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Co-Cultivation of Rumen Bacteria Enhances VitB12 (Cobalamin) Biosynthesis under Optimized Substrate Conditions

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Abstract

Introduction: Vitamin B12 (vitB12) micronutrient deficiency remains a significant global health issue, driving the search for new microbial sources capable of producing this essential nutrient. While industrial strains have been well studied, there is limited understanding of the genetic and metabolic diversity among culturable ruminal bacteria that may contribute to vitB12 synthesis. Given that most bacteria possess only partial sets of vitB12 biosynthesis genes, we hypothesized that ruminal bacteria rely on synergistic interactions to collectively establish a complete and functional vitB12 biosynthetic pathway.

Objectives: This study presents results on isolating, culturing, and genetically characterising the ruminal isolates while evaluating the impact of substrate supplementation on vitB12 production.

Methods: VitB12-producing bacteria isolates were isolated using Brain Heart Infusion Agar. These isolates were then phenotypically characterized, including biochemical and physical tests, while genotypic characterization was performed based on the 16S rRNA gene sequence. Followed by characterization of the vitB12 genetic elements in sequenced genomes from the NCBI public repository. We targeted the cob, hem, and cbi gene cluster and pdu operons. We then evaluated the presence of cobA, cobI, and cobM genes in our isolates. The isolates were screened for VitB12, using the quantitative analysis of vitamins in culture supernatants, done by using HPLC equipped with a UV detector, a gold-standard method for accurately measuring vitB12 and its derivatives. We enriched culture media using betaine and molasses substrates to improve the yield of VitB12 production.

Results: A total of 65 colonies were identified from 18 treatments in each of the three ruminal samples. From the 65 colonies, 25 were randomly selected and sub-cultured for morphological and biochemical characterization, of which 10 were from aerobic and 15 from anaerobic culture conditions. Our ruminal isolates showed multiple morphologies, suggesting the presence of different strains. Using homology comparisons, our isolates clustered with *Arthrobacter* spp., *Bacillus* spp., and *S. ruminantium* with similarities ranging from 95.1% to 100%. Our ruminal isolates expressed CobA and CobM, but not CobI. While most genes tend to be clustered in unique operons, there exists a diversity of genomic organization across different species. Overall, the ruminal isolates can produce vitB12 when co-cultured than when cultured individually. Individually, AnR2-C4 and AnR3-C4 did not produce a detectable amount of vitB12, but AnR2-C3 produced a quantifiable amount, averaging 0.082 mg/L. The combination of AR2-C3 and AnR2-C4 yielded a higher amount (3.116 mg/L) than AR2-C3 alone. Combination of the three, AR2-C3 + AnR2-C4 + AnR3-C4, yielded the highest quantity (4.481 mg/L). Supplementation of culture media with molasses and betaine enhanced the vitB12 yield.

Conclusions: This study demonstrates that ruminant-derived bacteria, particularly strains resembling *Ruminococcus* and *Lactobacillus reuteri*, are promising sources for vitB12 production. Using culturing, genomic analysis, and media enrichment, it showed how these bacteria work synergistically to support a functional B12 biosynthesis pathway. Genomic comparisons revealed species-specific adaptations in *cob* and *cbi* genes arrangements, reflecting their response to environmental and metabolic conditions. The limitation of a single isolate having an incomplete set of vitB12 genes is offset by the natural co-existence of multiple

strains in a single ecosystem. Additionally, supplementing culture media with molasses and betaine may further boost vitB12 yields.

Keywords: VitB12, rumen microbiome, bacterial isolates, 16S rRNA, cobalt, molasses, and betaine

1. Introduction

Vitamin B12 (vitB12, cobalamin) is an essential micronutrient in the human body, acting as a critical co-factor for DNA synthesis, neurological genesis and function, and erythrogenesis [1],[2]. Its biosynthesis is extraordinarily complex, requiring over 30 enzymatic steps, which makes chemical synthesis cost-prohibitive and positions microbial fermentation as the preferred industrial production method [2]. Only a small range of bacteria are capable of de novo synthesis of vitB12, among them are natural symbionts in ruminants [3]. Due to the rising demand for vitB12 in human nutrition and animal feed, manipulating the microbial fermentation through culture media improvement and genetic engineering is of significant economic and sustainability interest [3]. However, despite the vital role of vitB12 in human health, the diversity of ruminal bacteria and their vitB12 genetic determinants is not clearly understood.

In herbivores, the rumen represents a unique microbial ecosystem where diverse bacterial communities interact to degrade complex plant materials and synthesize essential metabolites, including vitB12 [4],[5]. Within this environment, bacteria such as *Bacillus* spp., *Arthrobacter* spp., *Prevotella* spp., and *Ruminococcus* spp. contribute to vitB12 biosynthesis through distinct metabolic pathways [3]. The metabolic diversity in the rumen allows for the possibility of synergistic interactions, where the different species can enhance complement, setting a complete vitB12 biosynthetic pathway [6].

Moreover, vitB12 biosynthesis is a multifaceted process that involves many steps of enzyme-mediated reactions and proceeds via two distinct pathways: aerobic and anaerobic. The primary distinction between the aerobic and anaerobic pathways of vitB12 biosynthesis lies in their reliance on oxygen-dependent enzymes and the time when cobalt atom is incorporated along the pathway. Aerobic bacteria are late cobalt incorporators, while anaerobic bacteria insert cobalt early in the pathway. The aerobic bacteria, such as *Pseudomonas denitrificans*, require molecular oxygen to drive several oxidative steps for ring contraction and cobalt insertion into the corrin ring, whereas anaerobic bacteria like *Propionibacterium shermani*i and *Lactobacillus reuteri* use alternative enzymes that bypass oxygen-dependent reactions [7]. Despite this fundamental difference, both pathways share key biosynthetic stages that begin with the formation of a modified tetrapyrrole precursor (uroporphyrinogen III), followed by sequential methylation of the ring to yield various precorrin intermediates. Cobalt is then chelated into the ring structure via specialized chelatases, forming the corrin macrocycle that characterizes vitB12 [8]. Subsequently, the macrocycle undergoes ring contraction, achieved through either an oxygen-dependent mechanism in aerobic organisms or an oxygen-independent process in anaerobic species, culminating in the conversion of precorrin intermediates into corrinoids with cobalt at their core. The final stages involve the adenosylation or methylation of the completed corrin structure, producing the active coenzyme forms of vitB12, such as adenosylcobalamin or methylcobalamin, which are essential for various metabolic functions, including methylation reactions and the conversion of methylmalonyl-CoA to succinyl-CoA [9].

Overall, vitB12 synthesis involves many enzymes, regulators, and transport proteins encoded by distinct clusters/operons of genes, including hem-cob-cbi-cbl and pdu genes [10]. In the aerobic pathway, key cobalamin genes designated cob genes mediate critical methylation and rearrangement reactions necessary for the assembly of the corrin ring. However, in oxygen-limited environments, the anaerobic pathway utilizes homologous genes, strategically designated cbi genes, to perform analogous functions [11]. In addition, the genetic elements responsible for vitB12 biosynthesis varies among bacterial species, with each organism possessing a distinct set of vitB12-active genes that reflect its evolutionary adaptation to specific environmental niches and substrate availabilities. This diversity in a genetic organization ultimately leads to significant variability in vitB12 production across microbial communities [10].

The de novo vitB12 bacterial culturing is a vital starting point in identifying and enriching novel microbial strains capable of vitB12 production. By cultivating environmental isolates under conditions that favour vitB12 synthesis, selecting robust bacterial populations before introducing optimized substrate conditions plays a crucial role in modulating both growth rates and vitamin yields. Molasses, rich in sugars, trace minerals, and nitrogen sources, provides the energy and cofactors needed for efficient bacterial metabolism [12]. Betaine, on the other hand, serves not only as a methyl donor for critical methylation reactions in vitB12 biosynthesis but also as an osmoprotectant that enhances cell viability under stress conditions [13]. The careful balance of these substrates is essential, as excessive concentrations may lead to metabolic inhibition or osmotic stress, thereby reducing overall fermentation efficiency.

Here we report the characterization of isolates recovered from ruminal samples acquired from commercial abattoirs in Nairobi City County, Kenya. We genotyped the ruminal colonies using 16S rRNA genes and used homology comparisons to identify which known species our colony sample isolates match. Further, we determined the presence of the different vitB12 active genes in our isolates. We assessed the culture, bacterial growth behaviours and their ability to produce measurable vitB12. We used molecular techniques to confirm the presence of cobA, cobI, and cobM in the ruminal isolates in conjunction with examining the genomic synteny of vitB12 synthesizing known genes, including the hem-cob-cbi-pdu operons. We report that the co-existence of multiple rumen symbiotic bacteria enables them to generate a full set of active vitB12 biosynthetic enzymes. The overall vitB12 productivity improves with substrate supplementation, but only to a limited proportion. Specifically, synergistic co-cultivation of rumen bacterial isolates under optimized substrate conditions, through the use of molasses and betaine, significantly enhances vitB12 biosynthesis. By leveraging the complementary metabolic capabilities of diverse rumen bacteria and fine-tuning substrate concentrations, key vitB12-producing bacteria that can be co-cultured can be identified and targeted for upscaling vitB12 productivity.

