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Bio-enrichment of vitamin B12 in plant-based food via fermentation

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ZUSAMMENFASSUNG

Menschen mit veganer Ernährungsweise haben häufig einen Vitamin-B12-Mangel. Der Trend zu rein bzw. überwiegend pflanzlichen Diäten stellt eine ernährungsphysiologische Herausforderung dar, besonders bei Bio-Produkten. So gekennzeichnete Produkte dürfen nicht mit Vitaminen supplementiert sein. Vitamin B12 entsteht in der Natur durch bakterielle Biosynthese. Daher wurde in der vorliegenden Arbeit geprüft, wie der Vitamin B12-Gehalt von pflanzlichen Lebensmitteln mittels Fermentation mit Vitamin-B12-produzierenden Bakterien erhöht werden kann. Auf diese Weise könnte Verbrauchern mit einer veganen oder vegetarischen Ernährungsweise ein über Fermentation Vitamin-angereichertes veganes Bio-Produkt zur Deckung des Vitamin B12 Bedarfs angeboten werden.

In einem ersten Schritt wurden verschiedene *Lactobacillus*-Stämme auf ihre Fähigkeit zur Biosynthese von Vitamin B12 in einem Vitamin-B12-freien Testmedium (VBAM) untersucht. Ausgewählte Stämme mit der höchsten Wirksamkeit wurden sowohl einzeln als auch in Kombination mit *Propionibacterium*-Stämmen in einer Co-Kultur-Fermentation unter Verwendung von verschiedenem Lebensmittel auf Haferbasis weiter getestet. Von entscheidender Bedeutung für die Studie war die Entwicklung und Optimierung von Quantifizierungsmethoden zum Nachweis von Vitamin B12. Zu diesem Zweck wurden mikrobiologische Assays und HPLC-MS/MS geprüft. Letztgenannte Methode ermöglichte eine genaue Messung des Vitamin-B12-Gehalts sowohl in der *in vitro* als auch nach Fermentation in Nährmedien und Lebensmitteln. Zusätzlich zu den experimentellen Fermentationsprozessen wurden genetische Studien durchgeführt. Damit sollten tiefere Erkenntnisse über die wichtigsten Gencluster gewonnen werden, die mit der Vitamin-B12-Biosynthese in den ausgewählten *Lactobacillus*-Stämmen und der Regulation von Genen während der Vitamin-B12-Produktion in Verbindung stehen.

Die Ergebnisse zeigen, dass nur wenige *Lactobacillus*-Stämme biologisch aktives Vitamin B12 produzieren können, wobei wichtige Vorläuferstoffe für die Synthese notwendig sind. Obwohl in den Genomen alle für die Vitamin B12 Produktion notwendigen Gene vorhanden sind, fehlt einigen Stämmen die Fähigkeit zur Vitamin B12 Synthese sowohl *in vitro* als auch in Lebensmitteln. Es konnte gezeigt werden, dass eine Co-Fermentation mit *Propionibacterium*-Stämmen zu einer schnelleren und erhöhten Vitamin B12 Produktion im Vergleich zur Monokultur-Fermentation mit *Lactobacillus*-Stämmen führte. In der Monokultur mit *Lactobacillus* kam es zu einer schnelleren Ansäuerung und pH-Absenkung als bei der Co-Fermentation. Die langsamere Ansäuerung bei der Co-Fermentation könnte allerdings die Stabilität des gebildeten Vitamin B12 unterstützen.

Zusammenfassend fördert diese Arbeit das Verständnis der mikrobiellen Vitamin-B12-Produktion von den in dieser Studie untersuchten *Lactobacillus* und *Propionibacterium*-Stämmen.

Eine Co-Fermentation mit B12-produzierenden *Lactobacillus*- und *Propionibacterium*-Stämmen kann den Vitamin-B12-Gehalt in pflanzlichen Lebensmitteln, wie Hafergetränken, nachweislich steigern und bietet einen Wettbewerbsvorteil bei der Entwicklung von Bio-Lebensmitteln. Diese Strategie der biologischen Anreicherung entspricht der Verordnung (EU) 2018/848, die chemisch synthetisierten Zusätze verbieten. Auf Grundlage dieser Erkenntnisse können gezielt innovative Lebensmittel für Verbraucher mit vollständig oder überwiegend pflanzlicher Ernährungsweise zum Schutz vor Vitamindefiziten entwickelt werden.

