flour doughs than in durum flour doughs, which was not surprising given
312 its higher amylase activity (0.19 units/g vs. 0.12 units/g). However, the amylase
313 activities likely decreased in the later part of fermentation due to the drop in pH, since
314 the optimal pH of cereal amylases is in the range of 4.5–5.5 (Muralikrishna and
315 Nirmala, 2005). With a lower initial level of available carbohydrates (11 g/100g)
316 compared to the content in the other flours (60–65 g/100g), the glucose content in
317 wheat bran dough was already depleted by day 3.

318 Heterofermentative species of LAB play a central role in spontaneous
319 fermentation and dominate the microbial ecology of sourdough (De Vuyst and
320 Neysens, 2005). In the present study, a low level of endogenous LAB was detected
321 before fermentation; they already dominated after day 1 and caused intensive
322 acidification, resulting in the inhibition of yeast, total aerobic bacteria, and total
323 *Enterobacteriaceae* growth (Table 2). LAB are able to utilize a wide range of
324 substrates as carbon sources (Juturu and Wu, 2015). The LA content in wheat bran
325 still increased from days 3 to 7 in wheat bran doughs even though glucose was no

326 longer detected.

327 Considering that *P. freudenreichii* exhibits a slow growth rate (Falentin et al.,
328 2010), an inoculation level of approximately 9.0 log CFU/g of *P. freudenreichii* DSM
329 20271 was used in this study. It has been suggested that growth of LAB could be
330 stimulated by propionic acid bacteria due to the consumption of LA and the
331 stimulatory effect of fatty acids produced by propionic acid bacteria (Smid and
332 Lacroix, 2013). In this study, a stimulatory effect of *P. freudenreichii* on LAB growth
333 was not observed in durum and wholewheat flour doughs. The stimulation of LAB
334 growth by *P. freudenreichii*, however, could be observed in the inoculated
335 wholewheat flour dough where the viable cell count of LAB was higher than in the
336 corresponding control dough on day 3 (*ca.* 9.0 log CFU/g and 8.5 log CFU/g,
337 respectively). Influence of the *P. freudenreichii* starter on the growth of other
338 microbial groups was not observed.

339 *P. freudenreichii* grew to higher cell densities in durum flour dough compared to
340 the other doughs in the first day of fermentation. However, the cell densities did not
341 increase from day 1 to 7, possibly due to the unfavorable pH for *P. freudenreichii*
342 (Deptula et al., 2017b). In the wholewheat flour dough, *P. freudenreichii* did not grow
343 despite a sufficient carbon source and favorable pH on day 1. In the wheat bran
344 dough, *P. freudenreichii* grew to lower cell density than in durum flour dough. No
345 growth of *P. freudenreichii* in wholewheat and growth to lower cell densities in wheat
346 bran doughs than in durum flour dough may be due to the presence of some *P.*
347 *freudenreichii* inhibiting components or endogenous microorganisms in the wheat

348 bran layer. For instance, it has been shown that flavonoids in buckwheat bran had
349 antibacterial activities against *Propionibacterium (Cutibacterium) acnes* (Wang et al.,
350 2013). However, to the best of our knowledge, there is no published information
351 available about *P. freudenreichii* inhibiting factors from wheat.

352 *P. freudenreichii* metabolizes LA as the preferred carbon source in cheese
353 fermentation to produce PA and AA as the main metabolites (Lee et al., 1974). In this
354 study, LA contents were lower in inoculated doughs than they were in corresponding
355 control doughs, indicating that *P. freudenreichii* consumed LA produced during the
356 fermentation. Since in control doughs PA amounts remained below detection limit, *P.*
357 *freudenreichii* starter is the likely source of PA in inoculated doughs. In this study, the
358 production of PA did not increase after day 3, which was likely due to the formation
359 of an acidic environment (pH < 4). In contrast, the AA contents still increased from
360 day 3 to 7 in all inoculated dough types, potentially produced by both *P.*
361 *freudenreichii* and LAB.