2. Objectives

We aimed to isolate, culture, and genetically characterize the ruminal isolates, while evaluating the impact of substrate supplementation on vitB12 production.

3. Methods

Samples collection

Study samples were collected from cattle rumen at a licensed commercial export abattoir called NEEMA LIVESTOCK AND SLAUGHTERING INVESTMENTS LTD (NL&SIC), located within Kasarani sub-county in Nairobi City County, Kenya. The identities of the animals were not revealed, thus making our sampling blind. Cattle selected for rumen sample collection were based on the healthy status of the animal. The rumen fluid was obtained fresh during the slaughtering process following ethical guidelines and approved protocols for animal handling [14]. The rumen fluid samples were randomly collected from the source with the help of the public health inspector and the general manager of the abattoir. The collected rumen fluid was immediately transferred to sterile anaerobic containers pre-flushed with nitrogen gas to maintain the anaerobic environment essential for microbial viability. Samples were transported in a cooler box with ice blocks to the PAUSTI molecular laboratory at Jomo Kenyatta University of Agriculture and Technology (JKUAT) and processed immediately.

Culturing of the rumen fluid bacteria

From each of the sample stocks of the rumen fluid, an aliquot of 1 ml was serially diluted ten-fold with sterile Phosphate-buffered saline (PBS), of a volume of 9 ml. The last three dilutions of each sample were plated by the spread method on nutrient agar with pH readjusted to 6.5 and incubated at 37°C for 48 hours, either under aerobic or gas pack anaerobic conditions [15]. Thereafter, the colonies were observed and characterized. The individual initial colonies were sub-cultured twice to obtain pure lines using the streaking method on Brain Heart infusion (BHI) media enriched with cobalt chloride hexahydrate to support the growth of vitB12-producing bacteria [16]. The media pH was adjusted to 6.5 to optimize microbial growth and enzymatic activity. The plates were incubated under different conditions as above at 37°C for 48 hours (see **Fig. S1 and S2**).

Morphological Characterization

The colony morphologies were observed based on form, elevation, margin, size, texture, surface, and opacity, with reference to standard microbiological techniques [17]. The colonies from the aerobic plates were observed under the biosafety cabinet with a source of white light for clear characterization. On the other hand, the anaerobic plates were observed under the atmosphere glove box to maintain the anaerobic conditions, and had a white light for clear observation.

Molecular characterization based on the 16S rRNA gene

The genomic DNA was isolated from pure cultures grown under both aerobic and anaerobic conditions using the Quick-DNA Fungal/Bacterial Miniprep Kit (D6005), following the manufacturer's protocol by ZYMO Research [18]. The JENWAY Genova Nano nanodrop spectrophotometer was employed to determine the concentration (17.086–111.53 ng/µL), purity, and potential contamination of the extracted genomic DNA. Genotyping was done by amplifying the 16S rRNA gene using a ProFlex Base PCR system with universal primers: fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTAC-CTTGTTACGACTT) (Frank et al., 2008). The total reaction volume for the PCR mixture was 50 µL, comprising 2 µL of purified bacterial DNA (17.086–111.53 ng/µL) as the template, 25 µL of One Taq 2x Master Mix, 1 µL of forward primer (1 µM), 1 µL of reverse primer (1 µM), and 21 µL of nuclease-free water. The genomic DNA from *Lactococcus* spp. was used as the positive control. The PCR cycling conditions in the ProFlex Base PCR system were programmed as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 3 minutes, annealing at 57°C for 30 seconds, and extension at 72°C for 1.5 minutes. The reaction concluded with a final extension step at 72°C for 5 minutes. The PCR products were resolved on a 1% (w/v) agarose gel in 1X TAE buffer stained with SafeView (ABM G108), and viewed against a 1-kilobase pair PR4100 molecular ladder. The amplicons were then sent to Macrogen Asia Pacific Pte. Ltd. for paired-end sequencing using the Sanger method.

The sequence reads were trimmed using trimmonatic version 0.39 [19] to remove background noise and refined using Geneious Biologics software version prime 2025.0 [20]. The resulting sequences were subjected to a homology search against the non-redundant bacterial nucleotide database at the National Center for Biotechnology Information (NCBI) database using the blastn algorithm at default settings (www.ncbi.nlm.nih.gov). The top blast hits were then selected as the corresponding homologs and retrieved for phylogenetic clustering. In total, 50 sequences (comprising the aerobic, anaerobic, and homologs) were multiply aligned using the MUSCLE version 5 alignment tool [21]. Thereafter, a phylogenetic tree was reconstructed using the maximum approach using the IQ-Tree tool version 2.0.7, which automatically chose the best fitting evolutionary model and reliably resampled 1000 bootstrap trees [22]. The final tree was rendered and visualized using FigTree Version 1.4.4 (http://tree.bio.ed.ac.uk/).

Molecular characterization based on the *Cob* genes

The genomic sequences used for designing primers for the specific genes were *Arthrobacter* spp., *L. reuteri*, and *S. rumimantium* downloaded from the NCBI databases (corresponding to phylogenetic clustering matching our selected isolates AR2-C3, AnR3-C4, and AnR2-C4). We amplified cob genes - cobA, cobM, and cobI using a ProFlex Base PCR system with primers tailored for each isolate, as shown in **Table S1** using the software called APE Tool [23]. The total reaction volume for the PCR mixture was 50 µL, comprising 2 µL of purified bacterial DNA (17.086–111.53 ng/µL) as the template, 25 µL of One Taq 2x Master Mix, 1 µL of forward primer (1 µM), 1 µL of reverse primer (1 µM), and 21 µL of nuclease-free water. The reaction mixture, excluding the DNA, served as the negative control. The PCR cycling conditions in the ProFlex Base PCR system were programmed as shown in Table S2, representing each isolate done in 35 cycles. Upon completion of the PCR reaction, the amplified PCR products were resolved on a 1% (w/v) agarose gel prepared in 1X TAE buffer and stained with SafeView (ABM G108). A 50 bp ladder, PR4005 Accuris SmartCheck™ DNA Ladder was used as a molecular weight marker.

Genomic synteny of vitB12 based on Hem, Cob, Cbi, and Pdu active genes

The whole-genome sequences of a select known bacteria capable of producing vitB12 were downloaded from NCBI databases and viewed for manual annotation of known vitB12-active genes using Artemis genome viewer tool

(https://www.sanger.ac.uk/tool/artemis/). Once an-notated, comparative genomics analyses were conducted to identify both orthologous genes and syntenic blocks. This step allowed for the highlighting of conserved regions within the cob and cbi gene clusters across the different bacterial species under investigation.

Quantification of the vitB12 production from the isolates

Quantitative analysis of vitB12 in culture supernatants was conducted using High-Performance Liquid Chromatography (HPLC) equipped with a UV detector, a gold-standard method for accurately measuring vitB12 and its derivatives [24]. To facilitate accurate quantification of vitB12, the samples were pre-treated with potassium cyanide under alkaline conditions to convert all corrinoid forms to cyanocobalamin for accurate quantification [25]. A freshly prepared potassium cyanide solution was added to the supernatant, and the mixture was adjusted to pH 10 using sodium bicarbonate as an appropriate buffer. The reaction mixture was then incubated at 60°C for 30 minutes (allowing the complete conversion of naturally occurring cobalamin forms (methylcobalamin and adenosylcobalamin) and intermediate corrinoids into the stable cyanocobalamin form). To ensure precision and reproducibility, culture supernatants were collected from bacterial cultures grown under optimized conditions for vitB12 production. The cultures were centrifuged at 2500 rpm for 20 minutes to remove cells and debris, yielding clear supernatants for analysis [26].

The pre-treated samples were filtered through 0.22 µm syringe filters to remove particulate matter and injected into the HPLC system. The system was equipped with a reverse-phase C18 column, which provided high resolution and separation of cyanocobalamin from other compounds in the sample. The mobile phase typically consisted of a mixture of methanol and an aqueous buffer adjusted to a suitable pH for optimal retention and resolution. Detection was performed using a UV detector set at 361 nm, the wavelength at which cyanocobalamin exhibits maximum absorbance. The retention time for cyanocobalamin was determined using an external standard, and a calibration curve was prepared using known concentrations of commercially available cyanocobalamin from Inqaba Biotec East Africa Limited. The peak area of cyanocobalamin in the samples was compared to the calibration curve to quantify the concentration of vitB12 produced by the bacterial isolates. The results were expressed as milligrams of cyanocobalamin per litter (mg/L) of culture supernatant [27].

