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Obtaining Novel Vitamin B_{12} Production Strains *Acetobacter malorum* HFD 3141 and *Acetobacter orientalis* HFD 3031 from Home-Fermented Sourdough

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Abstract: Vitamin B_{12} is a critical nutrient in vegan and vegetarian lifestyles as plant-based vitamin sources are rare. Traditional fermented foods could be enriched by adding vitamin B_{12}-producing bacteria to offer non-animal vitamin sources. The aim was to isolate a vitamin B_{12} producer that is capable of producing the human-active vitamin even at low pH values so that it can be used in fruit juice fortification. Therefore, fermented foods (homemade and industrial) and probiotics were screened for vitamin B_{12} production strains. A modified microbiological vitamin B_{12} assay based on *Lactobacillus delbrueckii* subsp. *lactis* DSM 20355 was used to identify vitamin B_{12}-containing samples and the presence of vitamin B_{12}-producing strains. The screening resulted in isolating several positive strains for vitamin B_{12} formation derived from sourdough. Mass spectrometry confirmed the biosynthesis of solely the human physiologically active form. Species identification carried out by the German Strain Collection of Microorganisms and Cell Cultures resulted in two species: *Acetobacter orientalis* and *Acetobacter malorum*, of which two isolates were further characterised. The potential for cobalamin biosynthesises in food matrixes was demonstrated for *A. malorum* HFD 3141 and *A. orientalis* HFD 3031 in apple juice at different pH values (2.85–3.80). The isolates synthesised up to 18.89 µg/L and 7.97 µg/L vitamin B_{12} at pH 3.80. The results of this study suggest that acetic acid bacteria (AAB) and fermented acetic acid foods are promising resources for vitamin B_{12} and its producers, which might have been overlooked in the past.

Keywords: cobalamin; pseudocobalamin; *Acetobacter*; acetic acid bacteria; food biofortification; hidden hunger

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1. Introduction

Even though cobalamin (vitamin B_{12}) is exclusively produced by certain bacteria and archaea, foods of animal origin (meat, eggs, and milk) represent the primary source of cobalamin in the human diet [1]. When omitting these, meeting the recommended daily intake levels can be challenging. The German Vegan Study (GVS) revealed a significant gap in the uptake of vitamin B_{12} in the vegan and vegetarian community [2]. The GVS found that male vegans consume only 0.84 ± 1.21 µg/day, and female vegans consume 0.78 ± 2.14 µg/day of cobalamin, which is lower than the EFSA's recommended daily intake of 4.0 µg [3]. Thus, the provision of vital nutrients via fermented, plant-based foods represents an auspicious and cost-effective approach to addressing this nutritional disparity [4].

Not all bacteria are capable of producing vitamin B_{12}, and it is therefore necessary to isolate and identify capable strains. Identified vitamin B_{12} producers in the literature belong to the genus *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Micromonspora*, *Mycobacterium*, *Nocardia*, *Protminobac-*

ter, *Proteus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus*, *Xanthomonas* [5], *Propionibacterium* (e.g., [5,6]), *Acetobacter* [7,8] *Gluconobacter* [8], and *Lactobacillus* (e.g., [9,10]). Whereas *Pseudomonas denitrificants* and *Propionibacterium freudenreichii* subsp. *shermanii* are used for industrial large-scale productions, research on food biofortification focuses on lactic acid bacteria (LAB) and propionic acid bacteria (PAB) [11], mainly *Propionibacterium freudenreichii* [4]. LAB and *P. freudenreichii* possess GRAS ("generally recognised as safe") status according to the Food and Drug Administration, so their application is understandable. *P. freudenreichii* strains are prototrophic for all amino acids and nucleotides [12], which means requirements for fermentation mediums are relatively low. The preferred pH value of PAB lies between 6 and 7 [13], and the tolerance ranges down to pH 5.0 [14]. As the name suggests, it produces the pungent propionate, characteristic of certain cheese types [15], but may not be desired in biofortified foods. LAB produce the less pungent lactic acid as the major metabolic end-product; they are often auxotrophic towards several amino acids and vitamins [16] and require nutrient-rich fermentation media. The pH optimum is strain-dependent and ranges from 5.5 to 6.2 for lactobacilli and 4.5 to 6.5 for most strains of the *Lactobacillus* genus [17].

Different approaches are used to isolate and identify vitamin B_{12}-producing microorganisms from complex matrixes or strain collections, including methods based on the media composition, presence of individual genes, or direct measurement of the vitamin in the fermentation medium. Most commonly, testing the growth potential of isolates on vitamin B_{12}-free media is used to grow potential production strains selectively. The hypothesis is that only bacteria can grow on the medium if they can produce the vitamin. Vitamin B_{12} functions as a coenzyme in various prokaryotic pathways, e.g., cobalamin-dependent methionine synthase [18], glycerol dehydratase, and ethanolamine ammonia lyase [19]. However, as not all bacteria rely on the cobalamin-dependent pathways and cobalamin-independent alternatives are present [19], this technique is not entirely discriminatory against non-producing organisms [20]. This method was successfully applied, isolating four *Lactobacillus* strains from Nukazuke (Japanese pickle) [9]; one *Bacillus* sp. strain from Tua-Noa (non-salted fermented soybean) [21]; and *Lactobacillus lactis*, *Levilactobacillus brevis*, and *Pediococcus pentosaceus* from industrial *Chlorella vulgaris* fermentations [22]. The presence of homologues of the *bluB/cobT2* fusion genes [23,24] or the *cbiK* gene [20,25] have been used as markers for identifying probable vitamin B_{12} producers. Those genes code for specific enzymes of the cobalamin synthesis: the enzymes BluB and CobT2 catalyse the formation of the lower-ligand DMBI (5,6-Dimethylbenzimidazole) of vitamin B_{12} as well as its activation and incorporation into the cobalamin molecule [24]. CbiK catalyses the incorporation of the cobalt ion into the cobalamin structure [26]. Bhushan et al. applied a three-phase (vitamin B_{12}-free medium, cobalt supplementation, and screening for *cbiK* gene) screening method to isolate lactobacilli from human samples (breast milk and faecal matter) [20]. The screening resulted in two *Lactiplantibacillus plantarum* strains. Although the isolation was successful, the authors conclude that the first two steps—or lactobacilli—need to be more selective and solely screen for the *cbiK* gene to be sufficient to identify promising strains. Kumari et al. followed the purposed single-phase identification by the *cbiK* gene and identified three vitamin B_{12}-producing strains (*Limosilactobacillus reuteri* F2, *Lactiplantibacillus plantarum* V7, and *Lacticaseibacillus rhamnosus* F5) isolated from infant faecal samples [25]. Hugenschmidt et al. screened PAB and LAB strains by directly measuring the cobalamin content of the culture media by HPLC, without restricting the strains tested using the above methods [6].

