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1 **Effect of the lower ligand precursors on vitamin B12 production by food-**

2 **grade propionibacteria**

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28 **ABSTRACT**

29 *Propionibacterium freudenreichii* is the only generally recognized safe (GRAS)
30 bacterium known to synthesize active vitamin B12 and offers previously untapped
31 potential for naturally fortifying foods with vitamin B12. Biosynthesis of the
32 lower ligand 5,6-dimethylbenizimidazole (DMBI) is often a key limiting factor in
33 the production of active vitamin B12 in Propionibacteria. Here, we studied the
34 effect of the natural food-grade precursors of DMBI [riboflavin (RF) and
35 nicotinamide (NAM)] on vitamin B12 production by 27 *P. freudenreichii* and 3
36 *Propionibacterium acidipropionici* strains in whey-based medium. We employed
37 sensitive and selective UHPLC and LC−MS/MS to confirm and quantify the
38 synthesized vitamin B12. In 12 *P. freudenreichii* strains, co-supplementation with
39 RF (40 µM) and NAM (27 mM) increased the volumetric yield of vitamin B12 up
40 to 4-fold compared to the control cultures. For the majority of these strains, the
41 production level with RF and NAM exceeded the yield obtained with DMBI
42 supplementation (100 µM). The significant positive correlation between RF
43 consumption and vitamin B12 production suggests that RF is proportionally
44 directed towards vitamin B12 biosynthesis. This study shows that the availability
45 of RF and NAM enhances the production of active vitamin B12 by *P.*
46 *freudenreichii* in a strain-dependent manner.

47

48 **Keywords**: Vitamin B12; *Propionibacterium freudenreichii*; fermentation;
49 riboflavin; nicotinamide

50

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52

53 **1 Introduction**

54 Propionibacteria are among the few bacteria and archaea that synthesize
55 vitamin B12 forms that are active for humans (hereafter called active B12)
56 (Martens, Barg, Warren, & Jahn, 2002). The use of *Propionibacterium*
57 *freudenreichii* in foods is currently limited to the manufacture of Swiss-type
58 cheeses primarily for the characteristic eyes and typical cheese flavour (Thierry et
59 al., 2011). As the only producer of active B12 that is safe for use in food (EFSA,
60 2009), this bacterium could be utilized for the *in situ* B12 fortification of foods
61 and food ingredients that lack or are deficient in vitamin B12 (Hugenholtz &
62 Smid, 2002). Genetically engineered strains of *P. freudenreichii* are used in the
63 commercial production of B12 in the pharmaceutical industry (Thierry et al.,
64 2011).

65 The lower α-ligand of B12 (5,6-dimethylbenzimidazole, DMBI) plays an
66 important role in the absorption of B12 in humans by enabling selective binding
67 of the vitamin to the intrinsic factor (a B12 transporter glycoprotein) (Alpers &
68 Russel-Jones, 1999; Andrews, Pratt, & Brown, 1991; Nielsen, Rasmussen,
69 Andersen, Nexø, & Moestrup, 2012). For example, B12 with DMBI as the lower
70 ligand has a 500-fold higher affinity for the intrinsic factor than pseudovitamin
71 B12, which has adenine as the lower ligand (Stupperich & Nexø, 1991). Several
72 microorganisms synthesize cobamides with other benzimidazoles, adenine or
73 phenols as the lower ligand (Watanabe, Yabuta, Tanioka, & Bito, 2013). These
74 compounds function as cofactors for the B12-dependent enzymes in these
75 organisms (Taga & Walker, 2008) but are not recognized by human intrinsic
76 factor (Stupperich & Nexø, 1991).

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77 *P. freudenreichii* primarily synthesizes active B12 forms with DMBI as the
78 lower ligand and adenosyl or methyl groups as the upper ligand
79 (adenosylcobalamin and methylcobalamin) but may also synthesize small
80 amounts of other cobamides, including pseudovitamin B12 (Quesada-Chanto et
81 al., 1998; Renz, 1999; Vorobjeva, 1999). Some bacteria (e.g., *Lactobacillus*
82 *reuteri*) exclusively synthesize pseudovitamin B12 even when grown with DMBI
83 supplementation (Crofts, Seth, Hazra, & Taga, 2013; Santos et al., 2007). In a
84 recent study, *P. freudenreichii* was shown to preferentially produce active B12 in
85 a process that was guided by the biosynthesis of DMBI (Deptula et al., 2015).
86 When the availability of DMBI was restricted and therefore no active vitamin
87 could be synthesised, *P. freudenreichii* mostly accumulated incomplete cobamide
88 and only a low level of pseudovitamin B12, even when supplemented with
89 adenine (Deptula et al., 2015). The clear preference for the incorporation of
90 DMBI as the lower ligand makes *P. freudenreichii* an ideal candidate for
91 exploitation in the fermentation fortification of foods with the active B12 vitamin.

92 The complete DMBI biosynthesis pathway in aerotolerant Propionibacteria
93 was unclear for a long time. Taga, Larsen, Howard-Jones, Walsh, & Walker (2007)
94 showed that the BluB enzyme from the soil bacterium *Sinorhizobium meliloti* was
95 responsible for DMBI synthesis from a reduced flavin mononucleotide in an
96 oxygenated environment. The first genome sequence of *P. freudenreichii* subsp.
97 *shermanii* (strain CIRM-BIA1) revealed the presence of a *bluB* homologue (the
98 fusion gene *bluB*/*cobT2*) (Falentin et al., 2010). Recently (Deptula et al., 2015),
99 the BluB/CobT2 fusion enzyme from *P. freudenreichii* DSM 4902 was
100 heterologously expressed, purified and characterized. The enzyme was confirmed
101 to be responsible for the synthesis of DMBI from the reduced flavin

102 mononucleotide and its activation into the nucleotide ready for attachment as a
103 lower ligand of active B12 (Deptula et al., 2015). Flavin mononucleotide and
104 flavin-adenine dinucleotide are derived from riboflavin (RF), which together with
105 RF are collectively known as vitamin B2.

