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

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Abstract

Many commensal gut microbes are recognized for their potential to synthesize vitamin B_{12}, offering a promising avenue to address deficiencies through probiotic supplementation. While bioinformatics tools aid in predicting B_{12} biosynthetic potential, empirical validation remains crucial to confirm production, identify cobalamin vitamers, and establish biosynthetic yields. This study investigates vitamin B_{12} production in three human colonic bacterial species: *Anaerobutyricum hallii* DSM 3353, *Roseburia faecis* DSM 16840, and *Anaerostipes caccae* DSM 14662, along with *Propionibacterium freudenreichii* DSM 4902 as a positive control. These strains were selected for their potential use as probiotics, based on speculated B_{12} production from prior bioinformatic analyses. Cultures were grown in M2GSC, chemically defined media (CDM), and Gorse extract medium (GEM). The composition of GEM was similar to CDM, except that the carbon and nitrogen sources were replaced with the protein-depleted liquid waste obtained after subjecting Gorse to a leaf protein extraction process. B_{12} yields were quantified using liquid chromatography with tandem mass spectrometry. The results suggested that the three butyrate-producing strains could indeed produce B_{12}, although the yields were notably low and were detected only in the cell lysates. Furthermore, B_{12} production was higher in GEM compared to M2GSC medium. The positive control, *P. freudenreichii* DSM 4902 produced B_{12} at concentrations ranging from 7 ng mL^{-1} to 12 ng mL^{-1}. Univariate-scaled Principal Component Analysis (PCA) of data from previous publications investigating B_{12} production in *P. freudenreichii* revealed that B_{12} yields diminished when the carbon source concentration was $\leq$30 g L^{-1}. In conclusion, the protein-depleted wastes from the leaf protein extraction process from Gorse can be valorised as a viable substrate for culturing B_{12}-producing colonic gut microbes. Furthermore, this is the first report attesting to the ability of *A. hallii*, *R. faecis*, and *A. caccae* to produce B_{12}. However, these microbes seem unsuitable for industrial applications owing to low B_{12} yields.

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

Introduction

The biological role and molecular structure of vitamin B_{12}

Vitamin B_{12} is involved in various enzymatic reactions, including isomerases, methyltransferases, and dehalogenases [1], with B_{12}-dependent dehalogenases being exclusive to bacteria [2]. It is a critical cofactor in the type 1 reaction, catalysing the isomerization of L-methylmalonyl-CoA to succinyl-CoA, that subsequently enters the Krebs cycle [3]. Furthermore, B_{12} is crucial for the type 2 reaction of methyl transfer from 5-methyltetrahydrofolate to homocysteine, to produce tetrahydrofolate and methionine [4]. Hindrance in this type 2 reaction owing to B_{12} deficiency results in the malformation of red blood corpuscles, and manifests as pernicious anaemia [5].

The bioactivity of vitamin B_{12} hinges on three critical ligands, namely, the central cobalt ion located in the upper corrin ring, the methyl side-group, and 5,6-dimethylbenzimidazole (DMB) (see Fig 1). The methyl side-group and DMB are attached to the cobalt ion with a coordinate bond. The cobalt ion switches from the oxidised intermediate (III) to the reduced (I) state during the transfer of the methyl-group to homocysteine to form methionine. The oxidised cobalamin is subsequently re-alkylated with n-methyl-THF. The exact mechanism has been described previously by Matthews *et al.* [6].

Dietary vitamin B_{12} however is generally present with the methyl, adenosyl, hydroxyl or cyano group associated with the central cobalt. The cyano vitamer is a semi-synthetic stable derivative of B_{12} commonly used in dietary and medical applications [7]. Other variants such as azidocobalamin, sulfocobalamin or nitrocobalamin are derivatives synthesised as intermediates for analytical purposes [8]. Regardless of the ingested vitamer, once B_{12} is absorbed in the body, it is converted to the hydroxyl-form, that is then re-alkylated to the methyl form; ultimately entering the methionine synthesis pathway as described previously [4]. There is variation in the second ligand moiety, where either the biologically active DMB, or its structural homologue, adenyl-ribofuranose phosphate, may be present. Only the DMB-based vitamer is capable of participating in the catalytic reaction facilitated by methionine synthase.