SUMMARY

Vitamin B12 deficiency is prevalent among individuals adhering to exclusively plant-based diets. The shift toward plant-based diets has highlighted the challenge of vitamin B12 deficiency, particularly with a preference for organic products. Products labelled as such may not be supplemented with vitamins. In nature, vitamin B12 is produced through bacterial biosynthesis. This study examined the potential of using fermentation with vitamin B12-producing bacteria as a sustainable solution for enhancing the vitamin B12 content in plant-based foods. This would allow consumers following vegan or vegetarian diets to access vegan organic products enriched with vitamin B12 to meet their nutritional needs.

The research was initiated with the screening of various *Lactobacillus* strains for their ability to biosynthesize vitamin B12 in a vitamin B12-free assay medium. Selected strains with the highest efficacy were tested further, both individually and in combination with *Propionibacterium* strains, in a co-culture fermentation using oat-based food matrices. Critical to the study was the development and optimization of quantification methodologies. For this, microbiological assays and HPLC-MS/MS were utilized to measure vitamin B12 levels accurately in both the selection phase and post-fermentation in both nutrient media and food matrices.

In addition to the experimental fermentation processes, genetic studies were conducted to gain deeper insight into the key gene clusters associated with vitamin B12 biosynthesis in the selected *Lactobacillus* strains and the regulation of genes during vitamin B12 production.

The findings demonstrate that only a limited number of *Lactobacillus* strains could produce biologically active vitamin B12, and this required the addition of key precursors. Even though some *Lactobacillus* strains possess the complete genome necessary for vitamin B12 production, they were found to lack the capacity to produce vitamin B12 and its active form both *in vitro* and in food. Furthermore, employing a co-fermentation strategy with *Propionibacterium* strains increased and accelerated vitamin B12 production compared to monoculture fermentation with the *Propionibacterium* strain. In contrast, unlike the rapid acidification observed with monoculture fermentation using *Lactobacillus* strains, the co-fermentation moderated the acidification, ensuring good stability of vitamin B12.

In conclusion, this research enhances the understanding of vitamin B12 production in microbial fermentation and aids in developing innovative, sustainable nutritional solutions to address dietary deficiencies in vegan and vegetarian consumers. The co-fermentation strategy with both B12 producing *Lactobacillus* and *Propionibacterium* strains can significantly enhance the vitamin B12 content in plant-based food matrices (e.g. oat-based beverage), providing a competitive advantage in developing organic food products. This bio-enrichment strategy aligns with Regulation (EU) 2018/848 (European Union, 2018) concerning organic food products, which prohibit chemically synthesized additives.

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LISTS OF ABBREVIATIONS

AdoCbl	deoxyadenosylcobalamin
ANI	orthologous average nucleotide identity
ANOVA	analysis of variance
Cbl	cobalamin
cDNA	complementary DNA
CDSs	coding sequences
CFU	colony forming unit
CLIA	chemiluminescence immunoassays
CNCbl	cyanocobalamin
CV	coefficient of variation
CVD	cardiovascular disease
dDDH	digital DNA-DNA hybridisation
DEGs	differentially expressed genes
DMBI	5,6-dimethylbenzimidazole
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assays
ESI	electrospray ionization
FDA	Food and Drug Administration
FPKM	fragments per kilobase million
GC-MS	gas chromatography-mass spectrometry
gDNA	genomic DNA
GLC	gas-liquid chromatography
GMOs	genetically modified organisms
HPLC-MS/MS	high-performance liquid chromatography - tandem mass spectrometry
HSPE	hybrid solid phase extraction
IAC	immunoaffinity chromatography
IS	internal standard
KCN	potassium cyanide
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	lactic acid bacteria
LC-MS/MS	liquid chromatography- tandem mass spectrometry
LLOD	lower limit of detection
LOQ	limit of quantitation
MBA	microbiological assay
MeCbl	methylcobalamin
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mRNA	messenger ribonucleic acid

ABBREVIATIONS (Cont.)