106 Natural strains of Propionibacteria differ greatly in their ability to synthesize
107 B12 (Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010). The B12 yield
108 from *P. freudenreichii* is dependent upon the availability of cobalt and DMBI
109 (Hugenschmidt, Schwenninger, & Lacroix, 2011). Earlier studies with *P.*
110 *freudenreichii* cell homogenates showed that DMBI was synthesized from RF
111 (Lingens, Schild, Vogler, & Renz, 1992; Renz & Weyhenmeyer, 1972) and that
112 its biosynthesis was stimulated by nicotinamide (NAM) (Hörig & Renz, 1980).
113 However, the effect of RF and NAM supplementation on B12 production by
114 Propionibacteria has not been studied. DMBI is added to bacterial fermentations
115 in the pharmaceutical industry to increase B12 yields (Martens et al., 2002);
116 however, this process is not possible in natural fortifications. DMBI should be
117 substituted with compounds approved for use in food (i.e., RF and NAM) or with
118 food components rich in these vitamins to enhance B12 production.

119 Therefore, the aim of the present work was to study the influence of RF and
120 NAM on B12 production by several Propionibacteria strains in whey-based
121 medium (WBM). Ultra-high performance liquid chromatography (UHPLC) and
122 liquid chromatography–tandem mass spectrometry (LC–MS/MS) were employed
123 for the accurate identification and quantification of synthesized B12 under the
124 influence of the B12 precursors.

125

126 **2 Materials and methods**

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127 *2.1 Chemicals and materials*

128 Cyanocobalamin was obtained from Supelco (Bellefonte, USA) and ethanol
129 was obtained from Altia (Rajamäki, Finland). Sodium hydroxide, acetic acid,
130 dipotassium hydrogen phosphate, potassium dihydrogen phosphate, magnesium
131 sulphate heptahydrate, manganese(II) sulphate monohydrate and granulated yeast
132 extract were purchased from Merck (Darmstadt, Germany). Sodium cyanide,
133 acetonitrile (HPLC grade), trifluoroacetic acid (TFA), formic acid, sodium D/L-
134 lactate syrup (60% w/w) and Tween 80 were obtained from Sigma-Aldrich
135 (Steinheim, Germany). RF, NAM and cobalt(II) chloride hexahydrate were
136 purchased from Sigma-Aldrich, and DMBI was obtained from Merck. Water
137 (hereafter called MilliQ water) was produced by the MilliQ Plus system (0.22 μm,
138 ≥ 18.2 MΩ cm; Millipore Corporation, Bedford, MA, USA).

139

140 *2.2 Propionibacteria strains*

141 A collection of 27 *P. freudenreichii* and three *P. acidipropionici* strains were
142 studied. Twenty *P. freudenreichii* strains (256–265, 283–292) with probable dairy
143 origins were obtained from the culture collection of Valio Ltd. (Helsinki, Finland).
144 Two type strains (*P. freudenreichii* subsp. *shermanii* DSM 4902 and *P.*
145 *freudenreichii* subsp. *freudenreichii* DSM 20271) with dairy origins were
146 obtained from DSMZ (Braunschweig, Germany) and named 281 and 282,
147 respectively. One *P. freudenreichii* strain (266) was an isolate from a cheese
148 starter culture (Chamlagain, Edelmann, Kariluoto, Ollilainen, & Piironen, 2015).
149 Four *P. freudenreichii* strains (274–277) and the *P. acidipropionici* strains (278–
150 280) with probable cereal origins were obtained from the culture collection of
151 Polttimo Ltd. (Lahti, Finland).

152

153 *2.3 Growth media*

154 WBM (pH 6.4) was prepared according to Hugenschmidt et al. (2010) using
155 demineralized whey powder (Demi 50; Valio Ltd., Helsinki, Finland) instead of
156 ultra-filtered whey permeate. The composition per L of the medium was 60 g of
157 whey extract (from whey powder), 10 g of yeast extract, 13 g of sodium D/L-
158 lactate syrup (60% w/w), 0.1 g of Tween 80, 0.2 g of magnesium sulphate, 0.02 g
159 of manganese(II) sulphate, and 100 mL of 1 M potassium phosphate buffer. To
160 prepare the whey extract, an acidified whey powder suspension (85.7 g/L; pH 5.0)
161 was autoclaved (121 °C; 15 min) and paper-filtered (10–15 µm). The whey extract
162 (700 mL) was mixed with a Tween 80-Mg-Mn solution (150 mL) and autoclaved
163 separately from the yeast extract, lactate and phosphate buffer solution (150 mL;
164 pH 6.6). Immediately prior to usage, the two parts were mixed to obtain 1 L of
165 medium. A filter-sterilized (0.2 µm) solution of cobalt chloride was added to the
166 medium (5 mg/L). The propionic agar medium was composed (per L) of 5 g of
167 tryptone (Sigma-Aldrich), 10 g of yeast extract (Becton, Dickinson) and 14 mL of
168 sodium lactate with the pH adjusted to 7.3 prior to autoclaving.

169

170 *2.4 Precursor supplementation*

171 Each strain was cultivated in triplicate in WBM with and without RF (40
172 µM), the co-addition of RF (40 µM) and NAM (27 mM) or DMBI (100 µM)
173 (concentrations based on Hörig & Renz, 1980; Hugenschmidt et al., 2011). The
174 time effect of RF and NAM supplementation (on days 0, 3 or 6) on B12
175 production was studied with strains 256 and 266. Finally, RF or RF and NAM

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176 were added on day 0 and DMBI was supplemented on day 6 per Hugenschmidt et
177 al. (2010). The stock solutions were prepared in MilliQ water and filter-sterilized.
178
179 *2.5 Culture preparation and fermentation*
180 Cultures of strains cryopreserved (−80 °C) in glycerol were propagated on
181 propionic agar medium and incubated anaerobically (Anaerocult C; Merck,
182 Darmstadt, Germany) for 3–4 days at 30 °C. Three individual colonies of each
183 strain were transferred into 5 mL of WBM and incubated for 3–4 days at 30 °C
184 under anaerobic conditions. The cultures were sub-cultured three times prior to
185 inoculation. To determine B12 production by the strains, 20 mL of WBM was
186 inoculated at 1% (v/v) and incubated at 30 °C for 72 h under anaerobic conditions,
187 followed by 96 h of aerobic incubation (with the tubes slightly opened under
188 sterile conditions to allow air in and then closed again) under shaking conditions
189 (150 rpm; Certomat H, Sartorious, France) (Hugenschmidt et al., 2010).
190 The fermented broths obtained after 168 h of incubation were centrifuged
191 (12,000 x *g*; 10 min) and the supernatants were collected. The cell pellets were re-
192 suspended in 10 mL of PBS buffer (pH 7.3; Oxoid, Hampshire, UK) and
193 recovered by centrifugation. The cell biomasses in the tubes were weighed and
194 stored at −20 °C prior to B12 analysis. The pH of the supernatants was measured;
195 then, the supernatants were syringe-filtered (0.2 μm; Pall, MI, USA) and stored at
196 −20 °C prior to analysis for residual RF, lactose and acids. The progress of
197 fermentation was monitored at 72 h and 168 h by measuring the optical density
198 (600 nm; OD_{600}) with a Novespec II spectrophotometer (Amersham Pharmacia
199 Biotech, NJ, USA). The pH was recorded with a pH metre (Radiometer Analytical,
200 Lyon, France).