After the identification of probable vitamin B_{12} producers, their vitamin B_{12} formation capacity is typically checked by a microbiological assay using *Lactobacillus leichmannii* ATCC 7830 (=*L. delbrueckii* subsp. *lactis* DSM 20355) [10,21,22] or chromatographic methods (liquid chromatography–tandem mass spectrometry (LC-MS/MS) [23] and ultra-fast liquid chromatography with a diode array detector (UFLC-DAD) [20]). The microbiological vitamin B_{12} assay is based on this vitamin B_{12} auxotrophic indicator strain, which is incubated with a defined sample volume in a vitamin B_{12}-free assay medium. The growth of the indicator

strain, determined by the optical density (OD), is a measure of the concentration of vitamin B_{12} present. Regarding nutritional physiology, it is crucial to consider the possibility that microorganisms can produce forms of cobalamin that have no physiological function in humans (pseudocobalamin). However, in microorganisms, these analogues can fulfil the same functional property as the real vitamin B_{12} [23]. While the indicator strain cannot discriminate between the human-active vitamin B_{12} and the inactive pseudovitamins, chromatographic procedures like LC-MS/MS allow for the distinction between the forms [23]. Human active and non-active vitamin B_{12} forms deviate by their lower axial ligand. The lower axial ligand of pseudovitamins is adenine, whereas DMBI is present in the human active form [7,23].

This study aimed to isolate and identify novel cobalamin-producing strains from complex fermentation products and mixed cultures using a vitamin B_{12}-free medium. The isolate must be capable of producing the physiological active vitamin B_{12} and demonstrate tolerance to low pH values. Application for food biofortification was tested on apple juice to reach claimable vitamin B_{12} concentrations without needing a pH adjustment or adding growth-promoting substances (e.g., yeast extract). A concentration of at least 1.9 µg vitamin B_{12}/L was targeted, as this is the requirement for health claims according to Regulation (EC) No. 1169/2011 of the European Parliament and Council (version as of 1 October 2018).

2. Materials and Methods

2.1. Used Culture Media for Enrichment and Isolation

mMBA (modified microbial vitamin B_{12} assay media): in total, 42.3 g of the microbial vitamin B_{12} assay media (M036, HiMedia, Thane, India) was supplemented with 5 mg/L Cobalt-(II)-chloride hexahydrate (Carl Roth, Karlsruhe, Germany) and 5 g/L Glycerol (VWR Chemicals, Darmstadt, Germany) for the enrichment culture. Before autoclaving (121 °C, 15 min), 9 mL of the media were aliquoted in 12 mL culture tubes with screw caps. For streaking plates, 1.5% agar was added.

PAB (*Propionibacterium* agar): DSMZ medium 91 was used for the targeted enrichment of propionic acid bacteria (10.0 g of casein peptone, vitamin-free (Carl Roth, Karlsruhe, Germany); 5.0 g of yeast extract; 10.0 g of Na-lactate (60%-*w*/*v*, ThermoScientific, Geel, Belgium); and 15.0 g of agar (Carl Roth, Karlsruhe, Germany) in 1000 mL demi. H_2O). In an anaerobic jar, agar plates were incubated under anaerobic conditions using Anaerocult A (Merck, Darmstadt, Germany).

2.2. Samples

Commercial samples of one sourdough starter (Seitenbacher Natur Sauerteig, Seitenbacher Naturkost, Buchen, Germany), one Sauerkraut juice ("Sauerkrautsaft", dmBio, Karlsruhe, Germany), and one fermented bread drink ("Original Kanne Brottrunk", Kanne Brottrunk GmbH und Co. Betriebsgesellschaft KG, Selm-Bork, Germany) were bought in local supermarkets; four raw milk samples were obtained from local dairy farms (area in and around Fulda, Germany); and four probiotics were ordered online. The contained species are listed below for the individual products; if they got reclassified during the taxonomic reorganisation in 2020 [27], the new species designation is given:

- "Symbio Extra RedCare", Shop-Apotheke B.V., Sevenum, Netherlands: *Lactobacillus acidophilus*, *Lacticaseibacillus paracasei*, *Lactococcus lactis*, *Bifidobacterium lactis*;
- "OMNi-BiOTiC® 10", APG Allergosan Pharma GmbH, Graz, Austria: *L. acidophilus* W55, *L. acidophilus* W37, *L. plantarum* W1, *B. lactis* W51, *Enterococcus faecium* W54, *L. paracasei* W20, *Lacticaseibacillus rhamnosus* W71, *Ligilactobacillus salivarius* W24, *L. plantarum* W62, *Bifidobacterium bifidum* W23;
- "BactoFlor 10/20", Dr. Wolz Zell GmbH, Geisenheim, Germany: *B. bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *L. acidophilus*, *L. paracasei*, *Limosilactobacillus reuteri*, *L. rhamnosus*, *L. plantarum*, *E. faecium*;

- "Darmflora plus select", Dr. Wolz Zell GmbH, Geisenheim, Germany: *L. acidophilus*, *Lacticaseibacillus casei*, *L. rhamnosus*, *L. plantarum*, *B. breve*, *Streptococcus thermophilus*, *B. bifidum*, *B. lactis*.

