Gorse (*Ulex europeaus*) wastes with 5,6-dimethyl benzimidazole supplementation can support growth of vitamin B_{12} producing commensal gut microbes.

Ajay Iyer [1¤], Eva C. Soto Martín[2], Gary A. Cameron [1], Petra Louis [1], Sylvia H. Duncan [1], Charles S. Bestwick [1], Wendy R. Russell [1*]

1 University of Aberdeen, Rowett Institute, Foresterhill, Scotland AB25 2ZD

2 University of Aberdeen, Institue of Medical Sciences, Foresterhill, Scotland AB25 2ZD

¤Current Address: Teagasc, Fermoy, Co Cork, Ireland, P61 C996.

* w.russell@abdn.ac.uk

Abstract

Many commensal gut microbes have the ability to synthesise vitamin B_{12} and have been investigated for potential supplementary applications in preventing deficiencies. Despite the availability of bioinformatics tools to identify biosynthetic potential of B_{12}, practical experimentation is required test if firstly, confirm B_{12} production, secondly, to identify the cobalamin vitamers produced, and lastly, to establish the potential biosynthetic yields. Here, the ability of three dominant human colonic bacterial species, namely, *Anaerobutyricum hallii* DSM 3353, *Roseburia faecis* DSM 16840, and *Anaerostipes caccae* DSM 14662, to produce vitamin B_{12} was investigated. *Propionibacterium freudenreichii* DSM 4902 was used as the positive control to test for B_{12} production. Additionally, the viability of the use of protein depleted liquid wastes from Gorse (*Ulex europeaus*) as a potential alternate source of carbon and nitrogen for bacterial growth in a chemically defined medium was also investigated.

These studies confirmed the ability of the three important butyrate producing strains to produce B_{12}, although yields were very low and expression was intracellular. B_{12} production was higher in Gorse medium compared to the routinely used M2GSC

growth medium. *P. freudenreichii* DSM 4902 was able to produce B_{12} at $7\,\mathrm{ng\,mL^{-1}}$ to $12\,\mathrm{ng\,mL^{-1}}$. A univariate-scaled Principal Component Analysis model revealed that B_{12} yields become low at carbon source concentrations $\leq 30\,\mathrm{g\,L^{-1}}$.

Keywords:

B_{12} production, cobalamin, gut commensals, circular economy.

Abbreviations:

CDM: chemically defined medium, GEM: Gorse extract medium, PDF: protein depleted fraction, PCA: principal component analysis

Introduction

Vitamin B_{12} is an essential cofactor for numerous enzymes in the human body. Humans cannot synthesize B_{12} *de novo* and must obtain it from their diet, with potential supplementation from colonic symbiotic microbes [1]. The recommended daily intake for B_{12} is 2.4 µg [2]. B_{12} is involved in various enzymatic reactions, including isomerases, methyltransferases, and dehalogenases [3] , with B_{12}-dependent dehalogenases being exclusive to bacteria [5]. In humans, B_{12} is crucial for the type 2 reaction of methyl transfer from 5-methyltetrahydrofolate to homocysteine, which produces tetrahydrofolate and methionine [6]. Additionally, B_{12} serves as a cofactor in the type 1 reaction that catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA, a process that enters the Krebs cycle [7]. The two critical ligands of cobalamin are the upper ligand comprising of the corrin ring centred around a cobalt ion, and the lower ligand composed of 5,6-dimethylbenzimidazole (DMB). There is also a variable side group consisting of either methyl, adenosyl, hydroxyl or cyano associated to the cobalt atom. Addition of a cyano-group is a stable semi-synthetic derivative of B_{12} that is commercially available [8]. The known B_{12} vitamers are shown in Figure 1. Without DMB, methyltransferase enzyme activity cannot be initiated [7]. The biosynthesis of B_{12} requires several enzymes [9, 10], which are only found in bacteria and archaea [11]. Other organisms acquire B_{12} through association with these organisms [12] or have developed alternative cofactors or independent pathways [9] to bypass the need for the

cofactor.