362 No vitamin B12 was detected in the control doughs, showing that vitamin B12
363 was only synthesized by inoculated *P. freudenreichii.* In an optimized medium
364 supplemented with cobalt and DMBI, vitamin B12 production depended on cell yield
365 (Hugenschmidt et al., 2011). In the present study, the lowest production of vitamin
366 B12 and the highest cell yield of *P. freudenreichii* was observed in the durum flour
367 dough. In contrast, a more than 2-fold level of production of vitamin B12 was evident
368 in wholewheat flour doughs, while no increase in the cell number of *P. freudenreichii*
369 was observed. In wheat bran doughs, about a 5-fold level of vitamin B12 content was

370 detected, with lower growth compared to those in durum flour dough. These results
371 can partly be explained by the higher content of cobalt in wheat bran: *ca.* 0.1 µg/g dw
372 compared to white flour *ca.*< 0.01 µg/g dw (Ekholm et al., 2007), since cobalt is a
373 limiting factor for vitamin B12 production by *P. freudenreichii* during fermentation
374 (Berry and Bullerman, 1966; Hugenschmidt et al., 2011). Notably, in durum flour
375 dough with added cobalt (0.6 µg/g, dw), more than 200 ng/g dw of vitamin B12 (vs.
376 *ca.* 33 ng/g dw without cobalt) was produced by *P. freudenreichii*.

377 In this study, the vitamin B12 produced during fermentation was composed of the
378 active form, with DMBI as a lower ligand (data not shown). Given that no
379 supplements were added, the DMBI in the biosynthesized vitamin was likely derived
380 from *de novo* biosynthesis by *P. freudenreichii.* Riboflavin has been demonstrated to
381 be the precursor for the *de novo* biosynthesis of DMBI in the presence of oxygen in *P.*
382 *freudenreichii* (Hollriegl et al., 1982). Furthermore, riboflavin could be used together
383 with nicotinamide to enhance production of vitamin B12 with some *P. freudenreichii*
384 strains and a significant positive correlation was observed when comparing riboflavin
385 consumption with B12 production (Chamlagain et al., 2016). The higher riboflavin
386 content in wheat bran (*ca.* 1.93 µg/g dw) and wholewheat flour (*ca.* 0.45 µg/g dw)
387 than in durum flour (*ca.* 0.18 µg/g dw) may partly explain the higher levels of vitamin
388 B12 produced in these matrices. In addition, riboflavin can be synthesized by *P.*
389 *freudenreichii* and certain LAB, such as *Lactobacillus plantarum*, *Lactobacillus*
390 *lactis*, *Lactobacillus fermentum* and *Leuconostoc mesenteroides*, commonly retrieved
391 from flour and sourdough microflora (Burgess et al., 2009; Capozzi et al., 2011;

392 Russo et al., 2014). Moreover, some strains of LAB isolated from wheat sourdough
393 were used to enhance the riboflavin content of sourdough and pasta (Capozzi et al.,
394 2011; Russo et al., 2014). In our study, content of riboflavin significantly increased
395 during fermentation in doughs of durum flour and wholewheat flour but not in wheat
396 bran dough (Figure 3). This difference may be due to a distinct microbiota of the
397 flours and bran or riboflavin synthesized by the inoculated *P. freudenreichii* in each
398 dough types. Furthermore, whether riboflavin is a factor for enhancing the production
399 of vitamin B12 in non-sterile wheat materials should be confirmed in future studies.
400 Bran is the main byproduct of the milling process, which is underutilized for food
401 purposes due to its negative effect on the rheological or sensory quality of products
402 (Prückler et al., 2014). However, the use of bran in food products has been increasing
403 recently due to its high levels of dietary fiber, good quality proteins, and many other
404 beneficial substances. Furthermore, some promising bioprocessing techniques have
405 been proposed to improve the technological and nutritional quality of bran (Coda et
406 al., 2015). In the present study, bran and bran-containing flour were confirmed as
407 potential substrates for plant-based vitamin B12 fortification. These fermented
408 vitamin B12-containing matrices could be used, e.g., in bread baking or in other food
409 products. According to a recent study (Edelmann et al., 2016), *in situ* synthesized
410 vitamin B12 was as stable as cyanocobalamin, the most stable form of vitamin B12, in
411 baking processes. No significant loss was observed in straight/sponge-dough
412 processes, whereas a loss of only 23% was reported in sourdough baking (Edelmann
413 et al., 2016). In this study, around 150 ng/g dw of vitamin B12 was synthesized in