201

202 *2.6 Vitamin B12 analysis*

203 The B12 content in the cell pellets was extracted in cyano form and analysed
204 using the previously reported extraction and UHPLC methods (Chamlagain et al.,
205 2015). Briefly, 0.1–0.2 g of cell pellet was extracted with a pH 4.5 extraction
206 buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid) in the presence of
207 sodium cyanide to obtain 25 mL of the extract. The extract was analysed with a
208 Waters Acquity UPLC system (Milford, MA, USA) equipped with a photodiode
209 array detector (PDA; 210–600 nm) using an Acquity HSS T3 C18 column (2.1 ×
210 100 mm, 1.8 μm). The mobile phase was a gradient flow of MilliQ water and
211 acetonitrile both containing 0.025% TFA. The chromatogram was obtained by
212 recording the absorbance at 361 nm. Six cyanocobalamin standards (0.015–0.75
213 ng/μL) were injected (10 μL in duplicate) to create a calibration curve for each
214 sample set. The volumetric B12 yield (μg/mL WBM) and cellular B12 yield (μg/g
215 wet cell mass) for each strain are reported as an average of three biological
216 replicate fermentations.

217 The identity of the cobamide in the extracts was confirmed with mass
218 spectrometry using an Esquire-LC quadrupole ion trap mass spectrometer with an
219 electrospray ionization (ESI) interface (Bruker Daltonics, Bremen, Germany) in
220 positive ion mode as previously described (Chamlagain et al., 2015). The mobile
221 phase for LC–MS contained 0.1% formic acid. Briefly, ions with an *m/z* range of
222 900–1400 were scanned, and tandem mass spectrometry (MS/MS) was performed
223 for ions with *m/z* 1356 ($[M+H]^+$ of cyanocobalamin) using helium as the collision
224 gas. The instrumental settings were as follows: nebulizer (nitrogen) 50.0 psi, dry

225 gas (nitrogen) 8.0 L/min, dry temperature 300 °C, capillary 4500 V, end plate
226 offset −250 V and trap drive 84.
227

228 *2.7 Measurement of riboflavin content*

229 Vitamin B2 was extracted as RF from the control and fermented WBM
230 without any supplementation and analysed using the European standard method
231 (EN 14152:2014) after optimization for UHPLC. The supernatant (0.5 mL) was
232 mixed with 15 mL of 0.1 M hydrochloric acid and extracted in a boiling water
233 bath for 60 min. After cooling, the extract was adjusted to pH 4.5 with 2.5 M
234 sodium acetate and incubated (37 °C; 24 h) with Taka-Diastase (50 mg; Pfaltz and
235 Bauer, CT, USA) and β-amylase (5 mg; Sigma-Aldrich). The analysis was
236 performed with a Waters UPLC system equipped with a fluorescence detector
237 using a Waters Acquity BEH C18 column (2.1 mm × 100 mm; 1.7 µm particles).
238 The mobile phase contained 20 mM ammonium acetate dissolved in 30% aqueous
239 methanol and eluted at a constant flow of 0.2 mL/min. The excitation and
240 emission wavelengths were set at 444 nm and 520 nm, respectively. An external
241 calibration curve was obtained by injecting six RF standards (0.01–1.0 ng/µL) in
242 10 µL in duplicate for the quantitation.
243

244 *2.8 Analysis of sugar and acids*

245 The cell-free fermented WBM and control media were appropriately diluted
246 with MilliQ water and filtered (0.45 µm; Pall, USA). Lactose, lactic acid and the
247 metabolites propionic acid and acetic acid in the samples were quantified with the
248 HPLC method reported by Hugenschmidt et al. (2010). The analysis was
249 performed using an HPLC system equipped with a pump (Waters 515), an

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250 autosampler, a UV detector (Waters 717) and a refractive index detector (HP
251 1047A, HP, USA) on an Aminex HPX-87H column (7.8 × 300 mm, 9 µm
252 particles; Bio-Rad, USA).
253

254 *2.9 Statistical analysis*

255 The average B12 yields (µg/mL) and cellular B12 yields (µg/g wet cell mass)
256 for each strain following supplementation with B12 precursors were compared by
257 one way analysis of variance (ANOVA) and Tukey's post hoc test using SPSS 22
258 (IBM Corporation, NY, USA). A p value < 0.05 was considered statistically
259 significant.
260

261 # 3 Results and discussion

262 *3.1 Growth characteristics*

263 The final cell densities measured as the optical density at 600 nm (OD_{600})
264 varied greatly between strains, ranging from $OD_{600} = 1.2$ to $OD_{600} = 17.8$ in un-
265 supplemented WBM (Fig. 1A). A low (< 5) final OD_{600} value coincided with a
266 high (> 6) final pH (Fig. 1B) that was indicative of the strain's inability to
267 metabolize lactose in the medium. These strains relied solely on sodium lactate as
268 a carbon source, which was also reflected in the smaller amounts of propionic and
269 acetic acids produced by these strains (Supplemental Fig. 1A). In *P.*
270 *freudenreichii*, lactose utilization is strain dependent and is one of the two
271 phenotypic criteria (together with nitrate reductase activity) used to divide the
272 species into two subspecies: subsp. *freudenreichii*, which is lactose negative (type
273 strain 282), and subsp. *shermanii*, which is lactose positive (type strain 281)
274 (Thierry et al., 2011). Five of the strains growing to higher final OD_{600} values

275 (261, 262, 264, 266 and 289) produced slime during fermentation, which
276 prevented the isolation of cells from the final culture as a pellet. *P. freudenreichii*
277 strains are known to produce exopolysaccharides consisting of glucose, galactose
278 or other sugars depending on the strain (Darilmaz & Gumustekin, 2012;
279 Nordmark, Yang, Huttunen, & Widmalm, 2005; Thierry et al., 2011). Therefore,
280 the measured final OD_{600} values for these slime-producing strains most likely do
281 not reflect the final cell densities.