Evaluation of culture media for vitB12 production

The initial media formulation for bacterial growth was based on a Brain Heart Infusion (BHI) medium, subjected to two substrates (molasses and betaine) of three different concentrations (0.005, 0.010, and 0.015g/ml). These substrates were selected based on their involvement in key biosynthetic pathways, which could enhance bacterial metabolism and growth efficiency [28].

Bacterial growth response of bacteria under improved media

To monitor bacterial growth, optical density 600nm (OD600) measurements were taken at regular intervals using a spectrophotometer, which allowed for the evaluation of bacterial proliferation over time. Measurements were taken at regular intervals to determine the growth curve. Additionally, bacterial cell viability was assessed using a cell counter machine, providing insight into the effect of different molasses and betaine concentrations on bacterial survival [29]. This was then followed by using HPLC machine to check which substrates managed to maximize the synthesis of vitB12, resulting in an enhanced media culture.

4. Results

Laboratory cultures and morphological characterization reveal multiple colonies

A total of 65 colonies were identified from 18 treatments in each of the three ruminal samples. Of these, 27 colonies, rep 41.5 %, were observed under aerobic conditions, while 38 colonies, rep 58.5 %, were observed under anaerobic conditions (**Fig.1**).

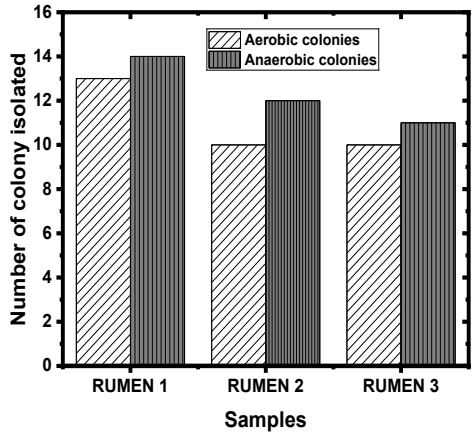

Figure 1. Average number of colonies from the ruminal samples grown under aerobic and anaerobic conditions. Each sample was plated in triplicate. There were a total of 65 colonies (rumen 1=27, rumen 2=20, and rumen 3=18). More colonies grew under anaerobic conditions.

From the 65 colonies, 25 were randomly selected and sub-cultured for morphological and biochemical characterization, of which 10 were from aerobic and 15 from anaerobic culture conditions. The sets of bacteria were sub-cultured on blood agar at 37°C for 48 hours in a biosafety cabinet for aerobic colonies and anaerobic colonies in an atmosphere glove box. In both set-ups, the sub-cultured colonies appeared either circular or irregular with smooth or undulated edges, and convex, flat, or raised elevations. The observed colonies can be placed into eight groups: under aerobic conditions (i) circular shape, entire margin with convex elevation, (ii) circular, entire with raised elevation, (iii) irregular, undulated, convex, (iv) irregular, undulated, and raised; and under anaerobic condition (v) circular, entire, and convex, (vi) circular, entire, and raised, and (vii) irregular, undulated, and flat and (viii) irregular, undulated, and raised (**Table 1**).

Table 1: Morphological characteristics of the colonies.

Colonies	Colony shape	Colony edge/ margin	Colony elevation
Rumen 1			
AR1.C1	Circular	Entire	Raised
AR1.C2	Circular	Entire/ smooth	Raised
AR1.C3	Irregular	Undulated	Convex
AR1.C4	Circular	Entire	Raised
AnR1.C1	Circular	Entire	Convex
AnR1.C2	Circular	Entire	Convex
AnR1.C3	Circular	Entire	Convex
AnR1.C4	Irregular	Undulated	Flat
AnR1.C5	Circular	Entire	Raised
AnR1.C6	Circular	Entire	Convex
Rumen 2			
AR2.C1	Circular	Entire	Raised
AR2.C2	Circular	Entire	Convex
AR2.C3	Circular	Entire	Convex
AR2.C4	Circular	Entire	Convex
AnR2.C1	Irregular	Undulated	Raised
AnR2.C2	Irregular	Undulated	Convex
AnR2.C3	Circular	Entire	Convex
AnR2.C4	Circular	Entire	Convex
AnR2.C5	Circular	Entire	Convex
Rumen 3			
AR3.C1	Irregular	Undulated	Raised
AR3.C2	Irregular	Undulated	Raised
AnR3.C1	Circular	Entire	Convex
AnR3.C2	Circular	Entire	Convex
AnR3.C3	Circular	Entire	Convex
AnR3.C4	Circular	Entire	Convex

Abbreviations: A = Aerobic, R = Rumen (1,2,3), An = Anaerobic, C = colony number

Molecular genotyping using the 16S rRNA gene suggests isolates are homologous to *Arthrobacter*, *Bacillus*, and *Selenomonas* species.

To identify the bacteria in the 25 colonies (10 from aerobic conditions and 15 from anaerobic conditions), genomic DNA was extracted, amplified using universal 16S rRNA primers, and sequenced. All 25 colonies were amplified and showed a size of about 1465 bp except colonies 1, 4, and 15 (**Fig. 2**). Using homology searches and phylogenetic analysis revealed isolates clustered with *Arthrobacter, Bacillus,* and *Selenomonas* species - including *Bacillus (Priestia) megaterium*, *B. cereus, Lactobacillus reuteri*, and *Selenomonas ruminantium* (**Fig. 3**).

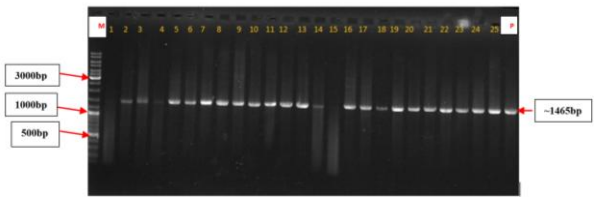

Figure 2: PCR amplifications of the 16S rRNA gene from the ruminal bacteria. The amplicons average 1465 bp. The DNA ladder (M) used was 1kb ladder-PR4100. Lane 1, 4, and 15 did not amplify. The genomic DNA from *Lactococcus* spp. was used as the positive control (P).

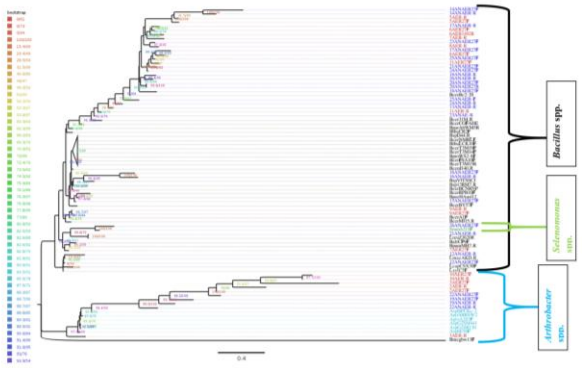

Figure 3. Evolutionary relationships between the 16S rRNA gene sequences of ruminal bacteria and their closely matching homologs from public databases. The major groups identified are species from the genera *Arthrobacter, Bacillus*, and *Selenomonas*. The ruminal samples grown under aerobic conditions are written in **red** font, while those under anaerobic conditions are in **blue** font. The homologs in **black** fonts are *Bacillus* spp. (including *Priestia megaterium*, *Lysinbacillus* spp., and *Lactobacillus* spp.), in **green** are the *Selenomonas* spp., and in **sky blue** are *Arthrobacter* spp. The legend represents bootstrap support values for the nodes as a percentage out of 1000 statistical bootstraps. The tree was generated using the IQTree Version 2.0.7 program [30] and visualized using FigTree Version 1.4.4 (http://tree.bio.ed.ac.uk/).

Different isolates encode different vitB12-active genes

We targeted to amplify three vitB12 active cob genes - CobA, CobM, and CobI - whose 16S rRNA genes clustered with *Arthrobacter* spp., *Lactobacillus reuteri*, and *Selenomonas* (**see Fig. 3**). We successfully amplified CobA and CobM in the genomic DNA of AR2-C3 (lane 1), AnR3-C4 (lane 2), and AnR2-C4 (lane 3) pure isolates. However, CobI did not amplify from the chosen isolates (**Fig. 4**). We mapped these genes in the vitB12 biosynthetic pathway reproduced here (**Fig. 5**).