fermented wheat bran dough by *P. freudenreichii*. If in straight-dough baking, 20% of wheat flour was replaced with B12-rich wheat bran, four slices of bread (120 g) would contain the recommended daily intake of vitamin B12 (2.4 μg). Furthermore, process optimization measures, such as adjusting the pH and adding ingredients with higher amounts of cobalt (e.g., yeast extract and buckwheat leaf flour; (Grembecka and Szefer, 2006), could be studied to enhance the production of vitamin B12 in wheat materials with *P. freudenreichii*.

5. Conclusion

In previous studies, vitamin B12 production in sterilized cereal matrices has been reported. However, this study demonstrated that nutritionally significant amounts of vitamin B12 can be produced in non-sterile wheat doughs by *P. freudenreichii*. Different wheat ingredients are thus promising option for *in situ* fortification of plant-based food with vitamin B12. The higher vitamin B12 production in bran and wholewheat flour suggests that outer layers of wheat grain might contain more potential precursors and other factors for vitamin B12 synthesis than white wheat flour. However, a safe use of these wheat matrices for B12 production requires controlling endogenous microbiota with appropriate co-culture fermentation.

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570 Table 1 Ingredients and yields of doughs.

Sample code	Substrate	Flour (g)	Water (g)	Dough yield[c]	Starter
DF_P[a]	Durum flour	9	21	333	*P. freudenreichii*
DF_P+Co[b]	Durum flour	9	21	333	*P. freudenreichii*
DF_C	Durum flour	9	21	333	-
WF_P	Wholewheat flour	9	21	333	*P. freudenreichii*
WF_C	Wholewheat flour	9	21	333	-
WB_P	Wheat bran	6	24	500	*P. freudenreichii*
WB_C	Wheat bran	6	24	500	-

571 [a] P means doughs inoculated with *Propionibacterium freudenreichii*; C means control
572 doughs.
573 [b] DF_P +Co= durum flour dough with addition of *P. freudenreichii* and 0.6 µg/g dw
574 of cobalt chloride.
575 [c] Dough yield is represented as the amount of dough prepared from 100 parts of flour.
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583 Table 2. Microbial counts of Propionibacteria (PAB) on YEL plate, presumptive
584 lactic acid bacteria (LAB) on MRS plate, total aerobic bacteria on PCA plate, total
585 *Enterobacteriaceae* on VRBGA plate and yeasts on YM plate (log cfu/g). The results
586 are expressed as the mean ± standard deviation (n=3).