282 The effect of supplementation on growth was strain- and supplement-
283 dependent. Generally, supplementation did not restrict the growth of the strains;
284 however, co-supplementation with RF and NAM together had a stimulating effect
285 on the growth of some strains (Fig. 1A), with a few producing slime in the
286 medium. Nevertheless, these supplements did not affect the final pH values of the
287 cultures (Fig. 1B), suggesting that there was no immediate effect of carbon
288 metabolism on acid production (Supplemental Fig. 1B).

289

290 *3.2 Confirmation of active B12 production with and without supplements*

291 The UHPLC−UV/Vis and LC−MS/MS analyses showed that all 27 studied
292 strains of *P. freudenreichii* produced active B12 in native WBM and with RF, RF
293 and NAM or DMBI supplementation. The cobamide extracted in its cyano form
294 from the cell biomasses of the strains grown in WBM and DMBI-supplemented
295 WBM eluted with the same retention time as cyanocobalamin (Fig. 2A) and had a
296 PDA spectrum (210–600 nm) identical to cyanocobalamin (data not shown). The
297 MS/MS spectra of the cobamide peak (Figs. 2C and 2D) were identical to the
298 fragmentation profile of cyanocobalamin (Fig. 2B). An analysis of the major
299 fragment ions (*m/z* 1209, 1124, 997 and 912 corresponding to $[M+H−DMBI]^+$,

300 [M+H−DMBI−CN−Co]$^{+}$, [M+H−DMBI−sugar−phosphate]$^{+}$ and
301 [M+H−DMBI−sugar−phosphate−CN−Co]$^{+}$, respectively) confirmed that cobamide
302 contained DMBI as the lower ligand as reported previously (Chamlagain et al.,
303 2015). Similarly, the extracted cobamide from media supplemented with RF or
304 RF and NAM was confirmed to be cyanocobalamin. In contrast, the
305 cyanocobalamin peak was detected in the cell extracts of the *P. acidipropionici*
306 strains only when grown with DMBI. The peak was not identified in the *P.*
307 *acidipropionici* strains grown with RF or with RF and NAM, confirming the
308 inability of these strains to synthesize DMBI *de novo* for active B12 production.
309 DMBI is usually added during commercial B12 production; however, its use is
310 not desirable for *in situ* B12 production in foods or for the production of B12
311 bioingredients with minimal downstream processing (Hugenschmidt et al., 2011),
312 thereby rendering *P. freudenreichii* a more suitable candidate for these
313 applications. However, the yield of active B12 and the response to
314 supplementation was strain dependent (Sections 3.3–3.5).
315

316 *3.3 Effect of riboflavin and nicotinamide supplementation time on B12 production*

317 The effect of the RF and NAM supplementation time on B12 production was
318 tested with two *P. freudenreichii* strains (Fig. 3). Strain 266 produced 2.4-fold
319 more B12 when the supplementation was performed on day 0 or day 3 compared
320 to day 6. In contrast, the B12 yield with strain 256 was not improved by RF and
321 NAM ($p > 0.05$) but was markedly increased (by 80%) by DMBI supplementation.
322 The results suggest that the availability of RF and NAM during the early stage of
323 fermentation improves B12 production from certain *P. freudenreichii* strains. This
324 finding is useful for the *in situ* B12 fortification of foods because native RF and

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325 niacin in food matrices are accessible from the beginning of the fermentation. In
326 contrast, DMBI is preferably supplied 24 h before the termination of fermentation
327 in industrial B12 production (Hugenholtz, Hunik, Santos, & Smid, 2002; Murooka,
328 Piao, Kiatpapan, & Yamashita, 2005) because early addition of DMBI is thought
329 to reduce the growth of *P. freudenreichii* and decrease the B12 yield (Marwaha,
330 Sethi, & Kennedy, 1983). Based on the results of this comparison, RF and NAM
331 were supplemented at the beginning of the fermentation in the latter experiment,
332 whereas DMBI was added on day 6 of the fermentation.
333

334 *3.4 B12 production in WBM without supplementation*

335 The B12 yield in WBM varied greatly between the studied *P. freudenreichii*
336 strains (Figs. 4A and 4B), suggesting strain-dependent B12 production. The
337 volumetric and cellular B12 yields ranged from 0.45 to 3.35 µg/mL (Fig. 4A) and
338 25 to 204 µg/g wet cell mass (Fig. 4B), respectively. For the six strains (261, 262,
339 264, 265, 266 and 289) that produced slime, the cellular B12 yields were not
340 accurate and therefore were not included in Fig. 4B. Because the produced B12
341 accumulate intracellularly in Propionibacteria, the volumetric B12 yield (µg/mL)
342 is directly affected by the amount of cell biomass obtained (data not shown) and
343 the cellular B12 yield (Fig. 4B). Frequently, higher cellular B12 yields were
344 obtained for strains growing to lower final OD_{600} values (Fig. 1A) that did not
345 metabolize lactose while the pH of the medium remained >6 (Fig. 1B). For
346 Propionibacteria, the culture medium pH greatly influences growth and B12
347 biosynthesis, with pH 6–7 optimal for growth (Hsu & Yang, 1991; Vorobjeva,
348 1999) and growth ceasing below pH 5 (Hettinga & Reinbold, 1972). Therefore,
349 the medium is usually maintained at approximately pH 7 during industrial B12

350 production by neutralizing the acids with alkali agents (Martens et al., 2002).
351 However, the control of pH to improve *in situ* B12 production in food matrices is
352 not preferred.