Harnessing microbes for industrial B_{12} production

The biosynthesis of B_{12} requires several enzymes, that are only found in the bacteria and archaea domains of life [9, 10]. The complete set of genes required for *de novo* production of cobalamins; particularly B_{12}, has been observed in numerous bacteria such as *Pseudomonas denitrificans*, *Bacillus megaterium*, *Rhodobacter capusulatus*, and *Thermosipho melanesiensis*, to name a few [11]. Many other bacteria such as *Escherichia coli*, *Kosmotoga olearia* and *Fervidobacterium nodosum* can only use salvage pathways to synthesise cobalamin from precursors. The population of these microbes capable of cobalamin production through salvage pathways plays an important role in the micro-ecology of the niche they inhabit, firstly, through commensalistic or even symbiotic association with primary B_{12} producers [9], and secondly, by facilitating tertiary associations with other microbes heterotrophic for the precursor. In industrial setups however, candidate selection of B_{12} producers is largely influenced by the genetic stability of the strain, bioconversion efficiency, B_{12} yields, extracellular production capability, and rapid turnover.

Many microbial species; either naturally, or through genetic engineering, are capable of over-synthesising vitamin B_{12}. The most commonly used species in industry are *Propionibacterium freudenreichii*, *Propionibacterium shermanii* and *Pseudomonas denitrificans*. Growth conditions are generally aerobic as *de novo* biosynthesis of vitamin B_{12} is an energetically demanding process, particularly the ligand DMB [9]. Consequently, there is great variability in