MRS	de Man, Rogosa and Sharpe
MS	mass spectrometry
OD	optical density
OHCbl	hydroxocobalamin
PAB	propionic acid bacterium
PBMS	plant-based meat substitutes
PC	principal component
PCA	plate count agar
PCA	principal component analysis
PPA	*Propionibacterium* agar
PPB	*Propionibacterium* broth
QPS	qualified presumption of safety
RIA	radioimmunoassay
RIN	RNA Integrity Number
RNA	ribonucleic acid
RP	reverse-phase
RPKM	reads per kilobase million
RSD	relative standard deviation
RT	retention time
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SD	standard deviation
SPE	solid-phase extraction
TLC	thin-Layer-Chromatography
TPM	transcripts per kilobase million
UHPLC-MS	ultra high-pressure liquid chromatography-mass spectrometry
UTR	untranslated region
UV	ultra-violet
VBAM	vitamin B12 assay medium

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1. INTRODUCTION

Vitamin B12, also known as cobalamin, is an essential nutrient that plays a critical role in various physiological functions, including DNA synthesis, red blood cell formation, and neurological function (Azzini et al., 2021, Institute of Medicine (U.S.), 1998). Unlike other vitamins, vitamin B12 is primarily found in animal-based foods such as meat, dairy, and eggs (Stabler, 2020). Consequently, individuals who are strictly adhering to vegetarian and vegan diets and do not supplement vitamin B12 are at a higher risk of vitamin B12 deficiency, since studies have shown that plant products do not contain vitamin B12 (Gilsing et al., 2010). Even fortified breakfast cereals are still not providing sufficient level of vitamin B12 (Green et al., 2017, Pawlak et al., 2013a). Traditional supplementation and fortification methods have been employed to address this deficiency, but these approaches often face challenges related to bioavailability, consumer acceptance, and sustainability (Allen, 2010, Watanabe, 2007).

Vitamin B12 is exclusively synthesized by microorganisms in nature (Watanabe, Bito, 2018b). Among all the microorganisms that potentially produce vitamin B12, *Propionibacterium shermannii* / *freudenreichii* (propionic acid bacterium, PAB) was well studied and is considered to be a high-level producer which can be used in food fermentations (Balabanova et al., 2021). PAB is able to produce high-levels of vitamin B12 (with GMOs production can reach up to 100 µg/L), however, it requires complex growth conditions, e.g., a two-stage fermentation, long growth period, slow acidification and a pH at the end of fermentation maintained at pH 6,5, which favours other opportunists to grow (Massoud et al., 2020, Xie et al., 2019).

On the other side, lactic acid bacteria (LAB) are well-known to have a long history of use as start culture for yogurt or fermented food applications in the food industry (Capozzi et al., 2012). However, LAB do not naturally produce sufficient levels of active vitamin B12. Indeed, biologically inactive vitamin B12 (pseudo vitamin B12) is the main metabolite generated in their metabolism pathways (Santos et al., 2007). Since lactate is an essential nutrient for PAB to utilise for growth and is also the major end product LAB metabolism (Hugenschmidt et al., 2011), a co-fermentation strategy might be a potential solution to enhance the B12 contents in fermented foods on the one hand, on the other hand, to increase the acidification process to maintain an adequate pH to guarantee food safety.

Furthermore, the use of fermentation technology for vitamin B12 enrichment promotes sustainability in food production (Calvillo et al., 2022, Piwowarek et al., 2022). Fermentation offers an alternative to complex and uneconomical chemical synthesis methods for vitamin B12 (Calvillo et al., 2022, Mohammed et al., 2014). By integrating microbial fermentation, for instance, in cereal matrices, the food industry can naturally fortify products with active B12, reducing reliance on synthetic supplements and fortificants (Chamlagain et al., 2018, Tripathi et al., 2024). This shift contributes to decreasing the overall environmental footprint associated with vitamin production, especially when compared to the higher carbon and water footprints linked to animal-based vitamin B12 sources (Bayindir-Gumus, Yardimci, 2025).