	Day 0		Day 1		Day 3		Day 7	
	Inoculated*	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
PAB								
DF	8.7±0.0 ^{a}	<2.0	9.6±0.1 ^{c}	<2.0	9.6±0.1 ^{c}	<2.0	9.5±0.1 ^{c}	<2.0
WF	8.7±0.1 ^{a}	<2.0	8.7±0.1 ^{a}	<2.0	8.7±0.0 ^{a}	<2.0	8.6±0.1 ^{a}	<2.0
WB	9.0±0.0 ^{b}	<2.0	9.4±0.1 ^{b}	<2.0	9.2±0.1 ^{b}	<2.0	9.2±0.1 ^{b}	<2.0
Presumptive LAB								
DF	<3.0	<3.0	9.2±0.0 ^{b}	9.2±0.0^{b}	8.8±0.0^{ab}	8.7±0.1^{ab}	8.4±0.1 ^{a}	8.5±0.2 ^{a}
WF	<3.0	<3.0	8.6±0.1 ^{a}	8.6±0.2^{a}	9.0±0.1 ^{b}	8.5±0.1 ^{a}	9.0±0.1 ^{b}	8.7±0.2^{ab}
WB	<3.0	<3.0	9.2±0.1 ^{b}	9.3±0.1^{b}	9.2±0.1 ^{c}	9.1±0.1^{bc}	9.0±0.1 ^{b}	9.0±0.1 ^{b}
Total aerobic bacteria								
DF	3.6±0.1 ^{b}	3.6±0.1^{b}	9.2±0.1 ^{b}	9.2±0.1^{b}	8.9±0.2^{ab}	9.1±0.1 ^{b}	8.5±0.1 ^{a}	8.3±0.1 ^{a}
WF	3.3±0.1^{a}	3.3±0.1^{a}	8.8±0.1 ^{a}	8.8±0.1^{a}	8.9±0.1^{ab}	8.6±0.3 ^{a}	9.0±0.1 ^{b}	8.8±0.2 ^{b}
WB	3.4±0.1 ^{a}	3.4±0.1^{a}	9.3±0.1 ^{b}	9.2±0.1^{b}	9.1±0.1 ^{b}	9.2±0.1 ^{b}	9.0±0.1 ^{b}	8.8±0.1 ^{b}
Total *Enterobacteriaceae*								
DF	4.9±0.0 ^{b}	4.9±0.0^{b}	6.8±0.1^{ab}	6.6±0.1^{a}	<2.0	<2.0	nd**	nd
WF	4.5±0.1 ^{a}	4.5±0.1^{a}	7.3±0.1 ^{c}	7.4±0.1^{c}	5.3±0.1 ^{b}	5.2±0.2 ^{b}	nd	nd
WB	4.5±0.1 ^{a}	4.5±0.1^{a}	6.9±0.0 ^{b}	6.8±0.1^{ab}	4.1±0.1 ^{a}	4.1±0.0 ^{a}	nd	nd
Yeasts								
DF	3.8±0.0	3.8±0.0	4.3±0.1 ^{a}	4.3±0.0^{a}	<2.0	<2.0	nd	nd
WF	<2.0	<2.0	4.8±0.1 ^{b}	4.9±0.2^{b}	<2.0	<2.0	nd	nd
WB	<2.0	<2.0	5.3±0.0 ^{c}	5.3±0.1^{c}	<2.0	<2.0	nd	nd

587 * "Inoculated" denotes doughs inoculated with *Propionibacterium freudenreichii*;
588 "Control" refers to doughs without inoculation.
589 ** nd = not detected.
590 DF = durum flour; WF = wholewheat flour; WB = wheat bran.
591 Values from the same day and microbial group bearing different superscripts (a–c) are
592 significantly different ($p < 0.05$).

593 Table 3. Vitamin B12 concentration (ng/g, dw) during fermentation. The results are

594 expressed as the mean ± standard deviation (n=3).

Time(d)	0	1	3	7
Inoculated*				
DF	19±2^{aw}	37±1 ^{ax}	40±3^{ax}	33±4 ^{ax}
DF+Co	17±1^{aw}	-**	-	203±24^{dx}
WF	17±2^{aw}	34±3 ^{ax}	78±8 ^{by}	87±10 ^{by}
WB	47±6^{bw}	81±8 ^{bx}	114±5 ^{cy}	155±17^{cz}
Control				
DF	nd***	nd	nd	nd
WF	nd	nd	nd	nd
WB	nd	nd	nd	nd

595 * "Inoculated" denotes doughs inoculated with *Propionibacterium freudenreichii*;

596 "Control" refers to doughs without inoculation.

597 ** Not studied.

598 *** nd = not detected.