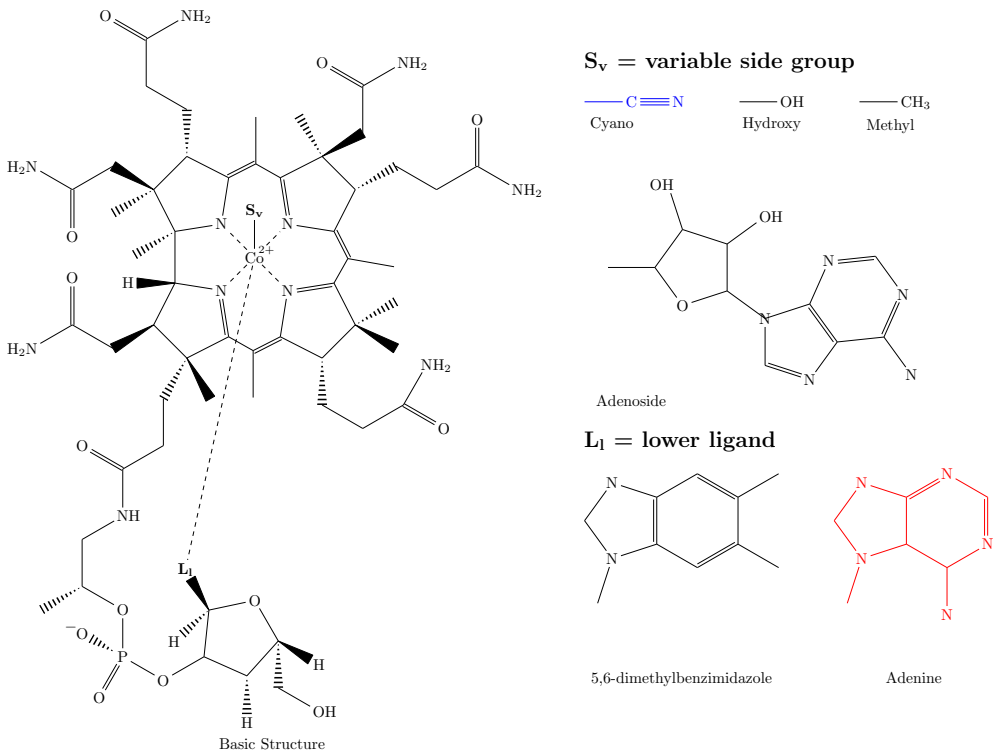

Fig 1. The variable ligands of B_{12} vitamers. Variable side-groups form coordinate bonds with the central cobalt ion. The cyano-group is marked in blue as it is a semi-synthetic derivate of B_{12}. The ligands at location L_l (secondary ligand) determine if the cobalamin vitamer is biologically active in humans with 5,6-dimethybenzimidazole (DMB) or inactive with adenine. B_{12} vitamers can be found with combinations of S_v and L_l ligands.

In a previous publication, biomass from invasive plant species, such as Gorse (*Ulex europaeus*), was utilized to produce high-quality leaf protein concentrates [13]. However, this process resulted in the production of a protein depleted fraction (PDF) containing bioactives, carbohydrates, and residual protein. In an effort to circularize the process and explore the development of probiotic products, three butyrate-producing microbial candidates, namely *Anaerobutyricum hallii* DSM 3353, *Roseburia faecis* DSM 16840, and *Anaerostipes caccae* DSM 14662, were investigated for their ability to grow on media comprising PDF as the sole carbon and nitrogen source. These microbial candidates were chosen due to their potential to produce vitamin B_{12} [14] and their purported beneficial impact in the human gut [15, 16].

The study aimed to investigate the growth capability of the three experimental

microbes on gorse leaf waste and their ability to synthesize biologically active B_{12} vitamers. In addition to PDF, the experimental media incorporated DMB to aid the vitamin B_{12} synthesis process. As a positive control, *Propionibacterium freudenreichii* DSM 4902, known for its documented ability to produce vitamin B_{12} and its application in probiotics, was used [17].

In addition to the experimental work outlined in this study, a systematic literature search was conducted to collect previous publications on vitamin B_{12} production by *P. freudenreichii*. The objective was to compare B_{12} yields with respect to growth media and conditions, aiming to identify overarching trends that could potentially enhance cobalamin production.