Four home-fermented sourdough samples and one home-fermented kimchi were tested. The samples were not prepared by the authors themselves but were made available following a request from personal contacts. Those sixteen samples were screened for vitamin B_{12} and vitamin B_{12}-producing strains. *Limosilactobacillus reuteri* DSM 20016 (ATCC 23272) was used as a positive control during the isolation procedure. This strain was selected as a positive control because it achieves fast growth on the mMBA, and the pseudocobalamin synthesis is well described in the literature [7,23]. A non-inoculated mMBA was used as a negative control.

2.3. Overview of the Isolation Procedure

The screening procedure is shown in Figure 1. In the first phase, the food products were screened for vitamin B_{12} presence by the MBA, and 100 µL of the sample was transferred to 9 mL of MRS pH 5.7 (Carl Roth, Karlsruhe, Germany) and incubated at 30 °C for 48 h to check for viable cells. When vitamin B_{12} was detected, the culture was washed 3-fold in saline solution, and 100 µL of the resuspended culture was transferred to the mMBA and incubated for 48 h at 30 °C. If no vitamin B_{12} was detected, the MRS culture of the sample was discarded.

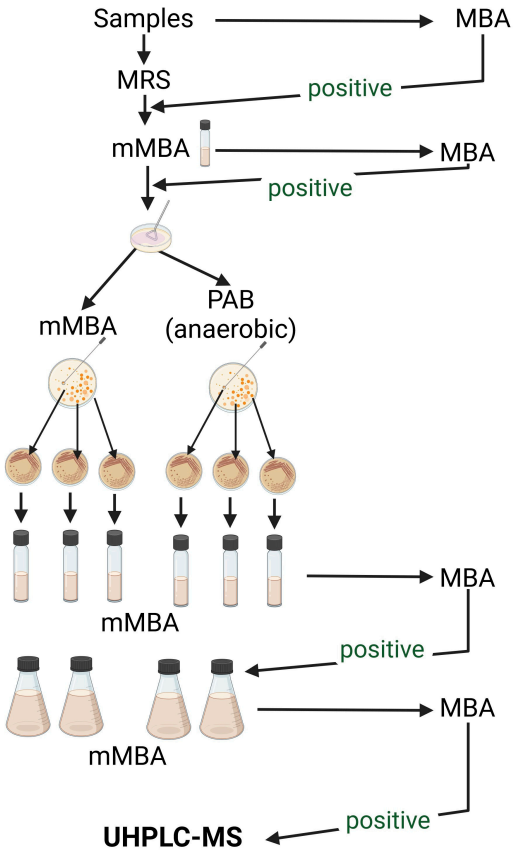

Figure 1. Isolation and screening procedure for cobalamin producers in different food samples and probiotics. MBA: microbial assay, PAB: *Propionibacterium* agar, and mMBA: modified microbial vitamin B_{12} assay medium. Created with BioRender.com, accessed on 20 June 2024.

Subsequently, five subcultures were made by transferring 100 µL in 9 mL of the mMBA after incubating at 30 °C for 48–72 h. The last subculture was used for the MBA to determine the presence of cobalamin producers in the microbial community. The positive MBA samples were spread-plated on the mMBA agar and PAB (anaerobic incubation) with

different dilutions and incubated for 72 to 96 h at 30 °C. A random number of colonies was streak-plated on the respective medium and incubated under the same conditions. Single colonies of the isolated colonies were transferred to the mMBA and tested once again for the growth of the indicator strain. Isolates positive for cobalamin were cultivated in 200 mL of the mMBA and subjected to LC-MS/MS analyses to determine the level of cobalamin present.

2.4. Sample Preparation

To examine the solid (food) samples and probiotics, 0.1 g of the sample or the content of one capsule was dissolved into 9 mL of saline solution; the following preparation was identical for the dissolved, liquid, and fermentation samples.

Part of the sample (4 mL) was transferred to an amber centrifuge tube and boiled for 10 min after adding 50 µL of 0.1% KCN (Carl Roth, Karlsruhe, Germany). This treatment ruptures the microbial cells; releases the cobalamin into the extracellular space; and converts it to its most stable form, cyanocobalamin [1]. After centrifugation at 4000 rpm for 10 min (Megafuge 1.0R, Heraeus, Hanau, Germany), 1 mL of supernatant was transferred to 9 mL of the vitamin B_{12} assay medium, prepared by dissolving 42.3 g/L of the test medium (M036, HiMedia, India) under heat. If the supernatant was turbid after centrifugation, the sample was filtered through a syringe filter (0.45 µm, PES 25 mm, WICOM, Heppenheim, Germany) before transferring to the test medium.

After autoclaving (121 °C, 15 min) and cooling down, the samples were inoculated with 50 µL of the washed and diluted indicator.