The ultimate objective of this study was to investigate a possible co-fermentation strategy to enhance vitamin B12 levels in a plant-based organic product. According to EU legislation EU 2018/848 (European Union, 2018) no additional synthesized nutrients should be added into the raw material and manufacturing process in the organic food sector; therefore, enriching vitamin B12 content through a natural fermentation process would be an excellent solution and unique selling point for the organic food manufacturers.

To achieve this objective, a comprehensive investigation into the biosynthesis of vitamin B12 must first be conducted, with particular emphasis placed on *Lactobacillus* species. This is because most *Lactobacillus* species are unable to produce appreciable levels of bioactive vitamin B12 and in general at a very low level (Santos et al., 2007, Varmanen et al., 2016). For example, *L. reuteri* can produce vitamin B12 under optimized conditions, but its production levels are generally low, unless specific medium compositions or genetic manipulations are applied (Santos et al., 2009, Torres et al., 2016, Y. Mohammed et al., 2014). Some specific strains, such as *Lactobacillus reuteri*, *Lactobacillus rossiae*, and *Lactobacillus coryniformis*, have been identified as capable of producing vitamin B12 (Angelis et al., 2014, Torres et al., 2016). However, further investigations were needed to identify whether these are able to produce bioactive vitamin B12.

Secondly, *in vitro* investigations were conducted in a laboratory setting to quantify the actual production of both the active and inactive forms of vitamin B12 among *Lactobacillus* strains. Furthermore, the suitability of fermentation and the possibility of natural biofortification in plant-based matrices (with oats as a case study) via a co-fermentation strategy with *Propionibacterium* species was investigated.

Moreover, to gain a deeper understanding of the mechanism of vitamin B12 biosynthesis among *Lactobacillus* producers, gene expression studies via transcriptomics, based on RNA sequencing, aimed to reveal the regulation of genes potentially involved in vitamin B12 biosynthesis in response to the addition of vitamin B12 precursors.

By achieving all the objectives mentioned above, the present study should provide a promising solution and a competitive advantage in developing plant-based organic food products, supporting the growing trend towards plant-based diets and preventing the relevant deficiencies to those consumer groups who adhering such diets.

2. LITERATURE REVIEW

2.1. Addressing vitamin B12 deficiencies: Increased vegetarian/vegan diet groups and biofortification

The growth of plant-based dairy alternatives market is driven by several factors. Firstly, ethical, environmental, and health concerns related to traditional dairy products have heightened consumer demand for plant-based alternatives (Harper et al., 2022). Additionally, individuals with dietary restrictions or health conditions, such as lactose intolerance or metabolic syndrome, are increasingly interested in these alternatives (Plamada et al., 2023). Moreover, plant-based beverage substitutes are naturally free from lactose and have lower amounts of cholesterol and fat compared to animal milk, making them more appealing to consumers (Abbou et al., 2025). Furthermore, the diverse availability of plant sources, including cereals, legumes, nuts, and seeds, enables the creation of various plant-based dairy alternatitives that can offer nutritional profiles comparable to those of animal-derived products (Romulo, 2022). Lastly, the marketing strategies of plant-based dairy alternatives, emphasizing sustainability and environmental commitment, have also contributed to their growth in the market (Ramsing et al., 2023). As a result, global consumption of milk substitutes has demonstrated steady growth over the past decade, as illustrated in Figure 1.