Materials and methods

All chemicals were purchased from Merck, (Darmstadt, Germany) unless stated otherwise. All water used was from the in house Milli-Q purification system. All experiments were performed in triplicate.

Media

Media preparation was carried out in an anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) set for 10 % H_2, 10 % CO_2, and 80 % N_2, with Milli-Q water which was first flushed with N_2 and autoclaved. The water was then allowed to equilibrate in the cabinet prior to preparation for 48 h.

Media preparation

The M2GSC medium was prepared as described previously [18]. The media was prepared, autoclaved, and allowed to equilibrate in an anaerobic cabinet for 48 h and then stored at 4 °C until further use. The recipe for M2GSC is provided in **Supplementary Text 1.1**.

The chemically defined medium (CDM) was prepared as described previously [14], except with the inclusion of 5,6-dimethylbenzimidazole (DMB, 20 mg L^{-1}), which was brought into the anaerobic cabinet and allowed to equilibrate for 2 h prior to preparation. Post preparation, the media was filter-sterilised using 0.2 µm filter

(Millipore, Merck, US) and stored at 4 °C until further use. The composition of the CDM is provided in **Supplementary Text 1.2**.

For the gorse extract medium (GEM) was prepared by replacing the carbon and nitrogen source of CDM with the gorse protein-depleted fraction (PDF). The composition of PDF is provided in a previous publication [13]. It contained residual protein (53.9 mg g^{-1} dry mass) and polysaccharides / sugars (glucose equivalent of 88.9 mg g^{-1} dry mass). Given that the plant mass was previously treated with cellulase as part of the protein extraction process, the carbohydrate profile of the PDF was expected to comprise of low molecular weight polysaccharides and glucose. Pectinase, xylanase or other cell wall constituent-targeting enzymes were not employed and those were thus expected to remain intact in the marc (left over plant mass) and corresponding digestion products (such as glucuronic acid, xylose, etc.) in the PDF were expected to be largely absent. GEM was prepared by replacing the carbon and nitrogen sources in CDM with PDF at 25 g L^{-1}. This meant that GEM contained about 1.35 g L^{-1} of proteins and 2.3 g L^{-1} of carbohydrates; values comparable to CDM.

Bacterial strains

Four bacterial strains were chosen for B_{12} production, namely, *P. freudenreichii* DSM 4902, *A. caccae* DSM 14662, *A. hallii* DSM 3353, and *R. faecis* DSM 16840, based on the work previously described [14]. *A. caccae* DSM 14662, *A. hallii* DSM 3353, and *R. faecis* DSM 16840 were available from the in-house culture stock. *P. freudenreichii* DSM 4902 was purchased from NCIMB (Aberdeen, Scotland, NCIMB 5959).

Culture revival and growth

Microbial species were revived from frozen stocks using M2GSC (5 mL) in Hungate tubes (sealable with rubber caps) in anaerobic conditions and incubated at 37 °C for five days. The strains were sub-cultured by inoculating 0.5 mL of the culture in 4 mL of fresh M2GSC and incubation for two days. Growth was measured with optical density at 650 nm using Novaspec-II visible spectrophotometer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Production of B_{12} samples

From the revived stock, 200 µL was inoculated in triplicate in M2GSC, CDM or GEM (10 mL) and gently mixed through repeated aspiration. The culture was statically incubated at 37 °C for 48 h. Sample (1 mL) was drawn at time points 0 h, 6 h, 12 h, 24 h, 30 h, 36 h and 48 h and centrifuged at 10 000 × *g* for 20 min at 4 °C (Biofuge (Fresco), Heraeus Instruments, Germany). The supernatant was recovered and analysed for B_{12} content. The pellet was resuspended in PBS buffer (1 mL) and slowly cooled to −80 °C by first placing them in an ice-bath during sample collection, followed by incubation in −20 °C freezer overnight. Samples were then in a −80 °C freezer for a couple of days. This was performed to facilitate gradual growth of ice crystals to pierce and rupture the bacterial cell wall. Samples were then thawed to 4 °C overnight and sonicated (MSE Soniprep 150, UK) in two 10 s bursts to rupture the cells. The cells were centrifuged at 10 000 × *g* for 20 min and the supernatant was recovered and analysed for B_{12} content.