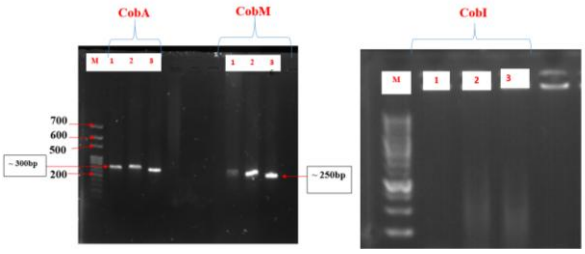

Figure 4: Amplifications of CobA, CobM, and CobI genes in the genomic DNA from the pure isolates (AR2-C3 (lane 1), AnR3-C4 (lane 2), and AnR2-C4 (lane 3)) that clustered with *Arthrobacter spp., Lactobacillus reuteri,* and *Selenomonas ruminantium*. CobA and CobM amplified while CobI looked indeterminate in all three isolates selected.

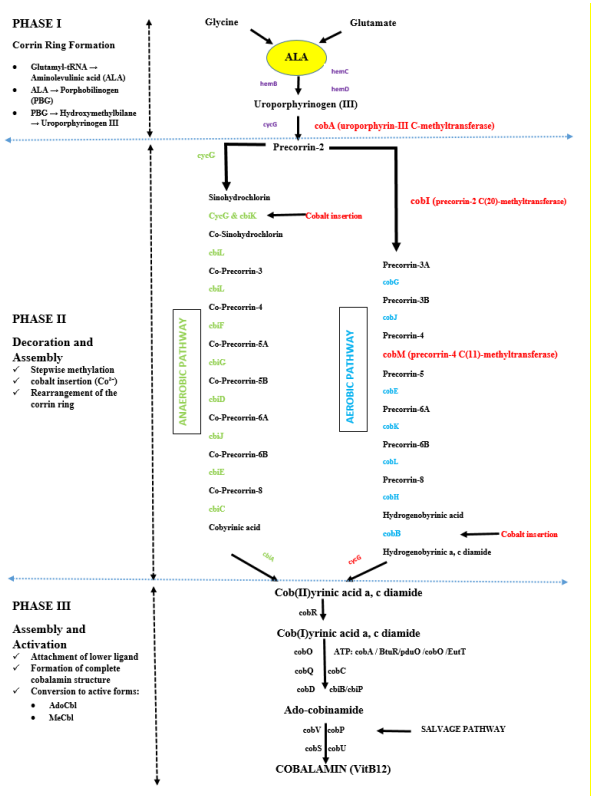

Figure 5. Vitamin B12 biosynthesis occurs via two distinct pathways depending on the organism's oxygen requirements: The **Aerobic Pathway** (Oxygen-dependent) and the **Anaerobic Pathway** (Oxygen-independent). Both pathways lead to the synthesis of vitB12, but they differ in the mechanism of cobalt insertion and ring contraction. The biosynthesis of vitB12 is divided into three distinct phases: **(I)** Corrin Ring Formation, **(II)** Decoration and Assembly, and **(III)** Activation and Transport. The enzyme CobA (uroporphyrin-III C-methyltransferase) catalyzes the initial methylation step, converting uroporphyrinogen III to precorrin-2. CobI (precorrin-2 C(20)-methyltransferase) is responsible for transferring a methyl group from S-adenosylmethionine (SAM) to precorrin-2, converting it into precorrin-3A. The enzyme CobM (precorrin-4 C(11)-methyltransferase) plays a critical role in methylation, contributing to the transformation of precorrin-4 to precorrin-5.

Comparative genomic synteny of vitB12-active genes

We then systematically examined the genomic organization of the vitB12 active genes in the genomes of selected vitB12-producing bacteria sourced from the NCBI repositories. We established that vitB12 is diverse at the nucleotide level, and rarely does a homology search using gene sequences from one species find their counterparts in another species. But relying on manual examination of the genomes, enabled us to identify the genes already assigned cobalamin name symbols and by homology their neighbors. While different genomes possess different sets of vitB12 genes, majority of the genes are organized in tandem syntenic blocks of distinct operons each

genome. Except for *L. reuteri* that have all the vitB12 active genes in a single operon on same strand, the other genomes have them scattered in different operons, with different neighbors on either of the strands (**Fig. S4**). Analysing the amino acids of the identified sequences show they form neat clusters of homology; highlighting the genes (cobA, cobM, and cobI) amplified in our ruminal isolates (**Fig. 6**).

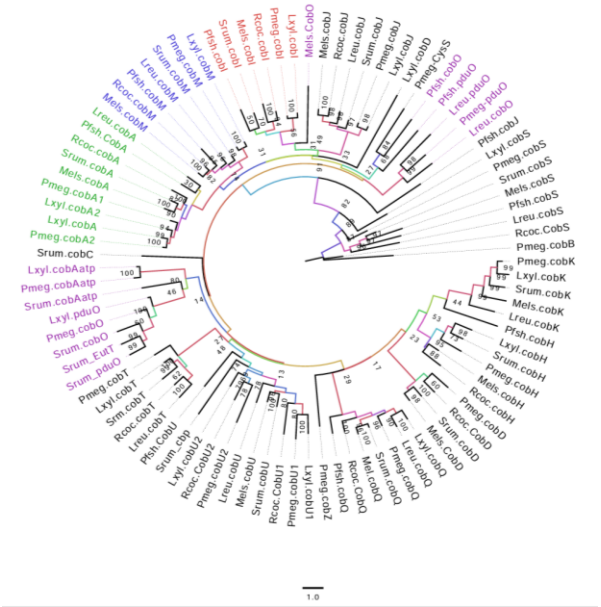

Figure 6. Phylogenetic tree analysis of the vitB12 biosynthesis Cob proteins retrieved from a select vitB12 bacterial genomes. Pfsh – *Propionibacterium freudenreichii* sub. *shermanii* (reference species), Pmeg – *Priestia megaterium,* Mels – *Megasphaera elsdenii,* Lreu – *Lactobacillus reuteri* (known anaerobic species), Srum – *Selenomonas ruminantium,* Rcoc – *Ruminococcus,* Lxyl – *Lysinibacillus xylanilyticus*. Despite the cob genes being highly divergent at DNA level, the orthologous active enzymes cluster into unique clades – e.g. CobA, CobM, and CobI (highlighted). The nomenclature CobA is also assigned to energy-dependent enzyme Ado-cobinamide synthases named ATP: CobA/BtuR/PduO/CobO. The analysis indicates that not all bacterial genomes code for all the cob genes, e.g. *L. reuteri* lacks CobI.

Evaluation of culture media requirements and maximal vitB12 productivity

We then determined if our isolates that at least showed the presence of CobA and CobM were able to synthesize vitB12 either individually or in combination. Individually, AnR2-C4 and AnR3-C4 did not produce a detectable amount of vitB12 but AR2-C3 produced a quantifiable amount averaging 0.082 mg/L (Fig. S3). The combination of AR2-C3 and AnR2-C4 yielded a higher amount (3.116 mg/L) than AR2-C3 alone (**Fig. 7A**). Combination of the three, AR2-C3 + AnR2-C4 + AnR3-C4 yielded the highest quantity (4.481 mg/L) (**Fig. 7B, C**).

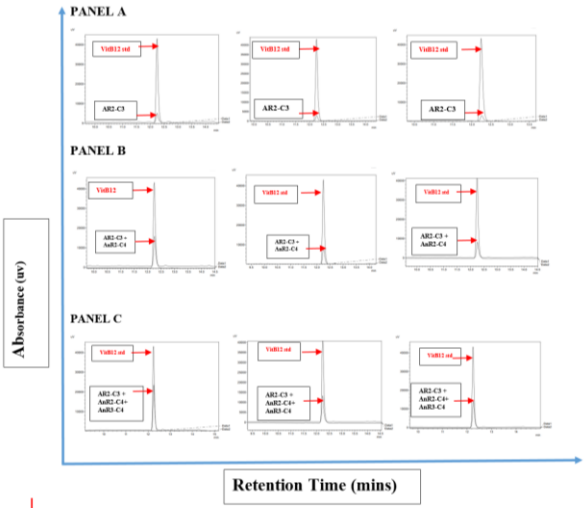

Figure 7: Quantification chromatographs of vitB12 synthesized by the ruminal isolates. Panel A chromatograph of isolate AR2-C3 (clustering with *Arthrobacter* spp.) indicating a small peak of vitB12 at an average concentration of 0.082 mg/L. Panel B is a combined chromatograph of AR2-C3 and AnR2-C4 (clustering with *Arthrobacter* spp., and *Lactobacillus reuteri* respectively) indicating peaks of vitB12 at an average concentration of 3.116 mg/L. Panel C, a combined chromatograph of isolates AR2-C3, AnR2-C4, and AnR3-C4 (clustering with *Arthrobacter* spp., *Lactobacillus reuteri*, and *Selenomonas ruminantium*, respectively) indicating peaks of vitB12 at an average concentration of 4.481 mg/L. The viB12 concentrations were relative to the commercial vitB12 standard (represented by the Data1 curve). The quantification was done on HPLC LC-2050C 3D (LIQUID CHROMATOGRAPH) of the Inspection Certificate of serial No. L22986103219 AE, P/N 228-65822-58, using Column C18.