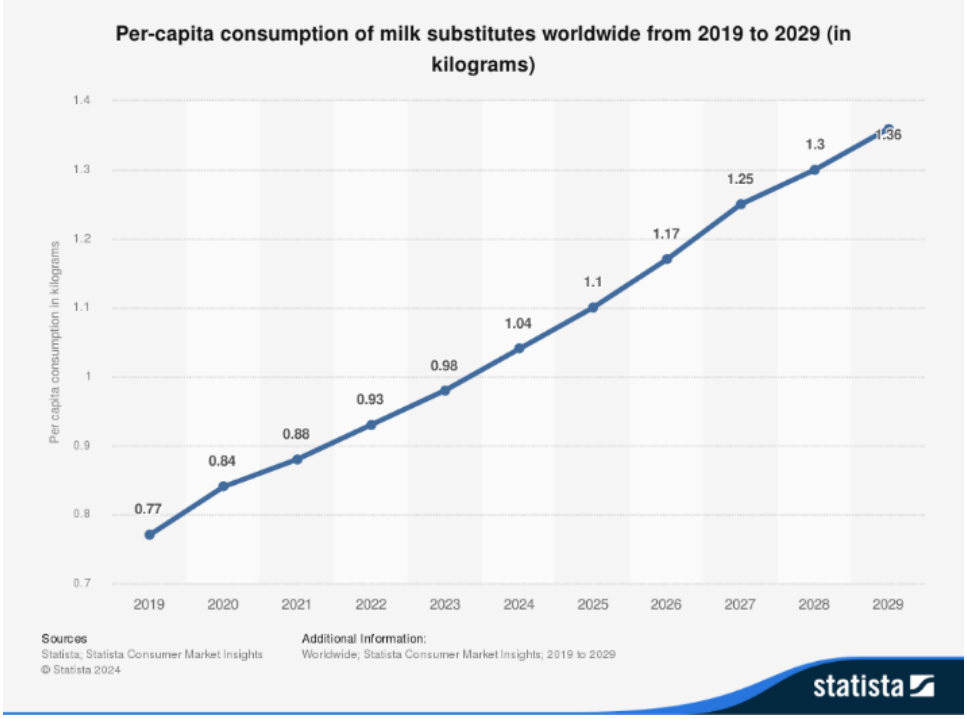

Figure 1 Per-capita consumption of milk substitutes worldwide. Adapted from "Per-capita consumption of milk substitutes worldwide from 2019 to 2029 (in kilograms)" by Statista, 2024. Copyright 2024 by Statista.

2.1.1. Vegetarian and vegan diet population and relevant deficiencies

Plant-based diets, predominantly comprising fruits, vegetables, nuts, and whole grains, are associated with a reduced risk of cardiovascular disease (CVD) (Hu, 2003, Satija, Hu, 2018). These diets provide an abundance of mono- and polyunsaturated fatty acids, omega-3 fatty acids, antioxidant vitamins, minerals, phytochemicals, fibre, and plant protein, all of which together offer protective effects against CVD (Hu, 2003).

Despite these benefits, plant-based diets, particularly vegan ones, can pose nutritional challenges. Essential nutrients like protein, iron, zinc, calcium, and vitamins B12, A, D, and long-chain omega-3 fatty acids might be deficient in the absence of animal products (Craig, 2009, Dwyer, Loew, 1994). Among these, the one of most concern is vitamin B12 deficiency, since it can only be reliably obtained from animal-source foods. For instance, vegans and vegetarians without appropriate dietary supplementation may face increased risks of malnutrition, especially among women and children (Green et al., 2017, Green, 2017, Kiely, 2021).

Research indicates that deficiencies are prevalent among these dietary groups, with deficiency rates among vegans varying from 60% to 80% (Herrmann, 2017, Woo et al., 2014). Lacto-ovo-vegetarians are also susceptible, with over 40% experiencing deficiencies of vitamin B12 (Pawlak et al., 2013b). Deficiency rates vary among different populations, with rates of 62% among pregnant women, 25-86% among children, 21-41% among adolescents, and 11-90% among the elderly (Pawlak et al., 2013b). In low- and middle-income countries, deficiency rates range from 26% to 51%. For example, studies in India report deficiency rates as high as 63.2% in early pregnancy (Finkelstein et al., 2024).

Notably, vitamin B12 deficiency can lead to serious health outcomes such as cognitive dysfunction and neurological disorders (Green et al., 2017). It is crucial for individuals adhering to plant-based diets to monitor and supplement essential nutrients, particularly vitamin B12. When effectively planned, including fortified foods, vegetarian diets can meet nutritional needs and support optimal health (Allen, 2009).