B_{12} Quantification using LC-MS

B_{12} estimation was performed using mass spectrophotometry in tandem with liquid chromatography as described elsewhere [19]. Calibration standards (all from Sigma-Aldrich; Saint Louis, USA) used were adenosylcobalamin, hydroxycobalamin, and methylcobalamin at concentrations 0.3 ng mL^{-1}, 1.0 ng mL^{-1}, 3.0 ng mL^{-1}, 10 ng mL^{-1}, 30 ng mL^{-1}, 100 ng mL^{-1} and 300 ng mL^{-1} in dimethyl sulphoxide (DMSO). Cyanocobalamin (internal standard) was used at 2 µg mL^{-1}. Samples or standards were injected at 100 µL with 10 µL internal standard. Details of the LC-MS setup is provided in **Supplementary Text 1.3**.

Literature review

To retrieve previous literature on B_{12} production in *P. freudenreichii*, the search structure:

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(propionibacterium AND (cobalamin OR B12))
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was used in PubMed and Scopus. The relevant titles were selected and analysed by eye to obtain values for B_{12} yield, the carbohydrate concentration used (Carbon g L^{-1}), fermentation time (Time h), cobalt salt used (mg L^{-1}) and added DMB (mg L^{-1}).

These values were used for the construction of a univariate-scaled PCA model to help compare and contextualise the B_{12} yields obtained in the work described herein. The details of the study and B_{12} are provided in **Supplementary Table 1**.

Statistical Analysis

All statistical analyses were performed using R (4.2.2) [20] using the tidyverse (2.0.0) [21] package for data processing and visualisation. Normality of the data distribution was checked using the Shapiro-Wilks test. The Carbon ($g\,L^{-1}$), Time (h), and B_{12} ($mg\,L^{-1}$) values were $\log_{10}$ transformed to achieve normality.

The k-means analysis followed by univariate-scaled principal component analysis was performed using the cluster (2.1.4) [22], and factoextra (1.0.7) [24] packages. A dimension reduction model was made using previously published B_{12} production with *P. freudenreichii*. A k-means analysis (MacQueen algorithm) was performed to observe clustering in the raw data. The raw data was then subjected to a univariate-scaled PCA analysis and the clusters established from k-means analysis was overlaid on the plot to observe their relative positions. This PCA model was then used to interpolate the relative position of the data points representing the experimental conditions described in our study. This was used to understand which cluster of experimental conditions our setup matched and compare the corresponding B_{12} yields. The raw data and codes can be found at `https://osf.io/3yb2r/` to reproduce the analyses.

Results and discussion

Growth profiles

Four bacterial strains, namely, *A. caccae* DSM 14662, *A. hallii* DSM 3353, *R. faecis* DSM 16840 and *P. freudenreichii* DSM 4902 were incubated in CDM, GEM and M2GSC over a period of 48 hours. The growth profiles are provided in Figure 2.

All tested strains demonstrated robust growth in M2GSC and GEM media, reaching Δ O.D values >1.0 throughout the incubation period. Conversely, CDM medium only supported the growth of *A. hallii* DSM 3353 and *P. freudenreichii* DSM 4902, with Δ O.D values <0.51.