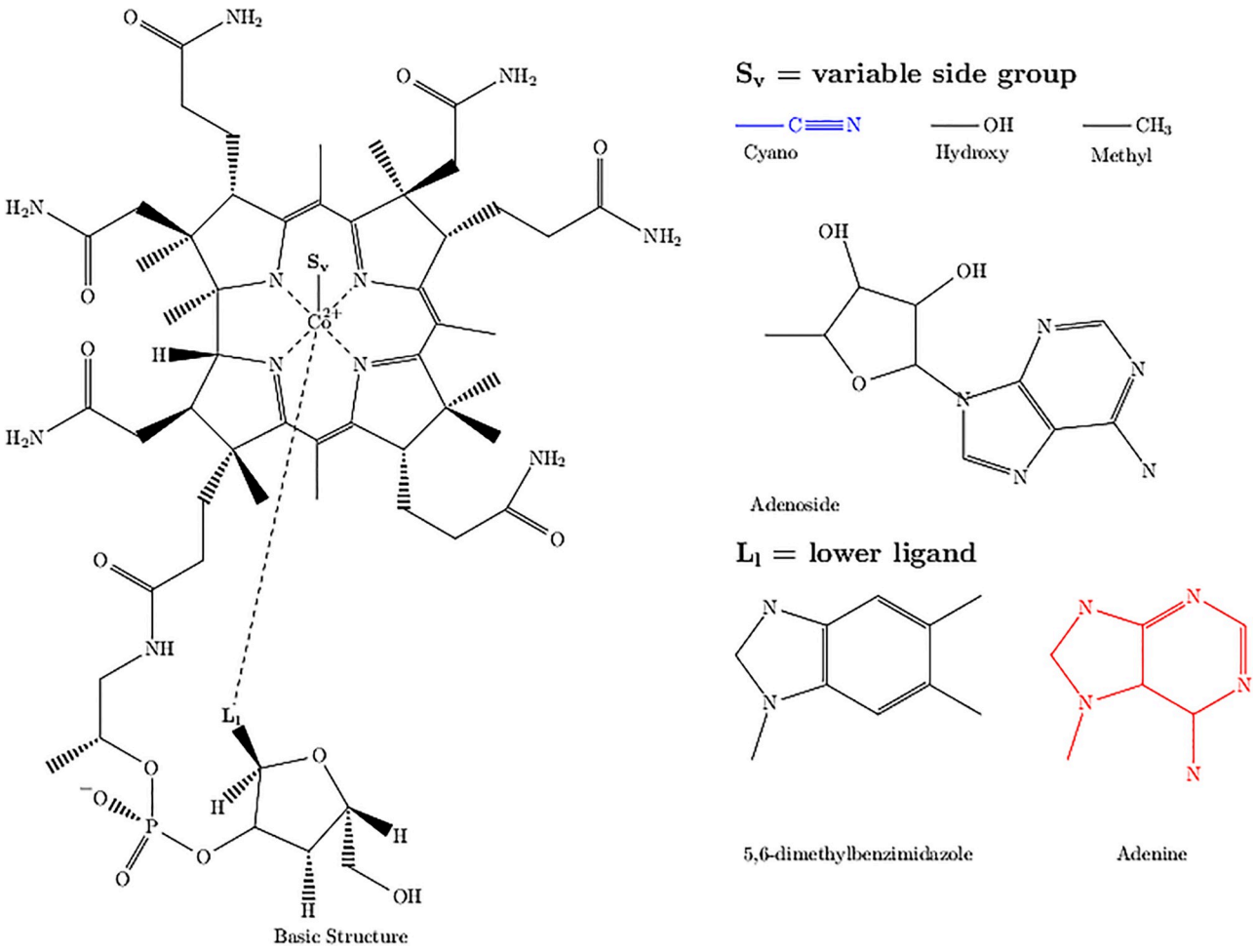

Fig 1. Structure of cobalamin vitamers. Variable side-groups (S_v) form coordinate bonds with the central cobalt ion. The ligands at location L_l determine if the cobalamin vitamer is biologically active in humans with 5,6-dimethybenzimidazole (DMB) or inactive with adenine (marked in red). The cyano-group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

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the substrates used depending on the metabolic capability of the microbe employed. For example, many media formulations use additional supplementation of cobalt, DMB, and in rare cases, haeme [12]. This helps cope with the increased demand for micronutrients and alleviates the metabolic burden of *de novo* DMB or corrin synthesis. Interestingly, this has lead to investigations on waste stream valorisation with cobalt and DMB supplementation for the growth of B_{12} producing bacteria. For example, *P. freudenreichii* has been shown to grow on apple pomace and potato wastes for large scale production of propionic acid, with vitamin B_{12} obtained as a secondary product in significant quantities [13, 14]. Such endeavours help generate high-value products and circularise waste streams otherwise fated for landfill or wastewater treatment plants.

B_{12} from Gorse wastes using gut microbes

Gorse (*Ulex europaeus*) is a shrub belonging to the Fabaceae family that has become invasive in many parts of the world [15]. However it is rich in proteins and plant-bioactives, and a leaf

protein extraction process was previously described to effectively utilise the biomass [16]. The process generated a protein depleted fraction (PDF) that contained residual proteins, plant bioactives, and carbohydrates as a by-product. In an effort to valorise this waste stream, 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 of PDF as the sole carbon and nitrogen source. These microbial candidates were chosen due to their potential to produce vitamin B_{12} [17] and their purported beneficial impact in the human gut [18, 19]. *Propionibacterium freudenreichii* DSM 4902, was used as a positive control for vitamin B_{12} 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

M2GSC. The recipe for M2GSC is provided in section 1.1 of S1 File, and was prepared as described previously by Cummings and Macfarlane [20]. The medium was allowed to equilibrate in the anaerobic cabinet for 48 h before use.

Chemically defined medium (CDM). CDM was prepared as described previously by Soto-Martín *et al* [17], except with the inclusion of 5,6-dimethylbenzimidazole (DMB, 20 mg L^{-1}). The medium was filter-sterilised using 0.2 μm filter (Millipore, Merck, US) and allowed to equilibrate in the anaerobic cabinet. The composition of the CDM is provided in section 1.2 of S1 File.