Evaluation of culture media enhances the productivity of vitB12

After establishing that our isolates when co-cultured can synthesize vitB12, we then next determined the best culturing media conditions that can yield the most vitB12. Therefore, in addition to the normal media variables supplemented with 1.0 mM cobalt chloride hexahydrate (temperature of 37°C, pH 6.5) cultured under anaerobic conditions, we added either molasses or betaine and evaluated performance via bacterial growth using optical density measurements, cell viability using cell counter machine, and vitB12 yield measured using HPLC as before. We found that at a concentration of 0.01g/mL betaine was a superior substrate for bacterial growth compared to molasses. The terminal growth rate after 12 hours for betaine was at OD_{600} = 2.5, while for molasses was at OD_{600} = 1.9. However, at higher substrate concentrations (0.015g/mL), the growth rates substantially reduced (**Fig. 8A** and **B**). Similarly, betaine

supported the highest cell counts (16.0 $\times 10^6$ cells/mL) relative to molasses (13.5 $\times 10^6$ cells/mL) at 12 hours, and cell viability declined at higher substrate concentrations in each case (**Fig. 8C and D**). Upon co-culturing the three isolates for 48 hours under anaerobic conditions, betaine yielded more vitB12 than molasses. However, at higher substrate concentrations (0.015g/ml), the vitB12 yields declined with both substrates (**Fig. 9**).

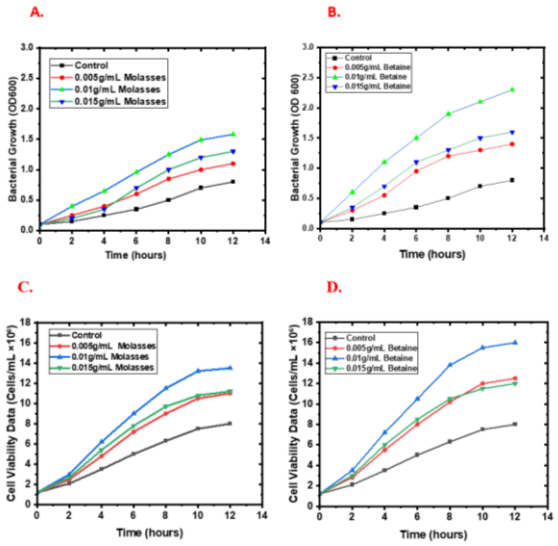

Figure 8: The effects of molasses and betaine substrates on the growth rates and viability of the combined vitB12-producing bacterial isolates. **A**. growth curves with molasses, **B**. growth curves with betaine substrates.**; C** cell viability with molasses and, **D.** cell viability with betaine. The growth rate OD600 and cell viability measurements were each taken at a time interval of two hours.

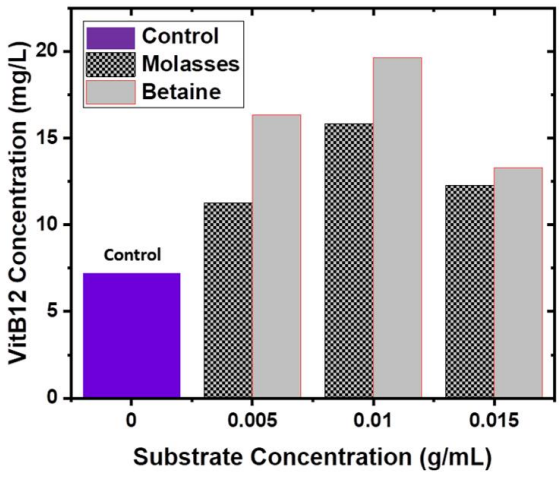

Figure 9: The HPLC VitB12 yields of the three co-cultured isolates with betaine and molasses-supplemented media. At all concentrations, Betaine yields higher VitB12 compared to molasses (Betaine peak values at 19.2 mg/L and Molasses at 15.8 mg/L).

5. Discussion

The rumen of herbivores is a natural microbial ecosystem, containing communities of bacteria, archaea, protozoa, and fungi that collaborate to break down complex plant materials [5][31]. Among these microorganisms, bacteria are the most abundant and play pivotal roles in fermenting carbohydrates, proteins, and lipids [32]. Diverse species of bacteria are known to inhabit the herbivore rumen, and concertedly contribute to the production of the vitB12 [33].

In this study, we cultured and characterized bacteria recovered from rumen fluid originally sourced from cattle at a commercial abattoir in Nairobi City County. Naturally, the rumen is an oxygen-limited ecosystem [34][35]. We cultured a total of 65 colonies under anaerobic and aerobic conditions, but observed more colony growth under anaerobic compared to aerobic conditions (**Fig.1**). These findings support prior reports that the majority of rumen bacteria are obligate or facultative anaerobes, optimising carbohydrate fermentation under low-oxygen tensions [32]. Typically, under experimental conditions, some facultative anaerobes exhibit growth when oxygen levels are minimal [34]. In contrast, strict anaerobes often fail to proliferate if oxygen is present [32], which explains the lower colony counts on our aerobic plates (**Fig.1**).

Morphological characterization revealed a diversity of eight possible groups of the colonies (**Table 1**). These different morphologies suggest the presence of multiple bacteria strains adapted to the ruminal micro-environment. These observations provide an initial measure of microbial diversity under varying oxygen availability. Therefore, employing both aerobic and anaerobic setups is key to capturing the broadest range of rumen microbiota.

Further, 16S rRNA genotyping revealed close homology between our isolates and known vitB12-synthesizing bacterial genera, including *Bacillus, Lactobacillus, Lysinibacillus*, and *Selenomonas* (**Fig. 3**), in correspondence to the previous report [36][37]. Notably, a subset of our isolates clustered with *Priestia megaterium* and *Selenomonas ruminantium*, both of which are well documented for their capacity to synthesize vitB12 [38]. Another group of isolates occupied a branch proximal to *Lactobacillus reuteri*, a species noted for its probiotic properties and, in certain strains, the ability to produce vitB12 [39]. The close

phylogenetic relationship indicated by short branch lengths and robust statistical support, reinforces the likelihood that these isolates possess similar biosynthetic pathways crucial to vitB12 production [3].

Rumen bacterial species prioritize available nutrient resources to synthesize specific products essential for their survival. In some cases, they synergistically combine metabolic pathways for example, in vitB12 biosynthesis, one species provides precursors while another completes the pathway to enhance overall efficiency [40]. This requires the activation and expression of different sets of genes. VitB12 biosynthesis requires the coordinated activity of several gene families (**see Fig. S4).** The cob gene family encodes enzymes that catalyse key methylation, adenosylation, and rearrangement reactions in the aerobic pathway, while the cbi gene family performs analogous roles under anaerobic conditions, ensuring proper corrin ring assembly [1][41]. Additionally, the hem gene family is involved in the early steps of tetrapyrrole biosynthesis, supplying essential precursors such as uroporphyrinogen III [41]. Moreover, the pdu gene family, which is primarily associated with propanediol utilization, is linked to vitB12-dependent metabolic processes, reflecting the intricate interplay between cobalamin synthesis and its functional integration within bacterial metabolism [41].

Except for a small lineage of bacteria known to de novo synthesize vitB12, the majority of bacteria do not possess a complete set of vitB12 active genes and therefore cannot synthesize vitB12 individually. For this reason, we hypothesized that for the bacteria co-existing in the rumen to biosynthesize vitB12, each of them would possess incomplete sets of the respective genes. We amplified CobA, CobM, from the genomic DNA of AR2-C3, AnR2-C4, and AnR3-C4 isolates while CobI did not amplify (**see Fig. 4**). The enzyme CobA (adenosylcobinamide phosphates, AdoCbi-P) is crucial in the conversion of uroporphyrinogen III to pre-corrin-2 in the initial stage of corrin ring assembly second phase of vitB12 synthesis [42]. Successful amplification of CobA in our isolates reveals it as one of the most conserved vitB12 active genes crucial in pre-divergence stages of aerobic and anaerobic pathways of cobalamin synthesis. Enzyme CobM (precorrin-4 methyltransferase) is crucial in the methylation and rearrangement processes prior to cobalt insertion in the aerobic pathway [37]. The homolog of CobM in the anaerobic sub-pathway is CbiE [41].