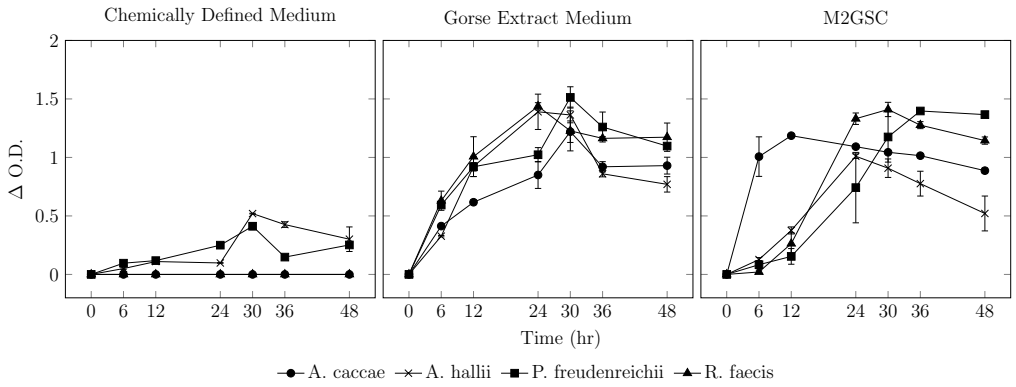

Fig 2. Growth profile of *A. caccae* DSM 14662, *A. hallii* DSM 3353, *P. freudenreichii* DSM 4902, and *R. faecis* DSM 16840 in chemically defined medium (CDM), gorse extract medium (GEM) and M2GSC.

In the experiments previously described by Soto Martín *et al.* [14], *A. caccae* DSM 14662 and *R. faecis* DSM 16840 were grown in different variants of CDM to test trophic capabilities. They were first grown in a semi-defined, vitamin-controlled version of the medium by incorporating vitamin-free casein acid hydrolysate fortified with L-tryptophan, L-serine, L-threonine, L-glutamine, and L-asparagine to check for auxotrophy for each individual vitamin. Subsequently, they were grown on amino-acid controlled CDM which was supplemented with all vitamins to check for auxotrophy for each amino acid. All three strains used here were able to grow well in both versions of CDM [14]. The reason for the discrepancies between the two studies could be due to the precultures, which were adapted to CDM medium for two passages before being used for auxotrophy tests by Soto Martín *et al.* [14], whereas they were grown in M2GSC medium in this study.

The GEM and CDM employed herein were identical in terms of their base composition of added vitamins (both expected to be devoid of natural cobalamins), minerals, micronutrients, and precursors, but differing solely in their carbohydrate and protein sources. While GEM contained residual proteins and peptides, CDM contained purified amino acids. This disparity may have contributed to the lack of growth by *A. caccae* DSM 14662 and *R. faecis* DSM 16840, in CDM. It is known that certain bacterial species have a preference for peptides as a nitrogen source rather than individual amino acids [25]; particularly in anaerobic conditions [26]. The uptake of individual amino acids is energetically demanding and requires active Na^{2+} mediated

transmembrane transport [27] and is the primary rate-limiting step in nitrogen uptake [26]. *A. caccae* DSM 14662 and *R. faecis* DSM 16840 are typically cultured in YCFA medium [28, 29]. But the cobalamin-devoid, free-amino-acid based CDM may have been energetically incompatible for their growth.

B_{12} production in experimental species

The extent of vitamin B_{12} production in the experimental species is shown in Table 1 below.

Table 1. Total B_{12} measured in experimental fractions at 48 h incubation

Species	Media	Lysate	Supernatant
A. caccae	GEM	16.10±7.59	N/D
	CDM	N/G	N/G
	M2GSC	N/D	N/D
A. hallii	GEM	7.40±5.90	N/D
	CDM	N/D	N/D
	M2GSC	N/D	N/D
P. freudenreichii	GEM	15.21±1.00	N/D
	CDM	N/D	N/D
	M2GSC	4.23±0.60[a]	7.95±1.40
R. faecis	GEM	16.34±0.92	N/D
	CDM	N/G	N/G
	M2GSC	0.77±0.12[b]	N/D

Values expressed in mean±standard deviation $\mu g\,L^{-1}$.
Marked values were significantly different from other yields measured across the microbial species for a given medium.
N/D = below detection level, N/G = no growth
B_{12} in all media at time 0 was N/D.

B_{12} vitamers were below detection levels at the 0 h time point for all of the media. In the case of M2GSC, *P. freudenreichii* DSM 4902 and *R. faecis* DSM 16840 were able to produce B_{12}. Although *P. freudenreichii* DSM 4902 showed B_{12} in the supernatant as well as the cell lysate fraction, this could be a result of cell-death given the late stage in the growth profile shown in Figure 2.