Gorse extract medium (GEM). 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 [16]. 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. Other monomers such as xylose, fucose, and glucuronic acid were expected to remain largely absent as xylanase, pectinase and other cell-wall digesting enzymes were not employed in the process.

The PDF was used at a final concentration of 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 by Soto-Martín *et al* [17]. *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 incubated 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 stored 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} was quantified using mass spectrometry in tandem with liquid chromatography as described elsewhere [21]. 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 section 1.3 of S1 File.

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 gL^{-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 **S1 Table** in S1 File.

Statistical analysis

All statistical analyses were performed using R (4.2.2) [22] using the tidyverse (2.0.0) [23] 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) [24], and factoextra (1.0.7) [25] packages. A dimension reduction model was made using previously published B_{12} yields from *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://doi.org/10.17605/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 h. The growth profiles are shown in Fig 2.

All tested strains demonstrated robust growth in M2GSC and GEM media, reaching ΔO.D values >1.0. In contrast, CDM medium only supported the growth of *A. hallii* DSM 3353 and *P. freudenreichii* DSM 4902, with ΔO.D values <0.51.

In previous experiments by Soto Martín *et al.* [17], *A. caccae* DSM 14662 and *R. faecis* DSM 16840 were grown in CDM variants to assess trophic capabilities. They were initially grown in a semi-defined, vitamin-controlled medium using 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 in an amino-acid controlled CDM 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 used by by Soto-Martín *et al* [17]. The growth profile in the panel for CDM in Fig 2 differs from previous reports, and may be explained by the pre-culture conditions. Soto-Martín *et al* subjected the microbes through two passages in CDM before measuring their growth, while in this study, the microbes were revived in M2GSC and directly inoculated in CDM.

The GEM and CDM used were identical in their base composition of added vitamins (as well as absence of natural cobalamins), minerals, micronutrients, and precursors. The key

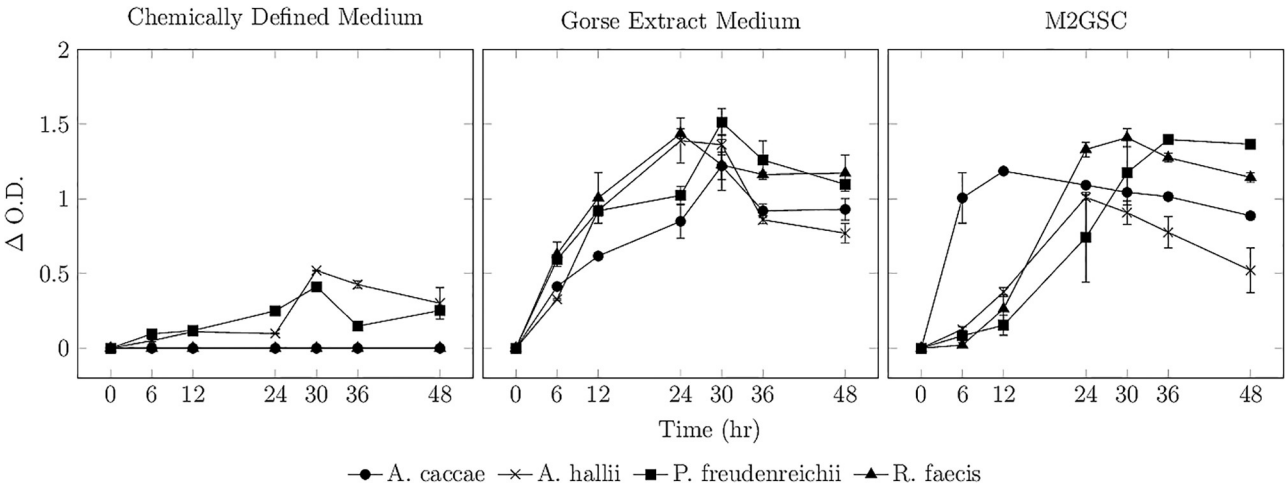

Fig 2. Microbial growth profile. Growth profile of *A. caccae* DSM 14662, *A. hallii* DSM 3353, *P. freudenreichii* DSM 4902, and *R. faecis* DSM 16840 in chemically defined medium (CDM), gorse extract medium (GEM) and M2GSC.