2.1.2. The dietary sources of B vitamins and bio-fortification

Dietary sources of B vitamins include a variety of foods such as meat, fish, eggs, dairy products, legumes, whole grains, and certain fermented foods. A detailed list of plant-based food sources which contains vitamin B12 is shown in Table 1.

Table 1 Vitamin B12 dietary source in plant-based foods.

Food product/supplement	**Vitamin B12 (µg/100 g)**	**Reference**
Spinach leaves (fertilized with cow manure)	0.14	(Mozafar, 1994)
Tempeh	0.7 - 8.0	(Nout, Rombouts, 1990)
Kimchi	0.1	(Kwak et al., 2010)
Tea leaves	0.1 - 1.2	(Kittaka-Katsura et al., 2004)
Black trumpet/golden chanterelle	1.09 - 2.65	(Watanabe et al., 2012)
Shiitake mushroom	5.61	(Bito et al., 2014)
Dried green laver/purple laver	32.3 - 133.8	(Miyamoto et al., 2009, Watanabe et al., 1999b)
Chlorella tablets	0.1 - 415	(Bito et al., 2016)
Spirulina tablets	127 - 244	(Watanabe et al., 1999a)

Notably, vitamin B12 is predominantly present in animal-derived foods, including meat, milk, eggs, fish, and shellfish (Watanabe, 2007, Watanabe et al., 2013). Certain plant-based items, such as dried laver (nori) and mushrooms, also contain vitamin B12 due to a symbiotic relationship with specific bacteria (Allen et al., 2018, Allen, Long, 1988, Watanabe et al., 2014). On the other hand, spirulina were often mistaken for supplementing vitamin B12, however, the corrinoid in blue-green algae is biologically inactive (Watanabe et al., 2013) The bioactivity of vitamin B12 can vary significantly among different food sources and is subject to alteration by cooking and storage methodologies (Watanabe, Bito, 2018a, 2018b).

For vegans and elderly populations who may have limited access to traditional sources of vitamin B12, fortified foods like breakfast cereals, as well as vitamin B12-enriched vegetables, serve as viable alternatives. Additionally, dried purple laver (nori) is highlighted as a particularly suitable plant-derived source of bioactive vitamin B12 for vegetarians (Watanabe et al., 2014).Some traditional fermented foods are a rich source of B vitamins. For example, fermented foods such as yogurt, kefir, sauerkraut, and tempeh can provide substantial quantities of B vitamins. Certain strains of *Lactobacillus* have demonstrated a remarkable ability to produce vitamin B12 in traditional food preparations such as yogurt and sourdough bread (Capozzi et al., 2012). Other studies have explored the enrichment of vitamin B12 using *P. freudenreichii* in various dairy products, including kefir and probiotic feta cheese (Angelopoulou et al., 2017, van Wyk et al., 2011). These fermented foods undergo a natural fermentation process, although the issue of the bioavailability was unclear due to the biological inactive form of analogues. The lactic acid bacteria present in these fermented foods are known to produce B vitamins such as vitamin B12, folic acid and vitamin B2 (LeBlanc et al., 2011).

Additionally, the use of folate-producing lactic acid bacteria in the fermentation of dairy products and other fermented foods has accordingly been found to increase the folate levels of these foods, making them even more beneficial as a dietary source of B vitamins (Levit et al., 2021, Mahara et al., 2019).

Applying a traditional fermentation approach, many studies have shifted the focus towards natural vitamin B12 fortification in plant-based foods. Different categories based on raw material type have emerged: 1.) cereal matrices, where *P. freudenreichii* enriches vitamin B12 in wheat flour and malt extracts (Xie et al., 2018); 2.) vegetable fermentation, involving the fermentation of hydrolysed tomato pomace or cabbage (known as kimchi) by *P. freudenreichii* (Haddadin et al., 2001, Ro et al., 1979); 3.) soybean products, such as *furu* (a traditional fermented soybean product) and soy-yoghurt, where *L. reuteri* and *P. freudenreichii* have been studied for vitamin B12 production (Bao et al., 2019, Gu et al., 2015); 4.) soy protein alternatives, like lupine, which aim to develop approaches to enrich vitamin B12 in non-soy plant protein products (Wolkers – Rooijackers et al., 2018). However, it should be noted that recent findings indicate that actually the pseudo-cobalamin form of vitamin B12, which is inactive, is produced by the majority strains of *Lactobacillus* (Santos et al., 2007).Thus, for future applications in the bio-fortification of B vitamins, particularly vitamin B12, selecting strains capable of producing biologically active compounds through an optimized fermentation process could help overcome deficiency problems.