Vitamin B_{12} was detected in the lysates of *A. caccae* DSM 14662 and *A. hallii* DSM 3353 cultured in the GEM medium, whereas no B_{12} synthesis was observed in the M2GSC medium, despite M2GSC being considered the undefined, yet "complete" growth medium. The inclusion of DMB in the GEM medium may have played a crucial

role in the production of vitamin B_{12}, as it was only added to GEM. A plausible explanation for the growth of *A. caccae* and *A. hallii* in M2GSC, but absence of corresponding detectable B_{12} in the lysates, may be via the synthesis of pseudo-B_{12} vitamers, that were not detectable by the LC/MS method employed in the study. This hypothesis may also explain why B_{12} levels were lower in the M2GSC compared to GEM in the case of *P. freudenreichii* DSM 4902 and *R. faecis* DSM 16840. While these microbes could successfully synthesise biologically active vitamers in anaerobic conditions, the addition of DMB may have eased the metabolic load otherwise required for its *de novo* synthesis, that resulted in an increase of total B_{12} production in GEM compared to M2GSC.

In this experiment, B_{12} measurement was performed using LC/MS, capable of detection at ng quantities in the sample. Physiologically, such quantities are far above the cellular requirement of the cells [30]. This suggests that the chosen bacterial species were able to overproduce B_{12}, but the experimental conditions, or perhaps the media design was unsuitable for any real commercial application. Moreover, this contributes towards confirmation of the speculation [31] that *A. hallii* (formerly *E. hallii*) could produce B_{12} vitamers in the gut. The three experimental microbes are unviable as sources of B_{12} for industrial applications.

Secondly for probiotic applications, the vitamin production requires to be in excess and in the extracellular environment. Since the chosen microbes could not satisfy both these requirements, they may not be good candidates for probiotic applications.

However, it was concerning to note the low B_{12} yields for *P. freudenreichii* DSM 4902 which was the positive control for this experiment. Generally, P. freudenreichii was expected to have yields in the range of $\geq 0.2\,\mu g\,mL^{-1}$, but corresponding values noted in Table 1 were about 13× lower. A comparison with other literature employing *P. freudenreichii* for B_{12} production was performed to investigate possible causes.

Component analyses using previous literature

From the search from the online databases terms, 1651 hits were returned from which 230 research titles were retrieved post title screening. A final list of 36 titles were selected based on the relevance of the research described therein, and the data points,

namely, concentration of the carbon source ($g\,L^{-1}$), cobalt ($mg\,L^{-1}$), DMB ($mg\,L^{-1}$) and total fermentation time (hours) were retrieved and is presented in **Supplementary Table 1**. This only included cases of batch culturing of *P. freudenreichii*.

The data in **Supplementary Table 1** was first subjected to k-means clustering to look for grouping patterns in the various experimental conditions that may correspond to B_{12} yields. Based on the "elbow method", three groups were deemed ideal to explain clustering in the data (Supplementary Codes). Next, the data was subjected to a univariate-scaled PCA analysis and the corresponding grouping schema was overlaid to visualise their clustering on the PCA plot, as shown in Figure 3.