2.5. Modified Microbial Vitamin B_{12} Assay (MBA)

For the modified vitamin B_{12} assay, *L. delbrueckii* subsp. *lactis* DSM 20355 was used as the indicator strain to determine cobalamin presence in the samples, the mixed culture, and the sequentially isolated cultures. The indicator strain is stored at −80 °C and reactivated (30 °C, 48–72 h) in 9 mL of microinoculum broth, consisting of 5.0 g of peptone, 2.0 g of yeast extract, 10 g of D-(+)-glucose, 2 g of KH_2PO_4, and 0.1 g of Tween 80 in 1000 mL demi. water (all components from Carl Roth, Karlsruhe, Germany). Before use in the microbiological assays, the indicator culture was washed (4000 rpm, 5 min; Megafuge 1.0R, Heraeus, Hanau, Germany) three times in saline solution (0.85% NaCl, Carl Roth, Karlsruhe, Germany) and diluted 10-fold.

A spectrophotometer (DR6000, Hach Lange, Düsseldorf, Germany) was used to measure the indicator's growth at λ = 600 nm (OD_{600}) after 48 h of incubation at 30 °C. Samples with an optical density at least twice the blank value were considered positive for vitamin B_{12}.

2.6. Growth Conditions and Determination of Bioactive Forms of Vitamin B_{12}

For LC-MS/MS analyses, the six isolates were cultivated in 200 mL of the mMBA (1%-*v*/*v* inoculum after cultivation on three subcultures in 9 mL of the mMBA) in an Erlenmeyer flask for seven days at 30 °C. *Acidipropionibacterium acidipropionici* DSM 20273 was cultured identically as a reference strain. Sample preparation was carried out according to the literature [28]. For this purpose, ^{15}N-cyanocobalamin was added as an internal standard. The standard was prepared and provided by Lenz et al. [28] following the regime reported by Wang et al. [29]. The samples were pre-digested with taka-diastase from *Aspergillus oryzae* and papain from papaya latex (both enzymes from Sigma-Aldrich, Darmstadt, Germany). Purification was carried out using immunoaffinity columns (EASI-EXTRACT® Vitamin B12; R-Biopharm, Darmstadt, Germany).

The distinction between the human active and pseudovitamin, using a UHPLC (ultra-high-pressure liquid chromatography) system coupled to an ion trap mass spectrometer, was ascertained by the authors describing the method [28].

2.7. Identification of the Isolated Production Strains

An analysis of the isolates was carried out using MALDI-TOF MS (matrix-assisted laser desorption ionisation-time of flight mass spectra) by DSMZ Services, Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (German Strain Collection of Microorganisms and Cell Cultures).

A. orientalis was initially described in 2001 by Lisdiyanti et al. [30]. The type of strain of the species is 21F-2^T (= NRIC 0481^T = IFO 16606^T = JCM 11195^T), isolated from canna flower. The strain produces 2-Keto-D-gluconic acid from glucose, but not 5-Keto-D-gluconic acid or 2,5-diketo-D-gluconic acid, and grows between pH 3.5 and 8.0 [30].

A. malorum was initially described in 2002 by Cleenwerck et al. [31]. The type of strain of the species is LMG 1746^T (= DSM 14337^T), isolated from rotting apple. It produces 2-Keto-D-gluconic acid from glucose but not 5-Keto-D-gluconic acid. The pH optimum ranges from pH 4.5 to 7.0 [32].

Reports of the species *A. orientalis* and *A. malorum* are rare, and none are associated with vitamin B_{12} synthesis. The PubMed search for "*Acetobacter orientalis*" returns 20 results, whereas the search for "*Acetobacter malorum*" returns 23 results.

2.8. Testing for Low pH Growth on Apple Juice

The determination of the growth capacity on low pH was carried out on apple juice adjusted to different pH values (pH 2.85, 3.05, 3.30 (original pH of the apple juice), 3.55, and 3.80). Apple juice was chosen because it is a regional product that is also consumed worldwide and available all year round at a relatively low price. Furthermore, the low pH inhibits the growth of most bacteria and therefore is a challenging medium. These pH values were selected to investigate the lower limits of the growth capacity and product formation potential, as this would be expected to differentiate the acetic acid bacteria from the LAB and PAB. The apple juice was purchased from a local supermarket ("rio d'oro", Aldi Süd, Fulda, Germany) and pH adjusted with hydrochloric acid or sodium hydroxide (Carl Roth, Karlsruhe, Germany). Before autoclaving (121 °C, 15 min), 30 mL of the prepared apple juices were aliquoted into 100 mL of laboratory glass bottles. Inoculated samples were carried out in triplicates and blanks in duplicates.

Before inoculation, the cultures were reactivated from storage (−80 °C, glycerol) in apple juice supplemented with 5 g/L yeast extract and pH adjusted to 5.6 (72 h, 30 °C). The reactivated cultures were subcultured twice with 0.1 L on 9.9 mL of pure apple juice (pH 3.30, 72 h, 30 °C) before transferring 300 µL (1%-v/v) to 30 mL of the fermentation media. Subcultures of the apple juice were carried out to enable adaptation to the low pH value of the apple juice. The cultures were incubated for 7 days at 30 °C in a linear shaking water bath (1083, GFL, Burgwedel, Germany). The optical density OD_{600} (DR6000, Hach Lange, Düsseldorf, Germany), pH (InLab Flex-Micro and SevenExcellence, Mettler Toledo, Gießen, Germany), and vitamin B_{12} concentration (MBA) were determined after incubation.

2.9. Quantification Procedure by Microbial Vitamin B_{12} Assay (MBA)

The microbiological B_{12} assay medium from Millipore (B3801, Darmstadt, Germany) was used for quantification. The manufacturer's manual was followed, with some modifications.