On the other hand, CobI is the first enzyme that converts pre-corrin-2 to precorrin 3 at the branch for aerobic pathway. Probably, the primers designed for CobI based on a reference sequence may not perfectly complement the target regions in all our bacterial isolates due to inherent sequence variability, leading to inefficient binding [1][43]. Another factor could be that the CobI gene may genuinely be absent or present in very low copy numbers in some of our samples, given the natural diversity in cobalamin biosynthesis pathways among rumen bacteria [40]. Different species possess distinct sets of vitB12-active genes, and it is possible that certain isolates do not harbour the CobI gene at all [44]. Alternatively, non-amplification of the cobI gene may indicate that our rumen bacterial isolates rely on an anaerobic rather than an aerobic pathway for vitB12 synthesis, given that cobI encodes the precorrin-2 methyltransferase specifically required in the aerobic route [37]. While the absence of cobI would suggest an anaerobic pathway, the concurrent detection of cobM complicates this inference. A closer genomic examination of active vitB12 operons (see **Fig. S4; Fig. 6**) rules out *S. ruminantium, P. megaterium*, and *M. elsdenii* and instead points toward *Ruminococcus* or *Lactobacillus reuteri* as the most likely matches. Notably, *Ruminococcus* lacks cobI but harbours cobM, whereas *M. elsdenii* carries cobI alongside cobM within the same operon. *P. shermanii* encodes cobI and cobM in tandem, *L. reuteri* also lacks cobI and retains cobM, *S. ruminantium* positions cobI in an operon separate from cobM, and *P. megaterium* features both genes as neighbors (see **Fig. S4**). These distinct gene arrangements clarify the genetic basis of vitB12 biosynthesis in each organism and strengthen the conclusion that the isolates in question are more closely aligned with *Ruminococcus* or *L. reuteri*.

The comparative genomic analysis revealed both conserved and variable arrangements of cob and cbi genes across the bacterial species, reflecting their distinct biosynthetic pathways and ecological adaptations [10]. In strains utilizing the aerobic route, such as *P. shermanii*, the cob genes were typically clustered in a linear arrangement with minimal interspersing of non-related genes. Conversely, isolates leveraging the anaerobic pathway, including *P. megaterium* and certain *Ruminococcus* species, displayed well-defined cbi

gene clusters arranged in tight operons, enabling efficient synthesis under low-oxygen conditions [10]. In some cases, additional regulatory elements and transposable sequences were observed near cob and cbi clusters, hinting at past genomic rearrangements that may have fine-tuned pathway expression under rumen-specific pressures. These structural differences highlight the evolutionary diversity of vitB12 biosynthesis and emphasize the potential for ecological niche-driven adaptation among rumen microbes [26].

The phylogenetic tree analysis of Cob proteins from selected vitB12-producing bacterial genomes (see **Fig. 6**) provides insights into the evolutionary relationships and functional clustering of these enzymes. Despite the high sequence divergence of cob genes at the DNA level, their encoded proteins group into distinct phylogenetic clades based on functional similarity. This suggests that, although genetic sequences vary significantly across different bacteria, natural selection has preserved the structure and function of key enzymes involved in vitB12 biosynthesis [3]. Notably, enzymes such as CobA (Uroporphyrinogen III methyltransferase), CobM (Precorrin-4 C(11)-methyltransferase), and CobI (Precorrin-2 C(20)-methyltransferase) form unique clusters, reinforcing their conserved roles in the path-way (see **Fig. 5**). The CobA designation is not exclusive to a single function but is also used for ATP: CobA/BtuR/PduO/CobO enzymes, which catalyse the synthesis of adenosylcobinamide, an important step in vitB12 metabolism.

Not all individual isolates possess the full set of vitB12 active genes and when co-cultured, they synergistically synthesize the vitB12 co-factor, yielding an average concentration of 4.481 mg/L (**Fig. 7**). This finding implies that some of our rumen fluid isolates are capable of synthesizing vitB12 independently, yet they do so inefficiently under the tested parameters as previously observed [7]. These results strongly suggest synergistic interactions within mixed cultures, in which complementary metabolic processes such as the provision of specific corrin ring intermediates or cobalt uptake mechanisms, enhance overall cobalamin biosynthesis [45][46]. Alternately, one species secretes enzymes or growth factors that stimulate its partner's metabolic output, further facilitating vitB12 production [47]. Such findings highlight the complexity of vitB12 biosynthesis in multi-species communities, paralleling the rumen's intricate ecology [7]. Metabolic pathways often span multiple organisms; thus, isolated cultivation may be inadequate for full vitB12 synthesis [7]. By replicating a more "rumen-like" environment in the laboratory through co-cultivation, the metabolic network is restored, enabling robust vitB12 formation [37].

In addition, the co-cultured bacterial isolates (AR2-C3, AnR2-C4, and AnR3-C4) were able to grow in brain heart infusion broth supplemented with different concentrations of molasses and betaine. The control setup confirmed that the bacteria could grow in the absence of both of these substrates but at a lower growth rate (**Fig. 8**). These findings align with previous studies indicating carbon and nitrogen source optimization is critical for microbial fermentation efficiency [2]. While culture media supplementation with either betaine or molasses significantly enhanced vitB12 production (**Fig. 9**), the enhancement declines at higher substrate concentrations, suggesting substrate inhibition or metabolic limitations, as previously observed that exceeding optimal substrate levels can lead to feedback inhibition and reduced metabolic efficiency, ultimately lowering vitB12 yields [44].

Conclusion

Overall, this research confirms that ruminant-derived bacterial isolates can serve as valuable sources for vitB12 production. Through de novo culturing, genomic analyses, and targeted culture media enrichment techniques, we identified candidate strains likely to be *Ruminococcus* and *Lactobacillus reuteri*, demonstrating that ruminal bacteria synergize to create a functional vitB12 biosynthesis pathway. Comparative genomic work further elucidated differences in cob and cbi gene arrangements, highlighting how each species adapts its vitB12 operons to specific environmental pressures and metabolic requirements. Supplementation of culture media with substrates such as molasses and betaine, potentially enhances vitB12 yields. These findings lay the groundwork for future metabolic engineering and industrial-scale fermentation efforts, with the ultimate goal of developing cost-effective, high-yield bacterial systems for vitB12 production in both food and feed applications.

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Authors' contributions

Boitumelo Tubutubu conducted the methodology, data curation and prepared the original draft. Edward G. Mamati and George F. Obiero supervised, reviewed and edited the study. All authors have read and approved the final version of the manuscript for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

References

[1] J. H. Martens, H. Barg, M. Warren, and D. Jahn, "Microbial production of vitamin B12," *Appl. Microbiol. Biotechnol.*, vol. 58, no. 3, pp. 275–285, 2002, doi: 10.1007/s00253-001-0902-7.

[2] H. Fang, J. Kang, and D. Zhang, "Microbial production of vitamin B12: A review and future perspectives," *Microb. Cell Fact.*, vol. 16, no. 1, pp. 1–14, 2017, doi: 10.1186/s12934-017-0631-y.

[3] L. Balabanova, L. Averianova, M. Marchenok, O. Son, and L. Tekutyeva, "Microbial and genetic resources for cobalamin (Vitamin b12) biosynthesis: From ecosystems to industrial biotechnology," *Int. J. Mol. Sci.*, vol. 22, no. 9, 2021, doi: 10.3390/ijms22094522.

[4] A. M. Vet, "Rumen microorganisms and fermentation Microorganismos y fermentación ruminal," vol. 361, pp. 349–361, 2014.

[5] H. G. Perez, C. K. Stevenson, J. M. Lourenco, and T. R. Callaway, "Understanding Rumen Microbiology: An Overview," *Encyclopedia*, vol. 4, no. 1, pp. 148–157, 2024, doi: 10.3390/encyclopedia4010013.

[6] A. Kumar, H. A. Palfrey, R. Pathak, P. J. Kadowitz, T. W. Gettys, and S. N. Murthy, "The metabolism and significance of homocysteine in nutrition and health," *Nutr. Metab.*, vol. 14, no. 1, pp. 1–12, 2017, doi: 10.1186/s12986-017-0233-z.

[7] Á. Calvillo, T. Pellicer, M. Carnicer, and A. Planas, "Bioprocess Strategies for Vitamin B12 Production by Microbial Fermentation and Its Market Applications," *Bioengineering*, vol. 9, no. 8, pp. 1–24, 2022, doi: 10.3390/bioengineering9080365.

[8] E. Raux, H. L. Schubert, and M. J. Warren, "Biosynthesis of cobalamin (vitamin B12): A bacterial conundrum," *Cell. Mol. Life Sci.*, vol. 57, no. 13–14, pp. 1880–1893, 2000, doi: 10.1007/PL00000670.