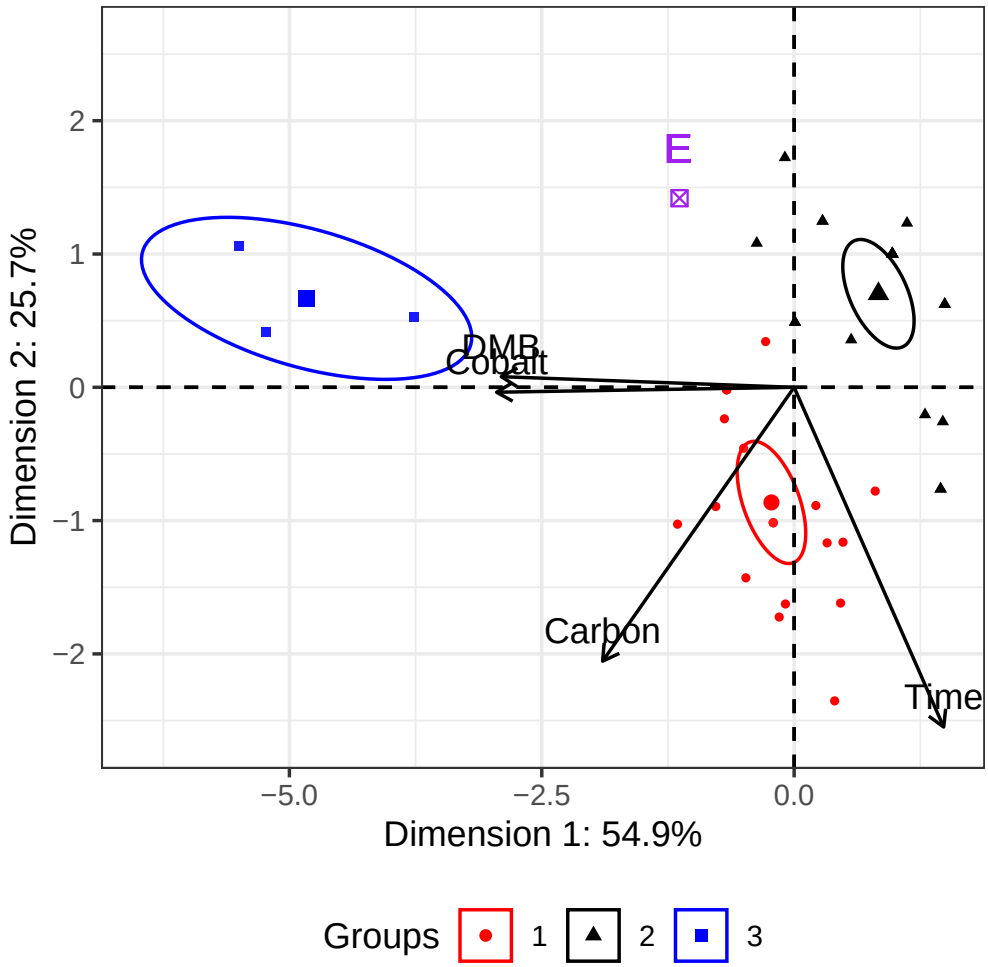

Fig 3. Univariate-scale PCA plot of the experimental conditions employed for B_{12} production using *P. freudenreichii* in previous publications. The point marked 'E' refers to the condition employed in this experiment. The position of the 'E' point was predicted by the model, which appears to associate to cluster 2. Ellipses represent 95 % confidence limits of each cluster.

In Figure 3, the PCA model could account for 80.6 % of the total observed variance in the data across Dimensions 1 and 2. The factors in the model, namely Carbon, Cobalt, DMB and Time accounted for 15.7 %, 37.9 %, 36.8 % and 9.6 % of the variance accounted in Dimension 1. In Dimension 2, Carbon, Cobalt, DMB and Time accounted for 39.4 %, >0.1 %, 0.1 % and 60.6 % of the variance.

Cluster 1, which is placed further from the centre along Dimension 2 represents conditions with media employing high concentrations of carbon source as well as a longer fermentation time, while Cluster 3, which is placed further from the centre along Dimension 1, represents high use of the precursors. The point labelled "E" represents the experimental conditions used in the experiment described here and plots the closest to the mid-point of Cluster 2. The median value of the conditions represented in each cluster is presented in Table 2.

Table 2. Median values of the experimental parameters and B_{12} yield represented in the clusters identified using k-means and PCA analysis.

Groups	**Carbon**	**Cobalt**	**DMB**	**Time**	**B_{12}**
Cluster 1	30 (25-35)	5.5 (5-12.7)	10 (0.9-15.0)	120 (72-16.0)	5.7 (1.3-16.1)
Cluster 2	10 (8.4-10)[a]	0 (0-0)	0 (0-0)	72 (72-102)	0.22 (0.16-2.21)
Cluster 3	40 (31.3-42.0)	40 (29-45)	70 (70-70)	48[b] (47.5-48.0)	6.7 (4.2-7.4)
Experimental	6[a]	10	20	48[b]	0.01

Values expressed as median (25 % quantile to 75 % quantile) to indicate the data distribution.
Carbon represents carbohydrates in the media ($g\,L^{-1}$).
Cobalt and DMB (5,6-dimethylbenzimidazole) are expressed in $mg\,L^{-1}$.
Time represents the total fermentation time (h).
B_{12} was the final reported yield ($mg\,L^{-1}$).
Values with the same superscript were statistically similar using one-sample t-test.