In total, 20 mL of the samples and 50 µL of KCN (1%) were mixed with 50 mL of the decomposition buffer solution (1.29 g of disodium hydrogen phosphate, 1.1 g of citric acid, and 1.0 g of sodium metabisulfite in 100 mL of distilled water) before autoclaving (121 °C, 15 min). After cooling, the pH was adjusted to 6.0, and the volume was filled up to 100 mL with water. In total, 1 mL of the centrifuged (4000 rpm, 10 min) sample was transferred to 5 mL of the assay medium and filled to 10 mL with water. The calibration concentrations of the vitamin B_{12} (Carl Roth, Karlsruhe, Germany) standard were set to 25, 50, 75, 100, 125, 150, and 175 pg/mL.

Before washing, dilution, and inoculation, 1 mL of the indicator culture was incubated in 9 mL of the test medium for 48 h to deplete the culture of cobalamin.

Determination was carried out in triplicate for the samples and standards. The optical density (OD_{600}) of the samples was measured against water. The sample concentration was determined using the semi-logarithmic plot of the optical density over the standard concentration.

3. Results

3.1. Isolation of Cobalamin Producers

Seven out of the sixteen product samples had positive responses on the first-performed MBA (Table 1), including all milk samples (n = 4), the bread drink, one probiotic, one home-fermented sourdough sample, plus the positive control. When checking the microbial community of those samples, only the sourdough sample tested positive for cobalamin. Eliminating some samples in the microbial community stage was unsurprising for most product categories.

Table 1. Summary of the positive and negative screened samples by MBA on the different phases of isolation.

Indicator Growth	Number of Products	Microbial Community	Isolates
Negative	9	6	15
Positive	7 *L. reuteri* DSM 20016	1 (sourdough) *L. reuteri* DSM 20016	6 *L. reuteri* DSM 20016

Vitamin B_{12} in milk is synthesised by the rumen microbiota of the cow and accumulated by the tissue [1]. It is still disputed whether the mammary gland is a sterile organ, whether microorganisms in raw milk are caused by contamination during sampling, or whether there is a milk microbiome [33]. However, it has been proven that the composition of the rumen microbiome is more diverse and differs significantly from the microorganisms in milk [34]. Determining cobalamin in the milk was expected, but detecting the responsible producers was not assumed. The one probiotic ("Darmflora plus select") that tested positive for cobalamin is supplemented with 2.5 µg of vitamin B_{12} per capsule. The positive result of the microbial community of the fermented bread drink was expected, as the bottle is labelled with a vitamin B_{12} content of 1.36 µg/L. The vitamin is produced by sourdough starters, which are not deactivated in the final product. However, the microorganisms responsible for the formation of vitamin B_{12} in the bread drink could not assert themselves in the second phase to such an extent that vitamin B_{12} could be measured.

As only the microbial community of one sourdough was positive for the vitamin, only colonies of these samples were isolated on agar plates. Six of the twenty-one randomly collected colonies were positive during the MBA analysis and, therefore, identified by the DSMZ after determining the vitamin B_{12} form present.

3.2. Identification of the Isolated Strains

The analysis of the six isolates sent to the DSMZ revealed that five isolates are very similar and can be assigned to the species *Acetobacter malorum*. In contrast, the sixth isolate is identified as *Acetobacter orientalis* HFD 3031. Accordingly, it is assumed that the isolates of *Acetobacter malorum* are a single strain that has been isolated several times. Therefore, only one strain, *A. malorum* HFD 3141, will be viewed in the following considerations.

3.3. LC-MS/MS Verification of Vitamin B_{12} Formation

LC-MS/MS was used to determine the form of vitamin B_{12} present. The fragmentation patterns of vitamin B_{12} can be clearly distinguished from those of the pseudovitamin in mass spectrometry (Figure 2).