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difference lay in their carbohydrate and protein sources. While GEM contained residual proteins and peptides, CDM contained purified amino acids. This difference may have contributed to the lack of growth by *A. caccae* DSM 14662 and *R. faecis* DSM 16840 in CDM. Certain bacterial species are known to prefer peptides as a nitrogen source rather than individual amino acids [26] under anaerobic conditions [27]. The uptake of individual amino acids is energetically demanding and requires active Na^{2+} mediated transmembrane transport [28] and is the primary rate-limiting step in nitrogen uptake [27]. *A. caccae* DSM 14662 and *R. faecis* DSM 16840 are typically cultured in YCFA medium [29, 30] and the cobalamin-devoid, free-amino-acid based CDM may have been energetically incompatible for their growth.

B_{12} production in experimental species

Vitamin B_{12} yields of the experimental and control bacterial candidates is shown in Table 1 below.

Vitamin B_{12} was below detection levels at the 0 h time point across all tested media. *P. freudenreichii* DSM 4902 and *R. faecis* DSM 16840 were able to produce B_{12} at the 48 h end-point in M2GSC as shown in Table 1. *P. freudenreichii* DSM 4902 showed B_{12} in the supernatant as well as the cell lysate fraction.

Vitamin B_{12} was detected in the lysates of *A. caccae* DSM 14662 and *A. hallii* DSM 3353 cultured in the GEM medium. In contrast, no B_{12} was observed in M2GSC, despite it being the ideal growth medium. DMB was exclusively added to GEM and was likely critical to B_{12} production. The growth of *A. caccae* and *A. hallii* in M2GSC without detectable B_{12} in the lysates could be attributed to the synthesis of pseudo-B_{12} vitamers, that were not detectable by the LC/MS method employed in the study. This may also explain the lowered B_{12} levels in M2GSC compared to GEM for *P. freudenreichii* DSM 4902 and *R. faecis* DSM 16840. Although these microbes demonstrated the ability to synthesize biologically active vitamers in anaerobic conditions, the addition of DMB likely alleviated the metabolic burden required for *de novo* synthesis, resulting in an increased total B_{12} production in GEM compared to M2GSC.

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 μg 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.

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In this experiment, B_{12} measurement was performed using LC/MS, capable of detection at ng quantities in the sample. Physiologically, such quantities are much higher than the metabolic requirement of the cells [31]. 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 helps confirm [32] that *A. hallii* (formerly *E. hallii*) could produce B_{12} vitamers in the gut. However, the three experimental microbes are unviable sources of B_{12} for industrial applications.

Next, for a strain to be viable candidate for probiotic applications towards B_{12} supplementation, it requires to secrete B_{12} in sufficiently large quantities in the extracellular environment. In all the three experimental gut microbes however, B_{12} was only detected in the cell lysates. This makes them unsuitable for application as probiotic B_{12} supplements.

Lastly, of particular concern was the observed low B_{12} yields for *P. freudenreichii* DSM 4902, which served as the positive control for this experiment. Generally, *P. freudenreichii* DSM 4902 has yields $\geq$0.2 μg mL^{-1}, yet the corresponding values noted in Table 1 were about 13× lower. A comparative analysis with other literature investigating *P. freudenreichii* for B_{12} production was conducted to explore potential causes.

Component analyses using previous literature

The systematic online searches yielded 1651 hits 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. The data points for the concentration of the carbon source (gL^{-1}), cobalt ($mg\ L^{-1}$), DMB ($mg\ L^{-1}$) and total fermentation time (h) were retrieved and is presented in S1 Table in S1 File. This only included cases of batch culturing of *P. freudenreichii*.