[9] Á. Calvillo, T. Pellicer, M. Carnicer, and A. Planas, "Developing a single-stage continuous process strategy for vitamin B12 production with Propionibacterium freudenreichii," *Microb. Cell Fact.*, vol. 22, no. 1, pp. 1–12, 2023, doi: 10.1186/s12934-023-02029-x.

[10] D. A. Rodionov, A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand, "Comparative Genomics of the Vitamin B12 Metabolism and Regulation in Prokaryotes," *J. Biol. Chem.*, vol. 278, no. 42, pp. 41148–41159, 2003, doi: 10.1074/jbc.M305837200.

[11] G. L. Holliday *et al.*, "Evolution of enzymes and pathways for the biosynthesis of cofactors," *Nat. Prod. Rep.*, vol. 24, no. 5, pp. 972–987, 2007, doi: 10.1039/b703107f.

[12] C. Saejung and L. Puensungnern, "Evaluation of Molasses-Based Medium as a Low Cost Medium for Carotenoids and Fatty Acid Production by Photosynthetic Bacteria," *Waste and Biomass Valorization*, vol. 11, no. 1, pp. 143–152, 2020, doi: 10.1007/s12649-018-0379-6.

[13] M. Cappuccilli *et al.*, "Vitamin B supplementation and nutritional intake of methyl donors in patients with chronic kidney disease: A critical review of the impact on epigenetic machinery," *Nutrients*, vol. 12, no. 5, pp. 1–20, 2020, doi: 10.3390/nu12051234.

[14] R. Fortina, S. Glorio Patrucco, S. Barbera, and S. Tassone, "Rumen Fluid from Slaughtered Animals: A Standardized Procedure for Sampling, Storage and Use in Digestibility Trials," *Methods Protoc.*, vol. 5, no. 4, 2022, doi: 10.3390/mps5040059.

[15] P. Kuppusamy *et al.*, "Low-carbohydrate tolerant LAB strains identified from rumen fluid: Investigation of probiotic activity and legume silage fermentation," *Microorganisms*, vol. 8, no. 7, pp. 1–17, 2020, doi: 10.3390/microorganisms8071044.

[16] S. D. Katz, "The Streak Plate Protocol," *Am. Soc. Microbiol.*, no. March, pp. 1–10, 2008, [Online]. Available: https://asm.org/ASM/media/Protocol-Images/The-Streak-Plate-Protocol.pdf?ext=.pdf

[17] A. C. Ogodo, D. I. Agwaranze, M. Daji, and R. E. Aso, "Microbial techniques and methods: Basic techniques and microscopy," *Anal. Tech.*

Biosci. from Basics to Appl., no. January 2022, pp. 201–220, 2022, doi: 10.1016/B978-0-12-822654-4.00003-8.

[18] Zymo Research, "Quick-DNA ™ Fungal / Bacterial Miniprep Kit. DNA from tough-to-lyse fungi and bacteria samples.," no. 888, pp. 1–11, 2021, [Online]. Available: https://files.zymoresearch.com/protocols/_d6005_quick-dna_fungal-bacterial_miniprep_kit.pdf

[19] A. M. Bolger, M. Lohse, and B. Usadel, "Genome analysis Trimmomatic : a flexible trimmer for Illumina sequence data," vol. 30, no. 15, pp. 2114–2120, 2014, doi: 10.1093/bioinformatics/btu170.

[20] M. Kearse *et al.*, "Geneious Basic : An integrated and extendable desktop software platform for the organization and analysis of sequence data," vol. 28, no. 12, pp. 1647–1649, 2012, doi: 10.1093/bioinformatics/bts199.

[21] R. C. Edgar, R. M. Drive, and M. Valley, "MUSCLE : multiple sequence alignment with high accuracy and high throughput," vol. 32, no. 5, pp. 1792–1797, 2004, doi: 10.1093/nar/gkh340.

[22] L. Nguyen, H. A. Schmidt, A. Von Haeseler, and B. Q. Minh, "IQ-TREE : A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies," vol. 32, no. 1, pp. 268–274, 2014, doi: 10.1093/molbev/msu300.

[23] M. W. Davis and E. M. Jorgensen, "ApE , A Plasmid Editor : A Freely Available DNA Manipulation and Visualization Program," vol. 2, no. February, pp. 1–15, 2022, doi: 10.3389/fbinf.2022.818619.

[24] C. L. Girard, R. Berthiaume, S. P. Stabler, and R. H. Allen, "Identification of cobalamin and cobalamin analogues along the gastrointestinal tract of dairy cows," *Arch. Anim. Nutr.*, vol. 63, no. 5, pp. 379–388, 2009, doi: 10.1080/17450390903020364.

[25] Lawrance Paul, "An Evaluation of Procedures for the Determination of Vitamin B12 in Foods, Supplements and Premixes using HPLC and UPLC after selective extraction with immunoaffinity cartridges," *Lgc*, 2011.

[26] P. Lawrance, "Vitamin B 12 - A review of analytical methods for use in food Vitamin B12 - A review of analytical methods for use in food," *Gov. Chem. Program. Rep.*, no. March, p. 8, 2015.

[27] A. Lebiedzińska, M. L. Marszałł, J. Kuta, and P. Szefer, "Reversed-phase high-performance liquid chromatography method with coulometric electrochemical and ultraviolet detection for the quantification of vitamins B1 (thiamine), B6 (pyridoxamine, pyridoxal and pyridoxine) and B12 in animal and plant foods," *J. Chromatogr. A*, vol. 1173, no. 1–2, pp. 71–80, 2007, doi: 10.1016/j.chroma.2007.09.072.

[28] K. Li, W. Peng, J. Zhou, and X. Cheng, "Establishment of beet molasses as the fermentation substrate for industrial vitamin B 12 production by Pseudomonas denitrificans," no. January, 2013, doi: 10.1002/jctb.4025.

[29] T. Berninger, Ó. González López, A. Bejarano, C. Preininger, and A. Sessitsch, "Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants," *Microb. Biotechnol.*, vol. 11, no. 2, pp. 277–301, 2018, doi: 10.1111/1751-7915.12880.

[30] L. T. Nguyen, H. A. Schmidt, A. Von Haeseler, and B. Q. Minh, "IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies," *Mol. Biol. Evol.*, vol. 32, no. 1, pp. 268–274, 2015, doi: 10.1093/molbev/msu300.

[31] L. M. Teixeira and V. L. C. Merquior, *The Prokaryotes: GAmma*. 2014.

[32] J. B. Russell and J. L. Rychlik, "Factors that alter rumen microbial ecology," *Science (80-.).*, vol. 292, no. 5519, pp. 1119–1122, 2001, doi: 10.1126/science.1058830.

[33] F. Watanabe and T. Bito, "Vitamin B12 sources and microbial interaction," *Exp. Biol. Med.*, vol. 243, no. 2, pp. 148–158, 2018, doi: 10.1177/1535370217746612.

[34] B. A. Dehority and A. A. Odenyo, "Influence of diet on the rumen protozoal fauna of indigenous African wild ruminants," *J. Eukaryot. Microbiol.*, vol. 50, no. 3, pp. 220–223, 2003, doi: 10.1111/j.1550-7408.2003.tb00121.x.

[35] K. Li, B. Shi, and R. Na, "The Colonization of Rumen Microbiota and Intervention in Pre-Weaned Ruminants," *Animals*, vol. 13, no. 6, 2023, doi: 10.3390/ani13060994.

[36] J. R. Roth, J. G. Lawrence, and T. A. Bobik, "Cobalamin (coenzyme B12): Synthesis and biological significance," *Annu. Rev. Microbiol.*, vol. 50, pp. 137–181, 1996, doi: 10.1146/annurev.micro.50.1.137.

[37] Y. Mohammed, B. Lee, Z. Kang, and G. Du, "Development of a two-step cultivation strategy for the production of vitamin B12 by Bacillus megaterium," *Microb. Cell Fact.*, vol. 13, no. 1, pp. 1–10, 2014, doi: 10.1186/s12934-014-0102-7.

[38] E. Raux *et al.*, "Salmonella typhimurium cobalamin (vitamin B12) biosynthetic genes: Functional studies in S. typhimurium and Escherichia coli," *J. Bacteriol.*, vol. 178, no. 3, pp. 753–767, 1996, doi: 10.1128/jb.178.3.753-767.1996.

[39] M. P. Taranto, J. L. Vera, J. Hugenholtz, G. F. De Valdez, and F. Sesma, "Lactobacillus reuteri CRL1098 produces cobalamin," *J. Bacteriol.*, vol. 185, no. 18, pp. 5643–5647, 2003, doi: 10.1128/JB.185.18.5643-5647.2003.

[40] K. A. Rea *et al.*, "Biosynthesis of cannflavins A and B from Cannabis sativa L," *Phytochemistry*, vol. 164, no. April, pp. 162–

171, 2019, doi: 10.1016/j.phytochem.2019.05.009.