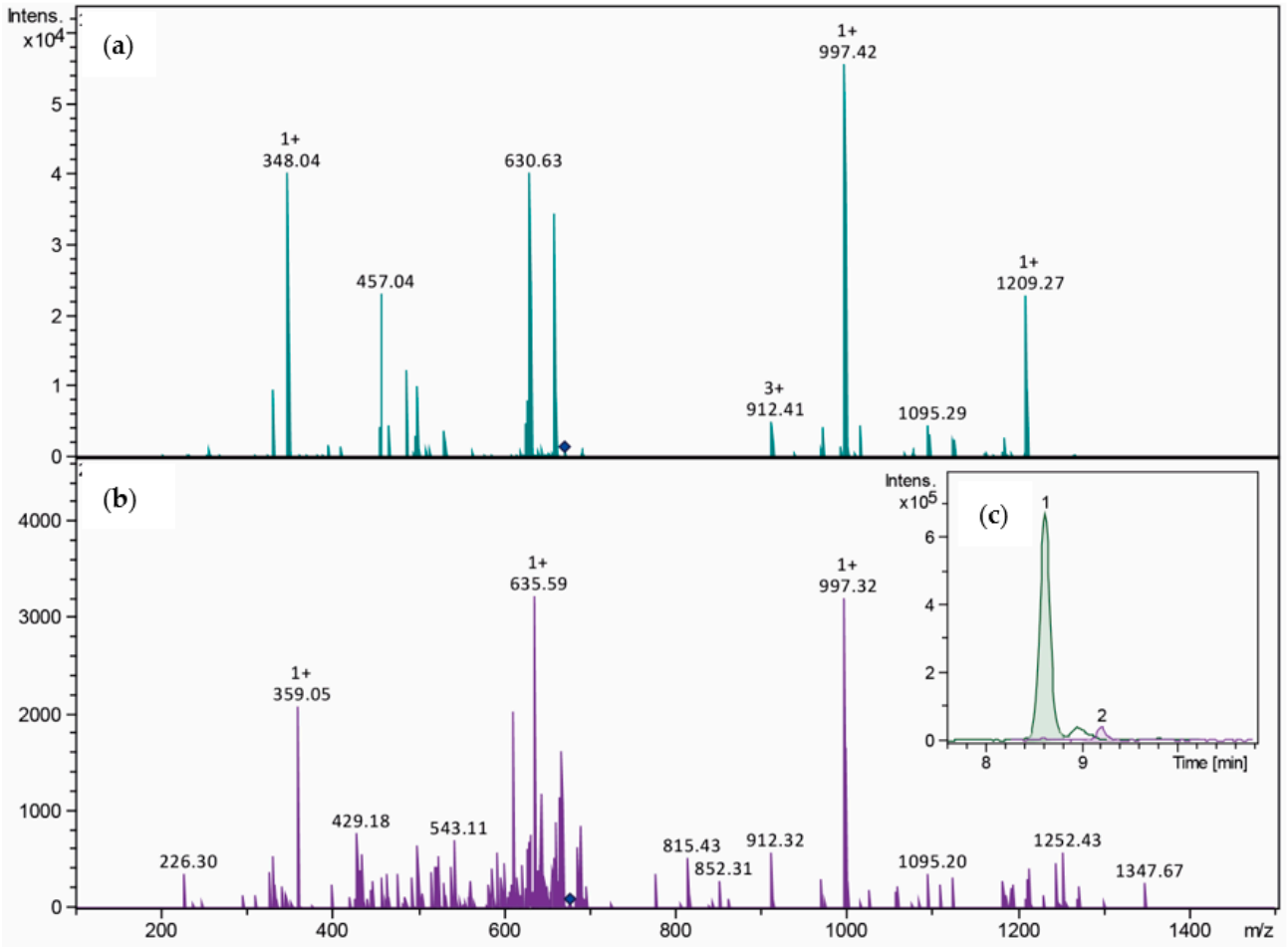

Figure 2. LC-MS/MS results of *A. acidipropionici* DSM 20273. (**a**) Fragmentation pattern of pseudocobalamin (green) containing characteristic *m/z* 348.04, and (**b**) vitamin B_{12} (purple) containing characteristic *m/z* 359.05. The rhombuses mark the respective parent ion of the two cobalamin forms of *m/z* 678.00 (vitamin B_{12}) and *m/z* 672.20 (pseudocobalamin). (**c**) EIC of vitamin B_{12} (*m/z* 678.00, purple) and pseudocobalamin (*m/z* 672.20, green) with their retention time differences.

Whereas *m/z* 997 can be found in the pseudo and active form (Figure 2a,b), the presence of the fragmentation ions *m/z* 348.04 $[Ade+sugar+PO_3]^+$ (pseudocobalamin) and *m/z* 359.05 $[DMBI+sugar+PO_3]^+$ (vitamin B_{12}) provides a clear marker of the vitamin form present, as these are fragments of the lower ligands [23]. Figure 2c shows the characteristic retention times for pseudocobalamin (peak 1) at 8.6 min and for vitamin B_{12} (peak 2) at 9.2 min. No peak could be detected at the retention time of 8.6 min (Figure 3a,c), but both isolates exhibited a peak at 9.2 min in the EIC. Neither the sample of *A. malorum* HFD 3141 nor *A. orientalis* HFD 3031 contained pseudocobalamin. The fragmentation pattern (Figure 3b,d) of *m/z* 678.00 shows the distinctive *m/z* 359.05 for the lower-ligand DMBI. It has, therefore, been shown that both of these isolates can produce vitamin B_{12}, but not the inactive form under given fermentation conditions.