353

354 *3.5 Effect of riboflavin and nicotinamide vs DMBI supplementation on B12*
355 *production*

356 RF supplementation alone did not increase B12 production by the strains ($p >$
357 0.05; Figs. 4A and 4B). This result is likely due to the relatively high level (2.5
358 μg/mL) of RF present in the WBM. We compared the consumption of native RF
359 from the medium by the studied strains. The analyses revealed that the *P.*
360 *freudenreichii* strains consumed 25% to 85% of the RF present in the WBM,
361 whereas the *P. acidipropionici* strains did not use the RF from the medium.
362 Indeed, a significant positive correlation ($r = 0.84$) was observed when RF
363 consumption was compared with B12 production (Fig. 5).

364 Supplementation of the WBM with both RF and NAM increased both the
365 volumetric and cellular B12 yields in several strains ($p < 0.05$; Figs. 4A and 4B),
366 including the *P. freudenreichii* subsp. *shermanii* type strain (281). Notably, this
367 enhancement in volumetric B12 production was more effective than DMBI
368 supplementation in seven of the strains (261–264, 266, 284 and 285) (Fig. 4A). To
369 the best of our knowledge, the enhancing effect of RF and NAM on B12
370 biosynthesis by metabolizing *P. freudenreichii* cell has not been reported
371 previously. For four strains (274, 275, 276 and 286) and the *P. freudenreichii*
372 subsp. *freudenreichii* type strain (282), the addition of RF and NAM significantly
373 decreased the volumetric B12 yield ($p < 0.05$) compared to the yield in WBM.

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374 DMBI supplementation enhanced the volumetric and cellular B12 yields ($p <$
375 0.05) of seven (256, 257, 281, 283, 288, 289 and 290) and five (256, 257, 281,
376 288 and 290) *P. freudenreichii* strains, respectively (Figs. 4A and 4B). The
377 increase in the volumetric yield ranged from 18% for strain 257 to 280% for type
378 strain 281. The enhancement in the cellular B12 yield was most effective for
379 strain 281, with a 3.2-fold higher concentration observed in the cells with DMBI
380 supplementation compared to the control culture (Fig. 4B). Altogether, these
381 results revealed that the availability of exogenous DMBI was not limiting for B12
382 production under the conditions used for specific *P. freudenreichii* strains.
383 Therefore, we speculated that the *P. freudenreichii* strains that did not respond to
384 DMBI supplementation were unable to uptake DMBI from the environment. The
385 DMBI transporter in *P. freudenreichii* has not been identified to date.

386 By including a number of strains, the present study provides evidence in
387 support of the current understanding that *P. freudenreichii* synthesizes DMBI
388 from RF (Hörig & Renz, 1977; Renz & Weyhenmeyer, 1972; Vorobjeva, 1999).
389 The results also indicate that the efficiency of DMBI synthesis in *P.*
390 *freudenreichii* is strain dependent. All three *P. acidipropionici* strains studied
391 were dependent on exogenous DMBI to produce even traces of active B12 (Figs
392 4A and 4B), suggesting the lack of the DMBI biosynthesis pathway in *P.*
393 *acidipropionici*. Indeed, genomic data (Parizzi et al., 2012) indicate that *P.*
394 *acidipropionici* lacks the *bluB/cobT2* gene responsible for DMBI biosynthesis and
395 activation in *P. freudenreichii* (Deptula et al. 2015).

396 In *P. freudenreichii*, nicotinate mononucleotide (NaMN) is the preferred
397 substrate for the activation of DMBI into α-ribazole-phosphate by the
398 BluB/CobT2 enzyme of the nucleotide loop assembly (Deptula et al., 2015;

399 Friedmann & Harris, 1965). NAM was rapidly converted into nicotinate in studies
400 on the DMBI-forming system using *P. freudenreichii* cells and cell homogenates,
401 suggesting that nicotinate was the actual stimulant of the DMBI-forming system
402 in *P. freudenreichii* (Hörig & Renz, 1980). In line with this finding, NAM was
403 found to be completely converted into nicotinate at the end of fermentation (data
404 not shown). Thus, the synthesis or regeneration of NaMN could have been
405 enhanced by the presence of excess nicotinate. Additionally, nicotinate might
406 allosterically regulate the enzyme involved in DMBI biosynthesis (Chen, Ailion,
407 Weyand, & Roth, 1995), which would be consistent with the greater effect of RF
408 and NAM supplementation over DMBI supplementation for the majority of the
409 strains. However, this hypothesis needs to be explored in follow-up studies.

410 In this study, the highest volumetric B12 yields (obtained with strain 288)
411 with and without DMBI supplementation were 5.3 μg/mL and 3.3 μg/mL,
412 respectively (Fig. 4A). The highest yield obtained with co-supplementation with
413 RF and NAM was 4.2 μg/mL (with strain 284). All of these values clearly exceed
414 those reported in a previous study using 100 natural strains of Propionibacteria,
415 where the maximum yield in DMBI-supplemented whey permeate was 2.5 μg/mL
416 (Hugenschmidt et al., 2010).

417

418 **4 Conclusion**

419 We used UHPLC–UV/Vis and LC–MS/MS and showed that all 27 *P.*
420 *freudenreichii* strains studied synthesized active vitamin B12 in whey-based
421 medium without DMBI supplementation, whereas the *P. acidipropionici* strains
422 were able to produce traces of B12 only when provided exogenous DMBI. B12
423 production by the *P. freudenreichii* strains was strain dependent. The yield from

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424 several strains was markedly increased by the addition of the lower ligand
425 precursors. The volumetric B12 yield increased up to 4-fold with co-
426 supplementation with RF and NAM, and for a number of strains the yield was
427 comparable or even higher than that achieved with DMBI. A significant positive
428 correlation between RF consumption and B12 production confirmed that the
429 DMBI ligand of B12 in *P. freudenreichii* was synthesized from RF. The increased
430 yield obtained with RF and NAM co-supplementation clearly indicates enhanced
431 *de novo* synthesis of DMBI and its activation into the nucleotide. The present
432 study suggests that improved *in situ* production of B12 in foods is possible
433 without the need for DMBI supplementation by selecting a *P. freudenreichii* strain
434 that better responds to RF and NAM.

435

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440 UHPLC and MS analyses.

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549

550 **Figure captions:**

551 **Fig. 1.** Effect of riboflavin (RF), co-supplementation with RF and nicotinamide
552 (NAM), and DMBI on the optical density (**4A**) and pH (**4B**) of whey-based

553 medium (WBM) fermented with 30 *Propionibacterium* strains. DMBI = 5,6-
554 dimethylbenzimidazole.