599 DF = Durum flour; DF + Co = durum flour dough with addition of 0.6 µg/g dw cobalt

600 chloride; dw = dry weight; WF = wholewheat flour; WB = wheat bran.

601 Values in the same row (w–z) and same column (a–d) bearing different superscripts

602 are significantly different ($p < 0.05$).

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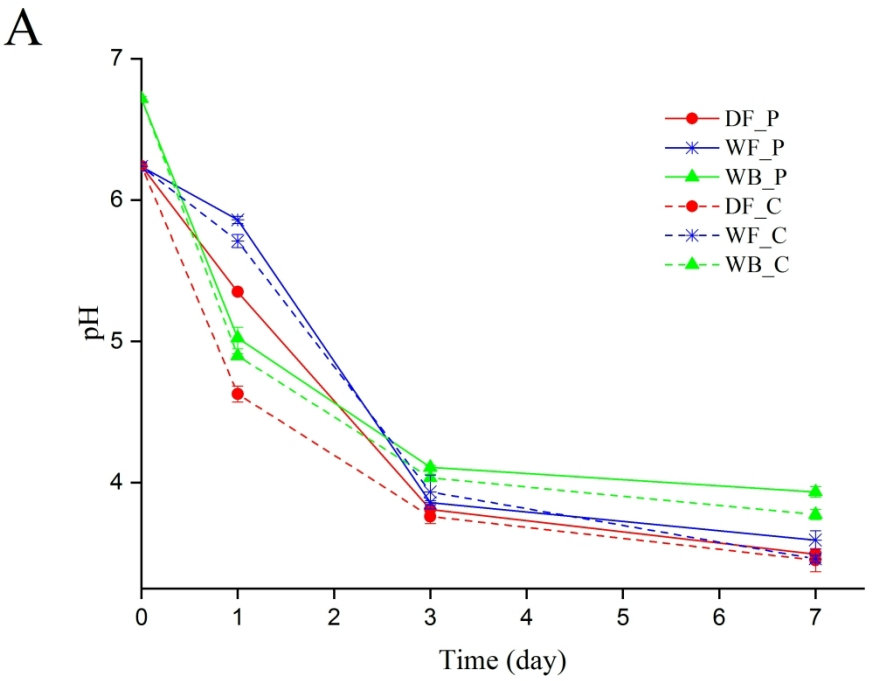
Figure legends

Fig. 1. Changes in pH value (A) and total titratable acidity (TTA) (B) during fermentation. Values are the means and standard deviations of three replicates. DF_P = durum flour dough with *Propionibacterium freudenreichii*; WF_P = wholewheat flour dough with *P. freudenreichii*; WB_P = wheat bran dough with *P. freudenreichii*. DF_C= durum flour control dough; WF_C = wholewheat flour control dough; WB_C = wheat bran control dough.

Fig. 2. Concentration (mg/g, dry matter) of glucose (A), lactic acid (B), propionic acid (C) and acetic acid (D) during fermentation. Values are means and standard deviations of 3 replicates. DF_P = durum flour dough with *P. freudenreichii*; WF_P = wholewheat flour dough with *P. freudenreichii*; WB_P = wheat bran dough with *P. freudenreichii*. DF_C = durum flour control dough; WF_C = wholewheat flour control dough; WB_C = wheat bran control dough.

Fig. 3 Concentration (µg/g, dry matter) of riboflavin in inoculated doughs at day 0 and day 7. Values are means and the error bar represents the range of the values from two biological replicates.