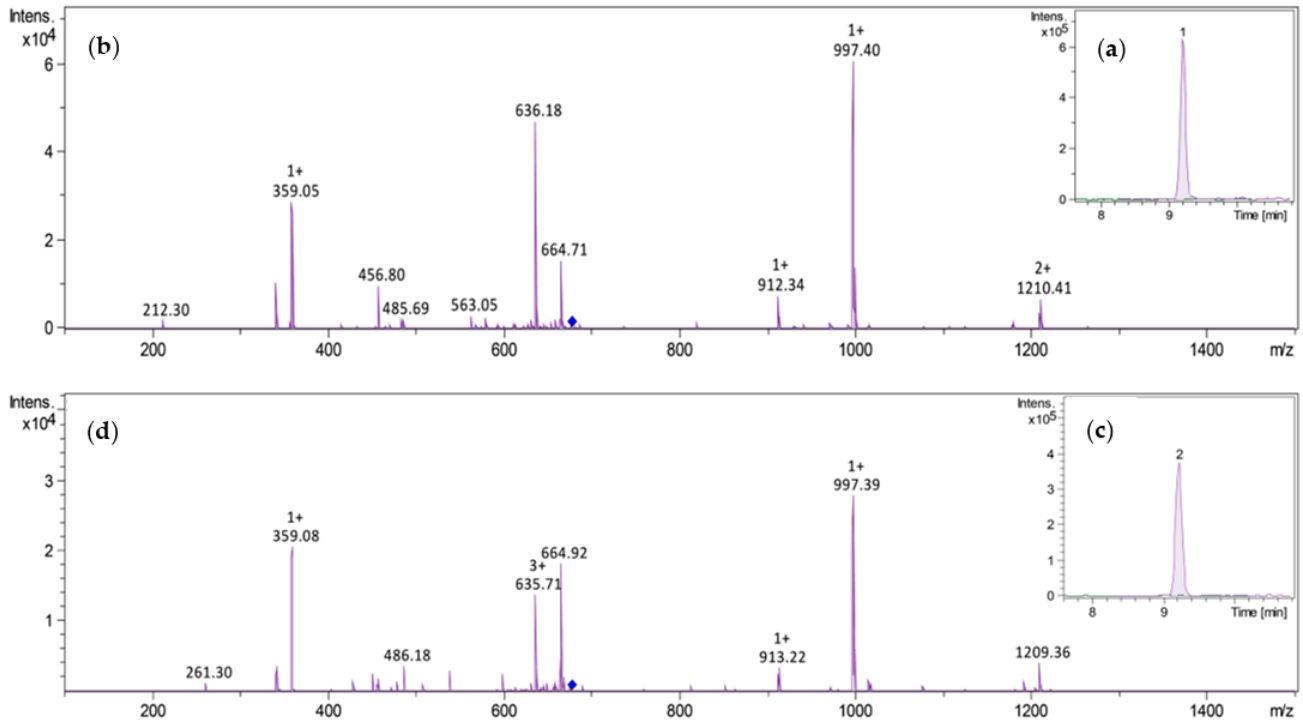

Figure 3. LC-MS/MS results of *A. orientalis* HFD 3031. (**a**) EIC of vitamin B_{12} (*m/z* 678.00) and pseudocobalamin (*m/z* 672.20) and (**b**) fragmentation pattern of vitamin B_{12} (purple) containing characteristic *m/z* 359.05. LC-MS/MS results of *A. malorum* HFD 3141. (**c**) EIC of vitamin B_{12} (*m/z* 678.00) and pseudocobalamin (*m/z* 672.20), and (**d**) fragmentation pattern of vitamin B_{12} (purple) containing characteristic *m/z* 359.05. The rhombuses mark the parent ion of vitamin B_{12} (*m/z* 678.00).

3.4. Low pH Tolerance Test

The results of the pH tolerance tests are displayed in Table 2. OD_{600} values are corrected by the turbidity input of the medium, i.e., minus the blank value, and the pH change from the initial value is presented. Both isolates, *A. orientalis* HFD 3031 and *A. malorum* HFD 3141, can grow on apple juice without supplementing amino acids or vitamins. The tolerance towards low pHs is similar for the two strains.

Table 2. Effect of initial pH on the change in OD_{600}, pH value, and vitamin B_{12} concentration after seven days.

Initial pH	*A. orientalis* HFD 3031			*A. malorum* HFD 3141		
	ΔOD_{600}	**ΔpH**	**Vitamin B_{12} [µg/L]**	**ΔOD_{600}**	**ΔpH**	**Vitamin B_{12} [µg/L]**
2.85	0.068 ± 0.24	−0.05	0.35 ± 0.90	0.000 ± 0.008	−0.05	<LOD *
3.05	0.193 ± 0.42	−0.05	2.68 ± 0.92	0.059 ± 0.018	−0.08	0.89 ± 0.92
3.30 (Original)	0.248 ± 0.063	−0.07	3.19 ± 0.13	0.069 ± 0.005	−0.18	0.21 ± 0.96
3.55	0.349 ± 0.064	−0.26	10.29 ± 0.50	0.088 ± 0.006	−0.22	3.81 ± 1.42
3.80	0.438 ± 0.026	−0.33	18.89 ± 2.33	0.181 ± 0.024	−0.38	7.97 ± 1.56

* value below Limit of Detection (LOD).

A slight increase in OD_{600} was observed at pH 2.85 for *A. orientalis* HFD 3031 and at pH 3.05 for *A. malorum* HFD 3141. As this increase occurs together with large standard deviations in OD_{600} and vitamin B_{12} concentrations, this is the threshold of the minimum tolerance of the respective strains. The tolerance range for the two isolates is therefore defined as 3.05 and 3.30, respectively. With an increasing initial pH value, the change in the optical density, pH drop, and vitamin B_{12} concentration increases. The highest growth and product synthesis was observed at the highest pH starting value for both

isolates. At pH 3.80, *A. orientalis* produces 18.89 µg/L vitamin B_{12} and *A. malorum* produces 7.97 µg/L. A further increase in growth and vitamin B_{12} synthesis at higher initial pH values can be hypothesised, as the optimal pH of *Acetobacter* sp. is 5.0–6.5 [35]. The decrease in pH during fermentation occurs due to the probable formation of gluconic acid from glucose [30,31]. Despite the unfavourable growth conditions in apple juice (low pH and poor amino acid composition), both strains were able to grow even at the original pH and produce considerable amounts of the vitamin.

4. Discussion

It was demonstrated that using a vitamin B_{12}-free medium to isolate cobalamin formers from complex fermentation products is viable. Although the enrichment and isolation experiments were aimed at PAB and LAB, two novel potent vitamin B_{12} strains belonging to AAB were isolated. Those strains were identified by MALDI-TOF MS as *A. malorum* and *A. orientails*. However, the identification of AAB is particularly challenging due to the high degree of similarity between the 16S rRNA gene sequences of closely related species. Wieme et al. (2014) could not distinguish *A. malorum* LMG 1746 from *Acetobacter cerevisiae* strains by MALDI-TOF MS analysis [36]. Even comparing the 16S rRNA of *A. malorum* and *A. cerevisiae* is not sufficient to clearly identify these species. Consequently, other methods, such as the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes, are recommended in the literature for the identification of AAB. This technique allows one to differentiate between those closely related species [37]. Consequently, it is possible that the current isolates, when identified by methods more tailored for AAB, must be reclassified. If similar trials were to be conducted, the trial program should include acetic acid fermented food sources and fermentation media specifically designed for AAB, like the designated pH 3.5 medium [38]. Although isolation and identification via the vitamin B_{12}-free medium are viable, subsequent analysis to determine the cobalamin form present is necessary in order to exclude pseudocobalamin synthesis.