555 **Fig. 2. A**) Example UHPLC−UV chromatograms of the cyanocobalamin standard
556 and extracts of the cell biomass obtained from 168-h fermented whey-based
557 medium (WBM) and from WBM supplemented with DMBI. LC−MS/MS spectra
558 of the cyanocobalamin standard (**B**) and the cobamide in cell extracts eluting at
559 the retention time of cyanocobalamin (3.27 min) from cell biomasses grown in
560 WBM (**C**) and WBM with DMBI supplementation (**D**). DMBI = 5,6-
561 dimethylbenzimidazole.

562 **Fig. 3.** Effect of the riboflavin (RF) and nicotinamide (NAM) addition time (days
563 0, 3 or 6) on vitamin B12 production by *P. freudenreichii* strains 256 and 266 in
564 whey-based medium (WBM). The results are expressed as the mean ± standard
565 deviation (n = 3), and columns with different letters differ significantly ($p < 0.05$).
566 DMBI = 5,6-dimethylbenzimidazole.

567 **Fig. 4.** Effect of riboflavin (RF), RF and nicotinamide (NAM), and DMBI
568 supplementation on the volumetric vitamin B12 yield (µg/mL) (**Fig. 4A**) and
569 cellular B12 yield (µg/g wet cell mass) (**Fig. 4B**) by 30 *Propionibacterium* strains
570 in whey-based medium (WBM). DMBI = 5,6-dimethylbenzimidazole. Error bars
571 represent the standard deviations of three biological replicate fermentations.
572 Strains 256 and 266 were not studied with RF supplementation.

573 *The B12 per gram of cells data are not shown for six strains (261, 262, 264, 265,
574 266 and 289) that were difficult to isolate as cell pellets.

575 **Fig. 5.** Correlation between riboflavin (RF) consumption and vitamin B12
576 production by 30 strains of Propionibacteria in whey-based medium (WBM).

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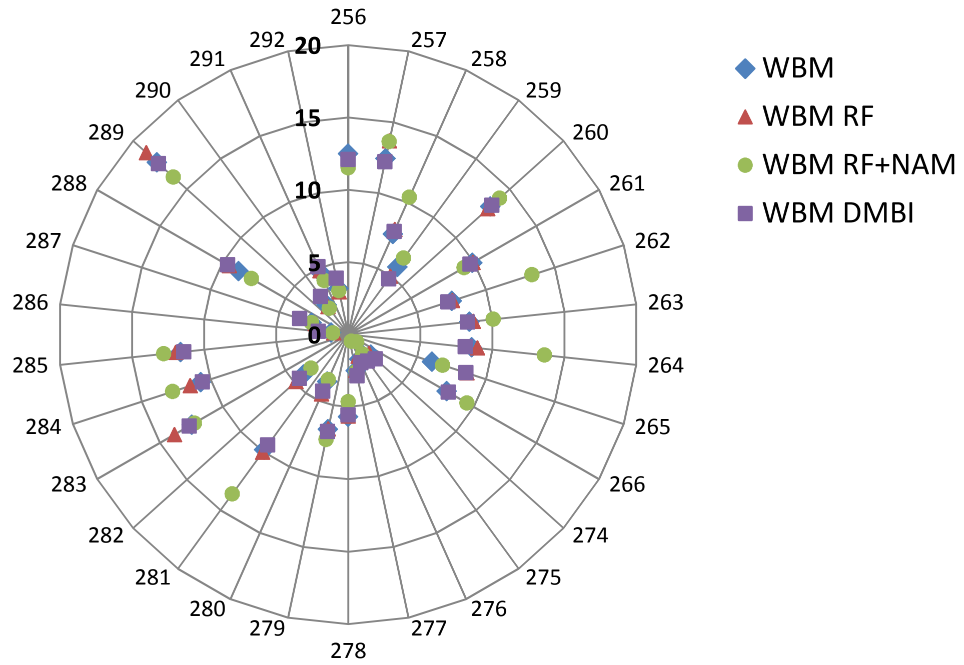

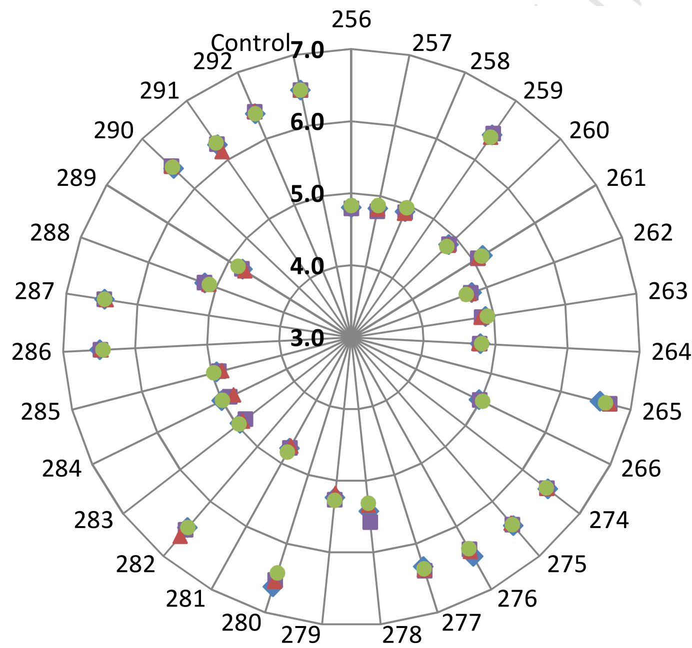

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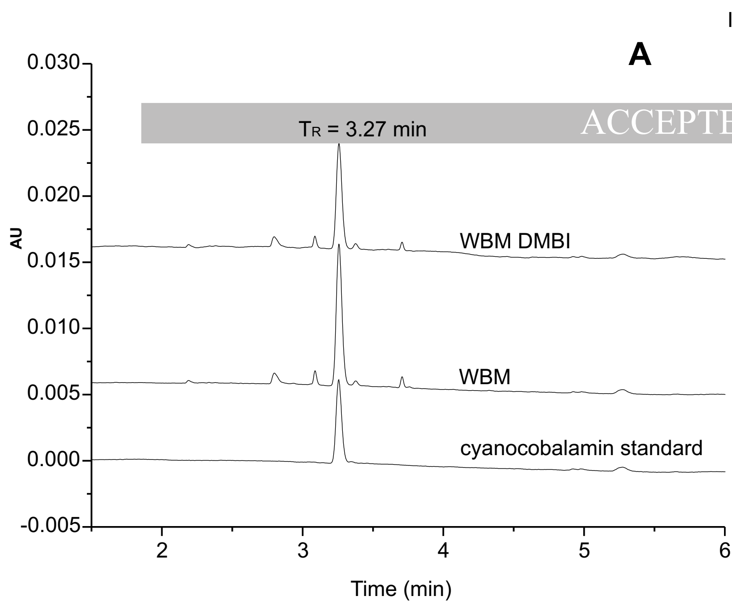