The data in **S1 Table** in S1 File 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 (S2 File). 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 Fig 3.

In Fig 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 represents conditions with media employing high concentrations of carbon source, as well as a longer fermentation time, Cluster 2 represents the absence of precursors and low carbon source concentrations, and Cluster 3 represents high use of the precursors.

The point labelled "E" represents the experimental conditions and plots the closest to the mid-point of Cluster 2 ($d = 0.718$) compared to Clusters 1 ($d = 2.57$) and 3 ($d = 5.86$), where d represents the Euclidean distance between the points. This implies that the experimental condition described in this work was similar to that represented by Cluster 2. The median value of the conditions represented in each cluster is presented in Table 2.

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

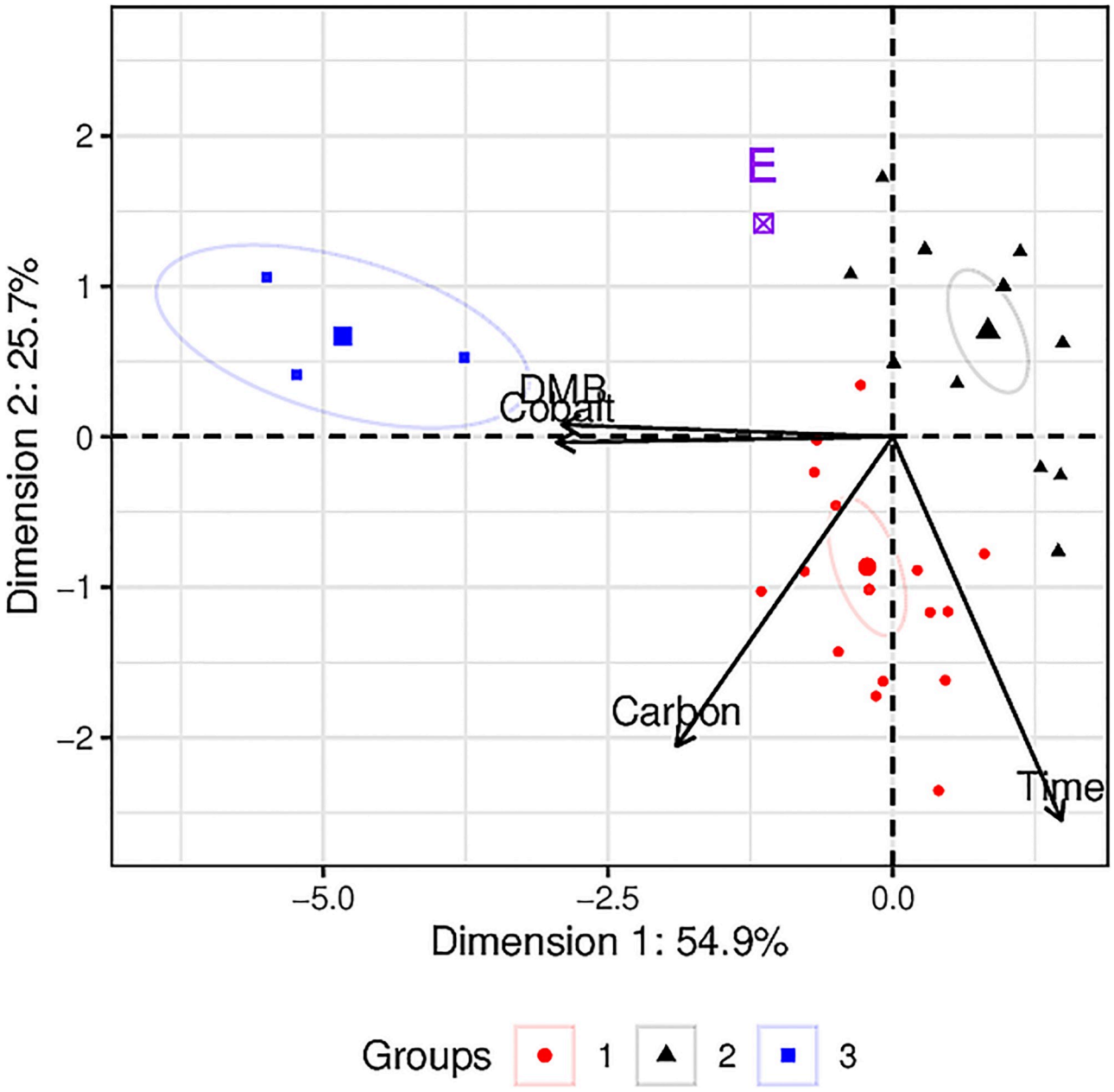

Fig 3. Dimension reduction of experimental conditions to elucidate their influence on B_{12} yields. Previously reported experimental conditions were clustered using k-means analysis. The clustering scheme was overlaid on a univariate-scale PCA model that was constructed using previously reported parameters for the growth of *Propionibacterium freudenreichii*, namely, 5,6-dimethylbenzimidazole (DMB, mg L^{-1}), cobalt (mg L^{-1}), total carbohydrates (Carbon, g L^{-1}), and incubation time (h). The point marked 'E' refers to the intrapolated position of the experimental conditions described herein and correspondingly, the expected B_{12} yields, which appears to associate to cluster 2. Ellipses represent 95% confidence limits of each cluster. Data and codes used for dimension reduction analyses is provided in S2 File.

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presented in Table 2. S1 Fig illustrates boxplots comparing the levels of Cobalt, Carbon, DMB, and Time for each cluster.

Thus the data from Table 2 suggest that the yields of B_{12} are impacted when the carbohydrate concentration in the media is > 30 g L^{-1}.

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.

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Limitations and scope for further work