The yield in Cluster 2 ($0.2\,mg\,L^{-1}$) was significantly lower than in Clusters 1 ($5.7\,mg\,L^{-1}$) and 3 ($6.7\,mg\,L^{-1}$), respectively ($F_{(2,\,44)} = 5.298$, $p = 0.009$). These findings suggest that the availability of carbon source (via carbohydrates), was lower in the experimental setup described here compared to previous publications. As presented in Table 1, B_{12} content was predominantly retained within the cell lysate, indicating that the microbes did not secrete B_{12} extracellularly. Consequently, to achieve desired B_{12} yields, it is imperative to supply essential nutrients and precursors at sufficiently high concentrations, as indicated by the values presented in Table 2. **Supplementary Figure 1** illustrates boxplots comparing the levels of Cobalt, Carbon, DMB, and Time for each cluster.

Considerations and limitations

The use of precursors in this experiment prevents distinguishing de novo cobalamin synthesis versus use of salvage pathways by the selected species. The production of DMB is an energetically costly step and consequently most B_{12} synthesis occurs under microaerophilic to aerobic conditions [32]. Alternatively, pseudo-vitamin B_{12} is produced, where DMB is replaced by adenyl-ribofuranose phosphate [33] as shown in Figure 1. For example, *A. hallii* DSM 3353 has been known to produce pseudo-vitamin B_{12} [34], but no studies have yet reported production of B_{12} vitamers with the DMB ligand. Additionally, other colonic microbes like *Lactobacillus reuteri* are known to produce pseudo-cobalamin under anaerobic conditions. In a consortium, there is the added dynamic of microbes synergistically utilising each other's metabolic intermediates to synthesize cobalamin [35]. It would be interesting to investigate whether anaerobic pseudo-B_{12} producers can incorporate externally added DMB in the growth medium to 'assemble' the bioactive B_{12} vitamer.

However, the main objective of this investigation was to provide a limited proof-of-concept demonstrating the ability of *A. caccae* DSM 14662, *A. hallii* DSM 3353, and *R. faecis* DSM 16840 to grow and produce vitamin B_{12} in a medium composed of gorse wastes. This may be extended to the growth of other potential vitamin synthesising colonic commensal microbes.

Conclusion

Three commensal gut microbes, namely, *A. hallii* DSM 3353, *R. faecis* DSM 16840 and *A. caccae* DSM 14662 appear to be capable of synthesising vitamin B_{12} in anaerobic conditions in gorse wastes supplemented with DMB. This is a proof-of-concept for further work using similar plant wastes with other commensal gut microbes, and there were many gaps which need to be addressed in subsequent experimental setups. These can be categorised into basic research and application research.

Basic research may entail the inclusion of additional known anaerobic pseudo-cobalamin producers (e.g., *L. reuteri* and non-pathogenic strains identified in a previous study [36]). There can be further exploration of the use of DMB on pseudo-vitamin producers to test for production of active B_{12} synthesis. This can be

extended by testing the addition of DMB to enhance B_{12} synthesis in a simulated colonic environment using volunteer faecal samples. This could pave the way for larger human applications considering the low toxicity value of DMB. Lastly, the LC/MS method requires to be modified to include the measurement of pseudo-vitamers.

In terms of application based research, further work is required to develop spray-drying or freeze-drying methods to formulate pre/synbiotic products using gorse waste. Furthermore, the list of invasive plants may be expanded to include species such as *Cytisus scoparius* (Broom) or *Pteridium aquilinum* (Bracken), whose waste can support B_{12}-producing microbial growth.

Supporting information

S1 Fig. Measures of experimental parameters in k-means clusters Median levels of B_{12} yield, carbon source, precursors, and fermentation time in the k-means groups, shown in Figure 3. This can be found in the Supplementary Materials file.

S1 Appendix. Supplementary text and details Details on media recipe and LC/MS conditions. This can be found in the Supplementary Materials file.

S1 Table. B_{12} yields in previous publications This can be found in the Supplementary Materials file.

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