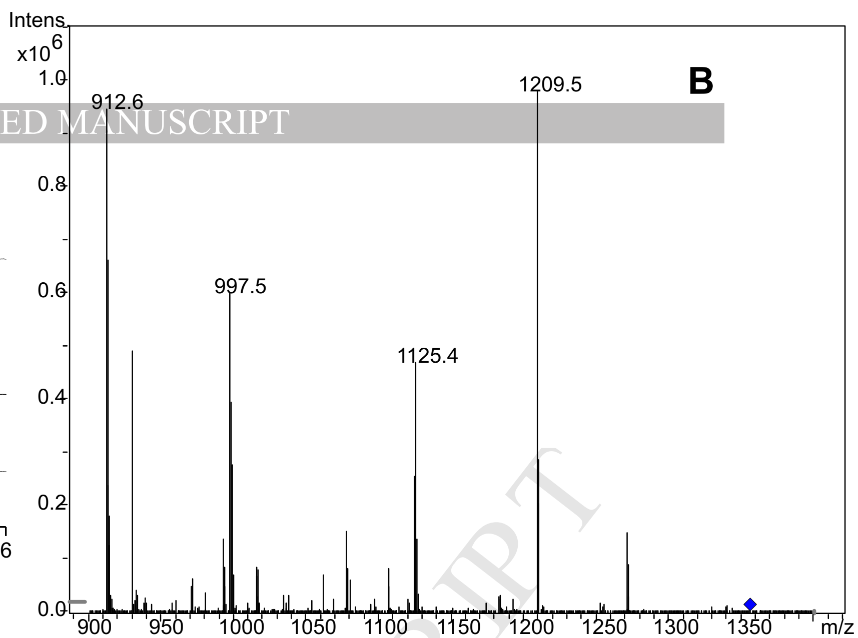

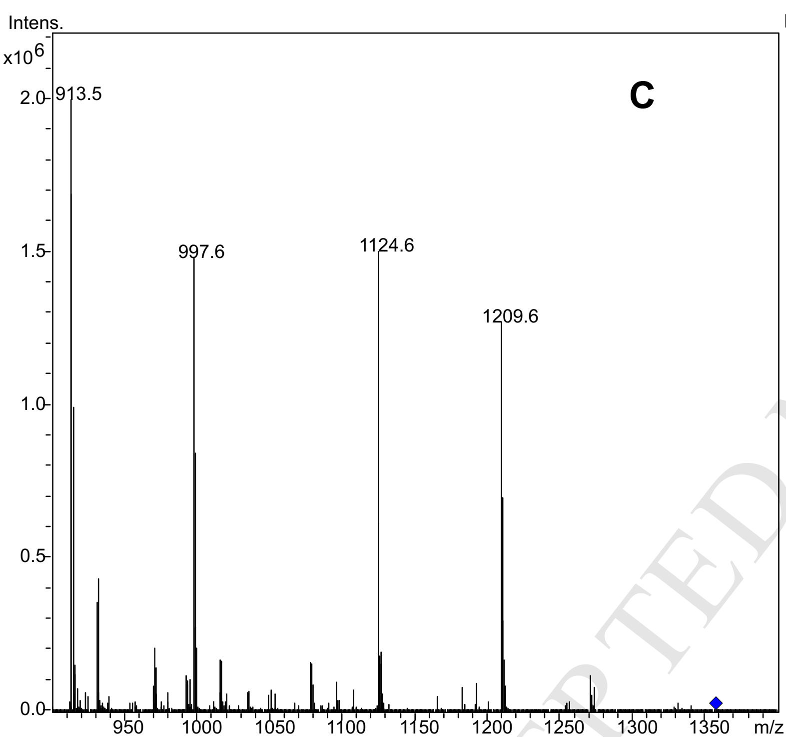

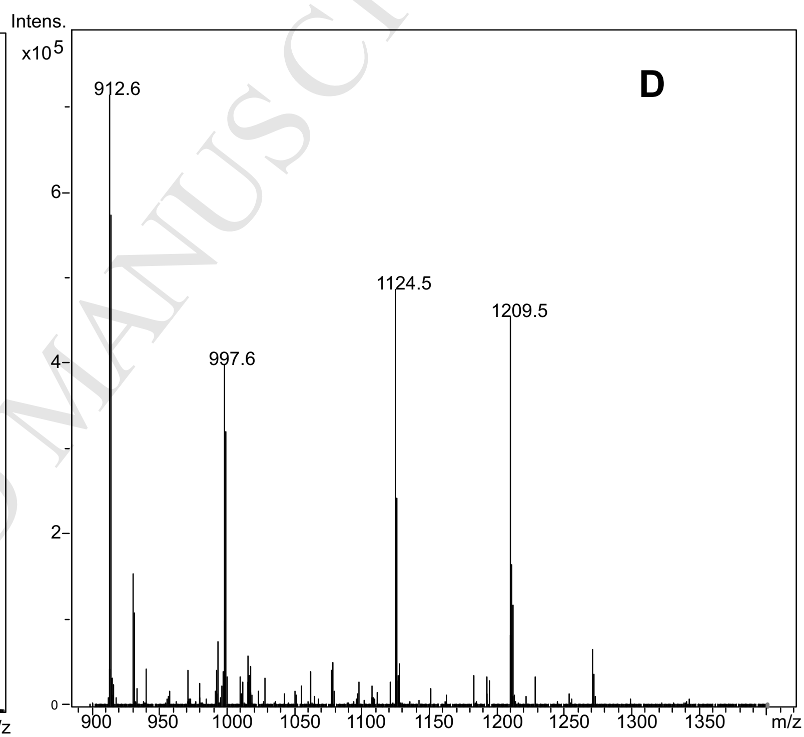

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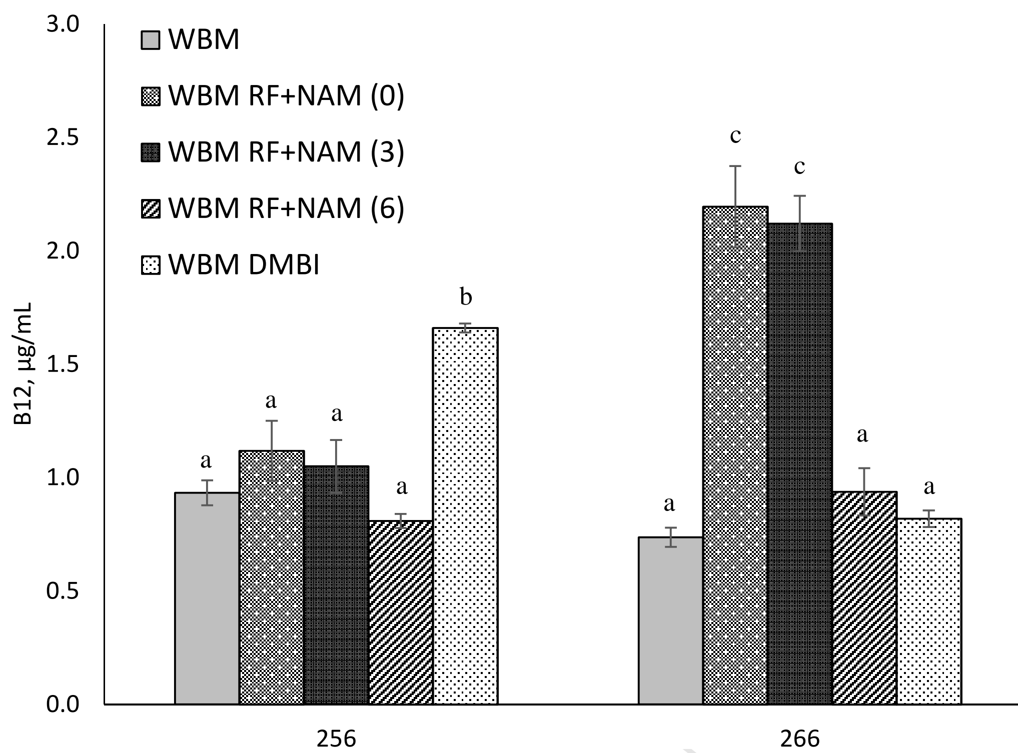

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A

B12, µg/mL

WBM
WBM RF
WBM RF+NAM
WBM DMBI

258 261 262 263 264 266 284 285 257 281 283 289 256 288 290 278 279 280 259 260 265 274 275 276 277 282 286 287 291 292

Increase with RF+NAM
Increase with DMBI
Production only with DMBI
No significant increase with supplementation

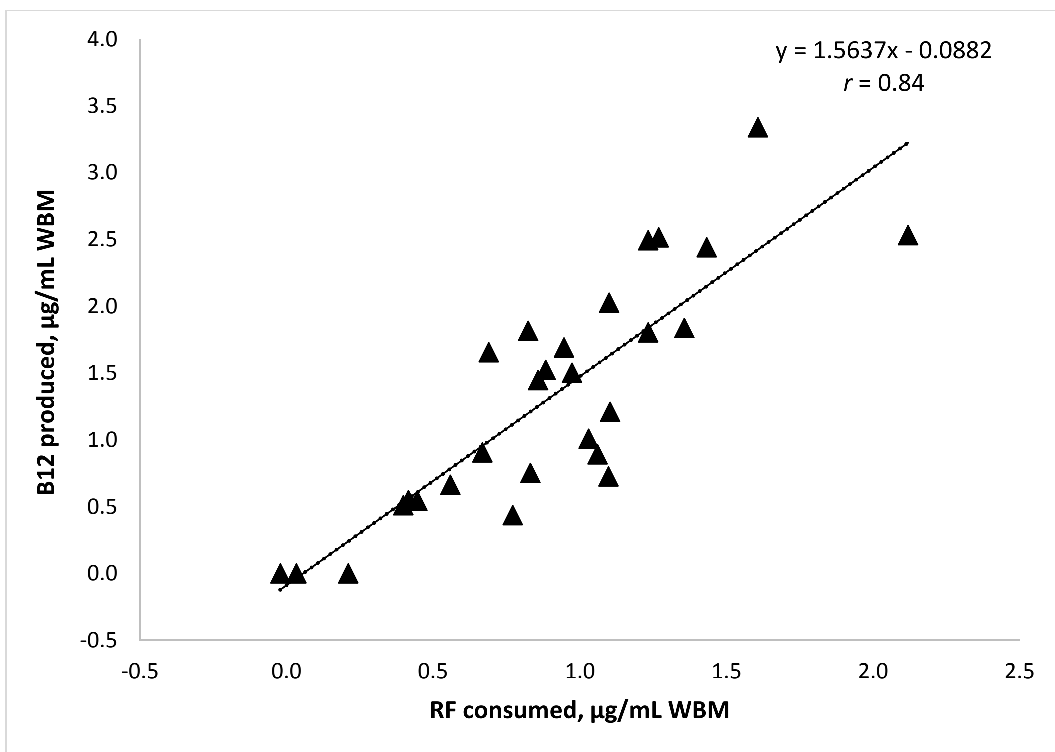
B

B12, µg/g wet cell mass

WBM
WBM RF
WBM RF+NAM
WBM DMBI

263 283 284 285 257 281 256 288 290 278 279 280 258 259 260 274 275 276 277 282 286 287 291 292 261 262 264 265 266 289

Increase with RF+NAM
Increase with DMBI
Production only with DMBI
No significant increase with supplementation
Data not shown*



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- UHPLC−UV/Vis−MS confirmed active vitamin B12 production by *Propionibacterium freudenreichii* strains.
- B12 yield was strain dependent.
- Supplementation with riboflavin and nicotinamide increased B12 yield up to 4-fold.
- Significant positive correlation was observed between riboflavin consumption and B12 production.
- Increasing *in situ* B12 production is possible with added natural precursors in food matrices.

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