The reason why only two other AAB [7,8] had been identified previous to our publication is that screened isolates are pre-selected by their genus or even species before screening. The pre-selection steps involve the use of readily genotypic identified strains (PAB and LAB) [6,20], Gram staining [9,20,22], morphological appearance under the microscope [9,22], catalase activity [20,22], and the detection of lactic acid and ethanol formation [9]. PAB are Gram-positive, non-motile, anaerobic to aerotolerant [39], and catalase-positive. LAB are Gram-positive, facultative anaerobes, and catalase-negative [40]. As AAB are Gram-negative, catalase-positive, obligate aerobes, and produce acetic acid from ethanol utilisation [41], they are filtered out beforehand. Forthcoming screening of readily isolated AAB strains or targeted isolation from food matrixes by the strategies mentioned above (e.g., vitamin B_{12}-free media and *cbiK* or *bluB*/*cobT2* genes as markers) will probably lead to the identification of more cobalamin producers within the AAB group. The screening by the specific genes involved in vitamin synthesis should ideally take into account the sequence variations of the homologues. Sequence variations in the *cobU/T*-like homologues have been shown to be responsible for the selectivity of different lower ligands incorporated into the cobamide [42–44]. Although there is a widespread preference for DMBI, other lower ligands can be incorporated into the cobamide depending on the homologue present and the environmental conditions [43]. Therefore, if DMBI is not provided by the fermentation medium or production strains, cobamides may be formed that do not fulfil a vitamin B_{12} function in humans. Screening and identification based on the presence of the specific enzymes of vitamin B_{12} synthesis should therefore include knowledge of the synthesis of DMBI and the selectivity of the homologues.

The biofortification of food by *Acetobacter* sp. bares a high potential for traditional food fermentations. Since the metabolic capacities are less restricted than those of LAB, they can grow in less nutrient-rich media, they have a high tolerance towards low pH, and pseudocobalamin was not detected in our samples. These features qualify *A. orientalis* HFD 3031 and *A. malorum* HFD 3141 for fortifying vitamin B_{12} in fermentation media

unsuited for other producers. Their ability to grow and produce vitamin B_{12} in apple juice was successfully demonstrated in this work. The concentration of 1.9 µg/L sufficient for health claims was exceeded by *A. orientalis* HFD 3031 (3.19 µg/L) at the original pH value of apple juice and by *A. malorum* HFD 3141 at pH 3.55 with 3.81 µg/L. However, the growth and final concentrations were relatively small, and measures to increase the concentrations should be investigated. In experiments on the enrichment of vitamin B_{12} by *L. reuteri* in furu (fermented tofu) [25,45] and soya milk [25], concentrations of 141.7 µg/kg wet weight and 132.2 µg/L, respectively, were achieved. During the fermentation of mature coconut water with *L. plantarum* DW12, a vitamin B_{12} concentration of 14 µg/mL could be achieved [46]. By the co-cultivation of *P. freudenreichii* and *Bifidobacterium animalis* subsp. *Lactis*, the vitamin B_{12} content reached up to 8.93 µg/L in soy whey, whereas in the single-culture fermentation of *P. freudenreichii*, 5.72 µg/L was detected [47]. The fermentation of solubilised wheat bran with *P. freudenreichii* resulted in the production of 55 µg/L vitamin B12 through co-fermentation with lactic acid bacteria and yeasts [48]. In most cases, the vitamin concentrations we achieve are similar to those found in the literature when fortifying foods, even though in some cases significantly higher levels are found. But the experiments conducted to assess the pH tolerance of the isolates in the low range indicate that the maximum potential for vitamin B_{12} synthesis has not yet been reached. Further investigation is required to ascertain the impact of different fermentation parameters, like higher pH values, different fruit juice types, fermentation temperatures, and aeration rates, on the synthesis. Studies on whether the addition of precursors (DMBI, riboflavin, nicotinamide, etc.) have a similar promoting effect as PAB [49] would also provide a deeper insight into the synthesis characteristics of the strains.

Fortifying plant foods by fermentation can provide relevant concentrations in vegan and vegetarian diets. Using strains with no capacity to produce analogues provides a reliable source of the physiologically active form. Incorporating *A. malorum* HFD 3141 and *A. orientalis* HFD 3031 in fermentation starter cultures, which naturally contain AAB, is promising. Scoby (kombucha), kefir grains (kefir and water kefir), vinegar mother (vinegar) [50], and sourdough [51] might be examples of the isolated strains' application. As AAB are already part of these fermentation processes, the product characteristics should not be altered drastically. Additionally, these listed foods fermented with AAB should be tested on their vitamin B_{12} content, as the data available in this area are considered insufficient.

This study demonstrates the need to broaden the search for potential strains in food biofortification with vitamin B_{12}. Previous authors focused on screening LAB and PAB for their cobalamin formation capacities. Our findings, as well as those of Bernhardt et al. [7] and Keto et al. [7,8], allow the conjecture that discovering more vitamin B_{12} producers within the AAB group is probable. The isolation of more AAB will give a wider variety of production features, like different food types, yields, pH tolerance, sugar utilisation, and nutrition requirements.

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