The primary focus of this study was to investigate the ability of PDF to support the growth of the three B_{12} producing anaerobic gut microbes. The secondary aim was to establish their B_{12} yields under these experimental conditions, and check if the vitamin was secreted in the extracellular medium. Understanding their ligand uptake or secretion mechanisms, or attempting to enhance yields for industrial application was beyond the scope of this work.

Consequently, there are some aspects of this experiment that warrant additional investigation. For example, *A. hallii* has previously been reported to produce pseudo-vitamin B_{12} [33]. Thus, while the present work could demonstrate that biologically active B_{12} was produced by *A. hallii*, it is difficult to conclude if the microbe can facultatively produce the bioactive vitamer if the ligands are externally supplied without a control medium devoid of DMB.

Expanding on the investigation, it is worth noting that other colonic microbes, such as *Lactobacillus reuteri*, are known to produce pseudo-cobalamin under anaerobic conditions. Such microbes present an interesting avenue for further investigation to understand facultative production of the bioactive B_{12} vitamer when ligands are externally supplied.

Lastly, the individual vitamers and pseudo-vitamers were not characterised. More work will be required to understand if there are unique stoichiometries in which these vitamers are produced.

Conclusion

The investigation could show for the first time that *A. hallii*, *R. faecis* and *A. caccae* produced vitamin B_{12}, albeit in very low quantities under our experimental conditions. While this is an important stepping-stone for further research on elucidating the salvage pathways for B_{12} biosynthesis, these bacterial candidates appear to be unsuitable for industrial-scale B_{12} production.

The protein depleted fraction (PDF) stands out as a valuable carbon and nitrogen source, capable of supporting the growth of gut microbes. The work presented in this publication sets the stage for developing circular processes to effectively utilise the Gorse biomass, and valorise the liquid waste stream to cultivate microbes with B_{12} production potential, and pave the way for sustainable probiotic products.

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 Fig

3. This can be found in the Supplementary Materials file.
(TIFF)

S1 File. Supplementary file containing recipe for M2GSC and CDM, and also S1 Table. All supplementary files and information is provided in this document.
(PDF)

S2 File. The codes used to analyse the data. The codes can use the data from the supplementary tables to reproduce the results.
(PDF)

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