2.1.3. Consumer favoured plant-based raw materials

Consumers in Europe have shown a preference for plant-based food alternatives that closely resemble the taste and texture of animal-based products. In terms of specific ingredients, consumers have expressed a desire for plant-based beverage and yogurt alternatives that have a less beany and sweet taste (Waehrens et al., 2023). For plant-based semi-hard cheese alternatives, consumers have indicated a need for improvements in terms of creating a more cheese-like, less artificial, and less bland product (Jaeger et al., 2024). Regarding plant-based beef alternatives, consumers are interested in products with a more umami taste and a taste and odour similar to beef (Caputo et al., 2023). Concerning the dairy alternatives, the major segmentation was devoted to cereals and legumes in the European market, that are gaining popularity due to their functional health benefits and which cater to consumers with dietary restrictions like lactose intolerance (Plamada et al., 2023). To create a more natural taste and increased versatile aroma, fermentation was considered as an effective strategy to not only improve the sensory properties but also increase the functionalities of plant-based alternative products (Hidalgo-Fuentes et al., 2024).

Apart from that, many countries around the globe traditionally produce fermented cereal products, like plant-based meat substitutes (PBMS)s, which enlarges the product category profile and enhances the innovation in the food industry. In many Asian countries, fermented foods were already an integral part of traditional culinary practices. For instance, many traditionally produced, fermented cereal products are based on rice, wheat, corn or sorghum as raw material (Blandino et al., 2003). In South Korea, a type of fermented beverage that called' Sikhye', uses rice as raw material with the addition of malt extract and sugar. Moreover, legume-based foods such as Chongkukjang which are based on a soybean paste and which are made with *Bacillus subtilis* and rice straw, are particularly prevalent (Patra et al., 2016).

2.1.4. Oat as a multi-functional plant protein source for probiotic applications

Regulatory bodies like the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) recognize several health-benefits of oats through approved health claims. Notably, β-glucan from oats is identified as a significant soluble dietary fibre. The cereals with the highest β-glucan levels are oats and barley (Manthey et al., 1999). Consuming 3 grams or more of β-glucan daily, as part of a diet low in saturated fats and cholesterol, can help reduce coronary heart disease risk (Clemens, van Klinken, 2014).

β-glucan performs a prebiotic function in the gastrointestinal tract, supporting beneficial microbial growth (Joyce et al., 2019). Moreover, oat proteins exhibit distinctive physico-chemical characteristics, featuring a protein content notably higher (12-20% more abundant) than that of other cereal grains (Boukid, 2021). Oat proteins are also rich in essential amino acids such as lysine and threonine, which are often scarce in other cereal grains (Tomičić et al., 2022), natural antioxidants such as avenanthramides and avenalumic acids are unique to oats (Xie et al., 2024). This enhanced protein quality is advantageous for those following plant-based diets or seeking alternative protein sources. Industrially, native isolated oat proteins exhibit good solubility and emulsifying properties across pH levels, beneficial for food applications (Spaen and Silva 2021). They also provide favourable sensory attributes due to their gelling properties compared to proteins from legumes and oilseeds (Boukid, 2021). Oat proteins can form heat-induced gels, and their gelling properties can be adjusted via enzymatic hydrolysis to create plant protein ingredients with desired functional properties (Brückner-Gühmann et al. 2021), the resulting gels exhibit a smooth and moist texture, primarily attributed to the heat-induced gelation of starch, which significantly influences their mouthfeel (Brückner-Gühmann et al., 2019).