24 **Fig. 1.**



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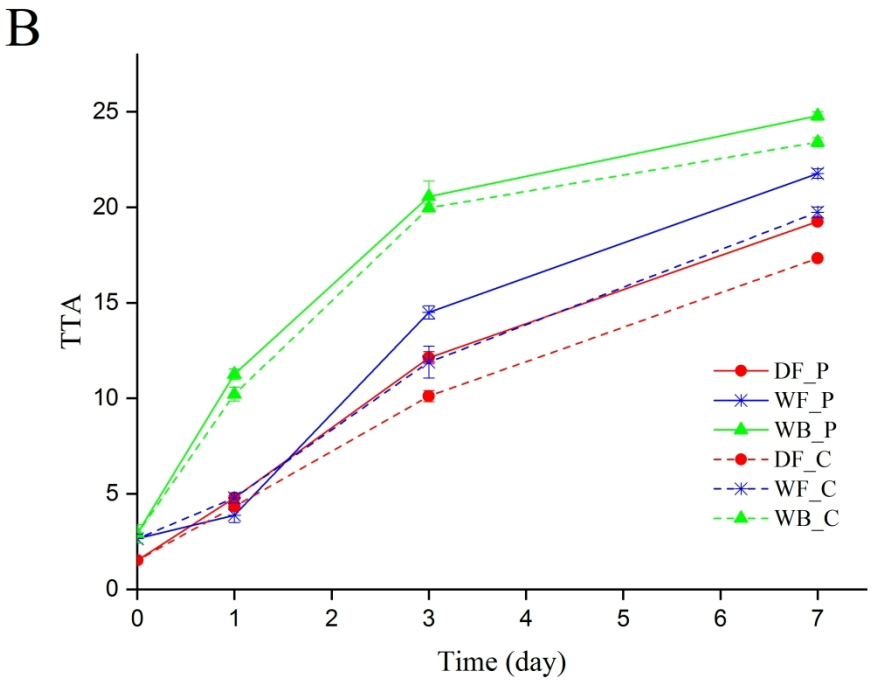

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Fig. 2.