[41] D. A. Rodionov, A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand, "Comparative Genomics of the Vitamin B12 Metabolism and Regulation in Prokaryotes," *J. Biol. Chem.*, vol. 278, no. 42, pp. 41148–41159, 2003, doi: 10.1074/jbc.M305837200.

[42] E. Raux, A. Lanois, M. J. Warren, A. Rambach, and C. Thermes, "Cobalamin (vitamin B12) biosynthesis: Identification and characterization of a Bacillus megaterium cobI operon," *Biochem. J.*, vol. 335, no. 1, pp. 159–166, 1998, doi: 10.1042/bj3350159.

[43] A. G. Vitreschak, D. A. Rodionov, A. A. Mironov, and M. S. Gelfand, "Regulation of the vitamin B12 metabolism and transport in bacteria by a conserved RNA structural element," *Rna*, vol. 9, no. 9, pp. 1084–1097, 2003, doi: 10.1261/rna.5710303.

[44] Y. Xu *et al.*, "Cobalamin (Vitamin B12) induced a shift in microbial composition and metabolic activity in an in vitrocolon simulation," *Front. Microbiol.*, vol. 9, no. NOV, pp. 1–9, 2018, doi: 10.3389/fmicb.2018.02780.

[45] J. He, V. F. Holmes, P. K. H. Lee, and L. Alvarez-Cohen, "Influence of vitamin B12 and cocultures on the growth of Dehalococcoides isolates in defined medium," *Appl. Environ. Microbiol.*, vol. 73, no. 9, pp. 2847–2853, 2007, doi: 10.1128/AEM.02574-06.

[46] E.-L. Nordmark, Z. Yang, E. Huttunen, and G. Widmalm, "Propionibacterium freudenreichii," *Biomacromolecules*, vol. 6, pp. 521–523, 2005.

[47] Y. Mathur, "Methylation reactions in the anaerobic biosynthesis of the lower ligand of Vitamin B 12," 2022.

Supplementary Information

Figure S1: shows the aerobic set-up design

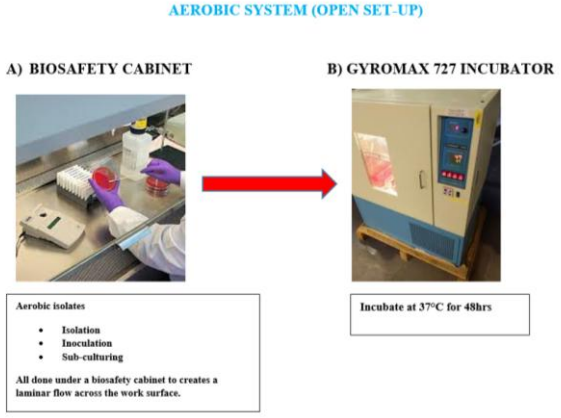

Figure S2: shows the anaerobic setup design

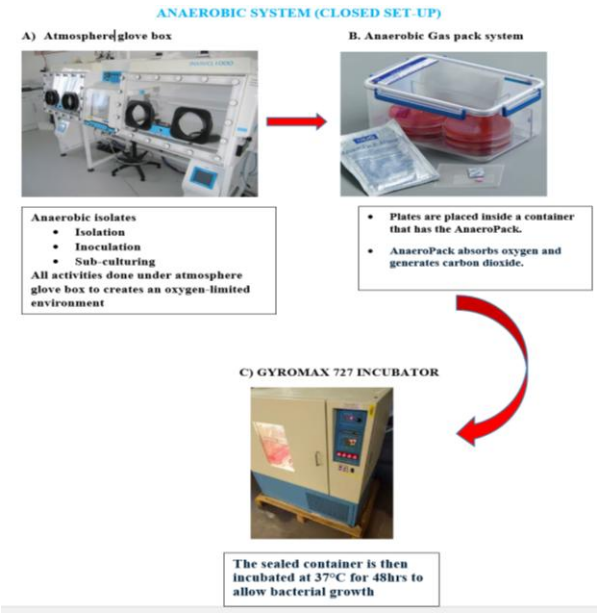

TABLE S1. PCR primers tailored for each bacteria isolate suspected of vitB12 synthesis

Isolate ID	Forward primer: cobA	Reverse primer: cobA
1. AnR2-C4	AAGACTGGGATAACTCCGGGAAAC	GAGCCGTGGGCTTTCACATC
2. AnR3-C4	GGGCTACTTACTGGTGRTGT	TCAAACACTCAGTGCCGCT
3. AR2-C3	TACCAGCGTGCTTTAATCGAG	ACCACATGACCAAAGCCAGC
Isolate ID	**Forward primer cobM**	**Reverse primer cobM**
1. AnR2-C4	CGAGCGAATGGATTAAGTAAGC	GAGCCGTGGGCTTTCACATC
2. AnR3-C4	CTTTATGAAGTTTAGCGGCGGACG	GGCTGCTGGCACGTAGTTAG
3. AR2-C3	GCCACATGGCTGACCATGAC	ATGGTGGAGAAATAAGGCAGGGTC
Isolate ID	**Forward primer cobI**	**Reverse primer cobI**
4. AnR2-C4	TACCAGCGTGCTTTAATCGAG	ACCACATGACCAAAGCCAGC
5. AnR3-C4	GCCACATGGCTGACCATGAC	ATGGTGGAGAAATAAGGCAGGGTC
6. AR2-C3	CCACATGGCTGACCATGACG	TGGTGGAGAAATAAGGCAGGGTC

TABLE S2. PCR cycling conditions tailored for each bacteria isolate suspected to have cob genes for vitB12 synthesis

cobA

Isolate ID		Initial denaturation	Denaturation	Annealing	Extension	Final extension
A.	An R2-C4	94°C for 5 mins	94°C for 3 mins	57°C for 30 sec	68°C for 1.5 mins	68°C for 5 mins
B.	An R3-C4	94°C for 5 min	94°C for 3 mins	50°C for 30 sec	68°C for 1.5 mins	68°C for 5 mins
C.	AR 2-C3	94°C for 5 min	94°C for 3 mins	55°C for 30 sec	68°C for 1.5 mins	68°C for 5 mins
			cobM			
D.	An R2-C4	95°C for 5 min	95°C for 3 mins	55°C for 30 sec	72°C for 1.5 mins	72°C for 5 mins
E.	An R3-C4	95°C for 5 min	95°C for 3 mins	52°C for 30 sec	68°C for 1.5 mins	68°C for 5 mins
F.	AR 2-C3	95°C for 5 min	95°C for 3 mins	54°C for 30 sec	72°C for 1.5 mins	72°C for 5 mins
			cobI			
G.	An R2-C4	95°C for 5 min	95°C for 3 mins	50°C for 30 sec	68°C for 1.5 mins	68°C for 5 mins
H.	An R3-C4	95°C for 5 min	95°C for 3 mins	55°C for 30 sec	72°C for 1.5 mins	72°C for 5 mins
I.	AR 2-C3	95°C for 5 min	95°C for 3 mins	53°C for 30 sec	72°C for 1.5 mins	72°C for 5 mins

Figure S3. Individually, AnR2-C4 and AnR3-C4 did not produce detectable amount of vitB12. Panel A; is isolate AnR2-C4 – clustering with Lactobacillus reuteri and Panel B; is isolate AnR3-C4 – clustered with Selenomonas ruminantium.

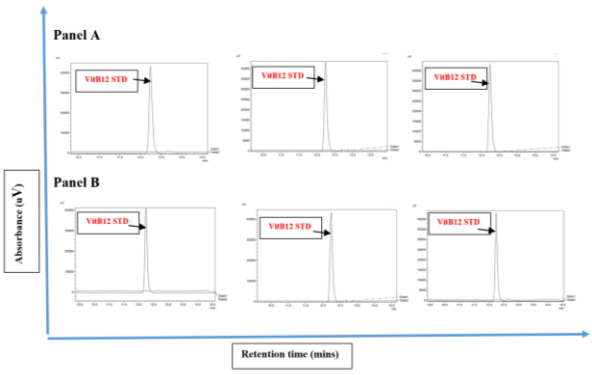

Figure S4. Schematic representation of the vitB12 biosynthesis gene clusters identified in different rumen-derived bacterial species, illustrating the arrangement of hem, cob, cbi, and pdu genes on their respective whole-genome scaffolds and contigs. Each arrow denotes a specific gene in the biosynthetic pathway, with its orientation indicating the direction of transcription. This highlights both aerobic (cob) and anaerobic (cbi) gene clusters, revealing variations in gene organization and spacing among the isolates. These arrangements emphasize the genetic diversity and potential adaptations for vitB12 production across the analysed bacterial strains.