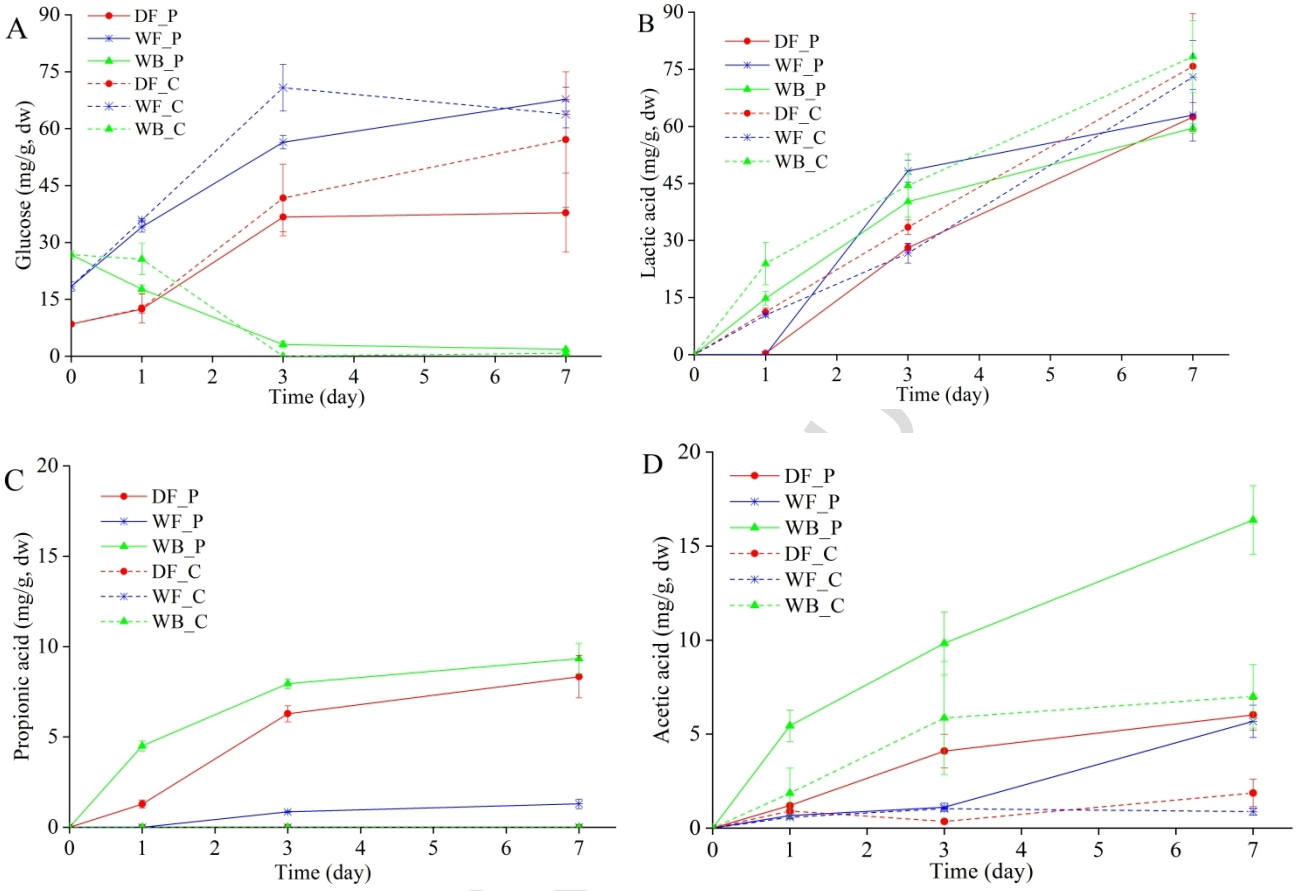
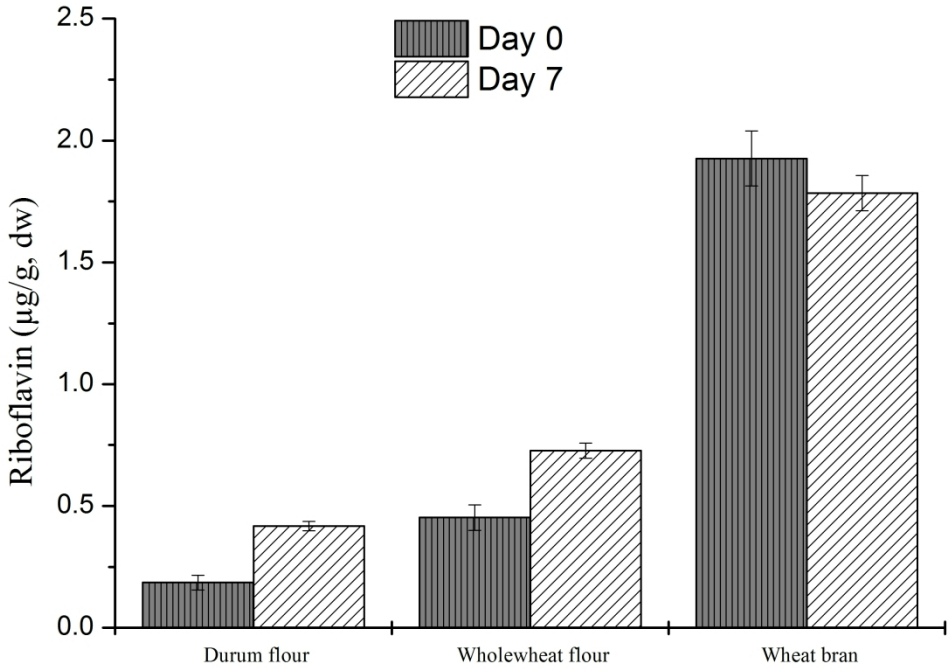


Fig. 3.



Highlights:

- Non-sterile wheat matrices maybe used for *in situ* production of vitamin B12.
- *P. freudenreichii* produced physiologically relevant amounts of vitamin B12.
- Wheat bran allowed higher production of vitamin B12 than other flours.
- Bran layer possibly contains precursors for vitamin B12 biosynthesis.