Oat flour and oat bran have been found to have a protein quality comparable to dehulled soya bean meal and could potentially replace soy proteins in non-GMO food applications (Hahn et al. 1990) and contain significant fat, predominantly unsaturated fatty acids. The nutritional profile of oat is presented in Table 2.

Differences in lipid content and fatty acid composition occur across oat varieties, with higher fat content typically found in dehulled and naked oats versus hulled types (Kouřimská et al. 2018). Some oat genotypes present fewer antinutritional factors, making them more suitable for human consumption (Kaur et al. 2019).

Table 2 Nutritional composition of oat. (Adopted from (Rasane et al., 2015))

Component of oat	Availability in oat (%)
Starch	60 %
Protein	Total: 11–15 % Globulins: 80 % of total protein Prolamins: 15 % of total protein Glutelin: 5–66%of total protein Albumin: 1–12 % of total protein
Lipid	5–9 %
Dietary fibres	*β*-glucan: 2.3–8.5 %
Trace Minerals	Calcium: 0.54 % Iron: 0.047 %
Vitamins	Thiamine: 0.002 % Riboflavin: 0.001 % Niacin: 0.032 %

Oats contain approximately 12-17% protein by dry weight (Amagliani et al., 2021). While this is less than certain animal protein sources like meat and dairy, oats' balanced essential amino acid profile, including lysine, threonine, and tryptophan, stands out compared to other plant proteins such as rice or wheat (Klose, Arendt, 2012). Most cereals, including barley, wheat, and rye, contain prolamin proteins as their main storage protein. These proteins are problematic due to their allergenicity, caused by their high proline and glutamine content. In contrast, the major storage protein in oats is globulin, comprising up to 80% of the total protein. (Shewry et al., 2002), as shown in Table 3. This composition makes oats more nutritionally complete and characterizes them as a sustainable protein source. Oats are also suitable for people with celiac disease, as they are gluten-free (Angelov et al., 2005). Therefore, functional food applications based on oat-matrix could also contribute to the gluten-free product market.

Table 3 Protein profile of oat compared to other plant species, adopted from (Amagliani et al., 2021).

Family	Species	Protein (% dry basis)	Albumins (%)	Globulins (%)	Glutelins (%)	Prolamins (%)
Pulses	Pea	24–28	18–25	55–65	3–4	4–5
	Chickpea	21–26	8–12	53–60	19–24	3–7
	Faba bean	32–34	1–3	70–78	12–20	2–4
Oilseeds	Soy	~40		85–90		
	Canola	21–26	20	60		
Cereals	Rice	6–8	8–10	6–17	75–83	2–7
	Oat	12–17	1–12	50–80	<10	4–15

2.2. Microbial production of vitamin B12 and biofortification

2.2.1. Structure and characteristics of vitamin B12

The term vitamin B12 is widely used to describe compounds of the cobalamin group. The structure is the most complex and it has the largest molecular mass (1.3 -1.6 kDa) among all the other vitamins (Martens et al., 2002). The molecular structure is shown as in Figure 2, the central corrin ring contains four ligands coordinated to a central cobalt ion, along with a lower (alpha) ligand connected to **5,6-dimethylbenzimidazole (DMBI)** and an upper (beta) ligand bonded to various groups. These include the **CN^- group**, forming cyanocobalamin (CNCbl), which is defined as vitamin B12; the **OH^- group**, forming hydroxocobalamin (OHCbl); **CH_3^- group**, forming methylcobalamin (MeCbl); and the **5'-deoxyadenosylcobalamin group**, forming deoxyadenosylcobalamin (AdoCbl) (Martens et al., 2002, Piwowarek et al., 2018).
Cyanocobalamin, which contains a CN^- group, is commonly produced during industrial extraction processes using microbes. In contrast, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) are the natural, active forms of vitamin B12 that the body uses in important metabolic functions. An active vitamin B12 synthesis requires the presence of DMBI as the lower ligand of the macrocyclic ring. Notably, when DMBI is replaced by adenine at this position, the compound formed is known as **pseudovitamin B12**. This form of vitamin can be synthesized by several anaerobic bacteria; however, it is inactive in the biological system (Piwowarek et al., 2018).

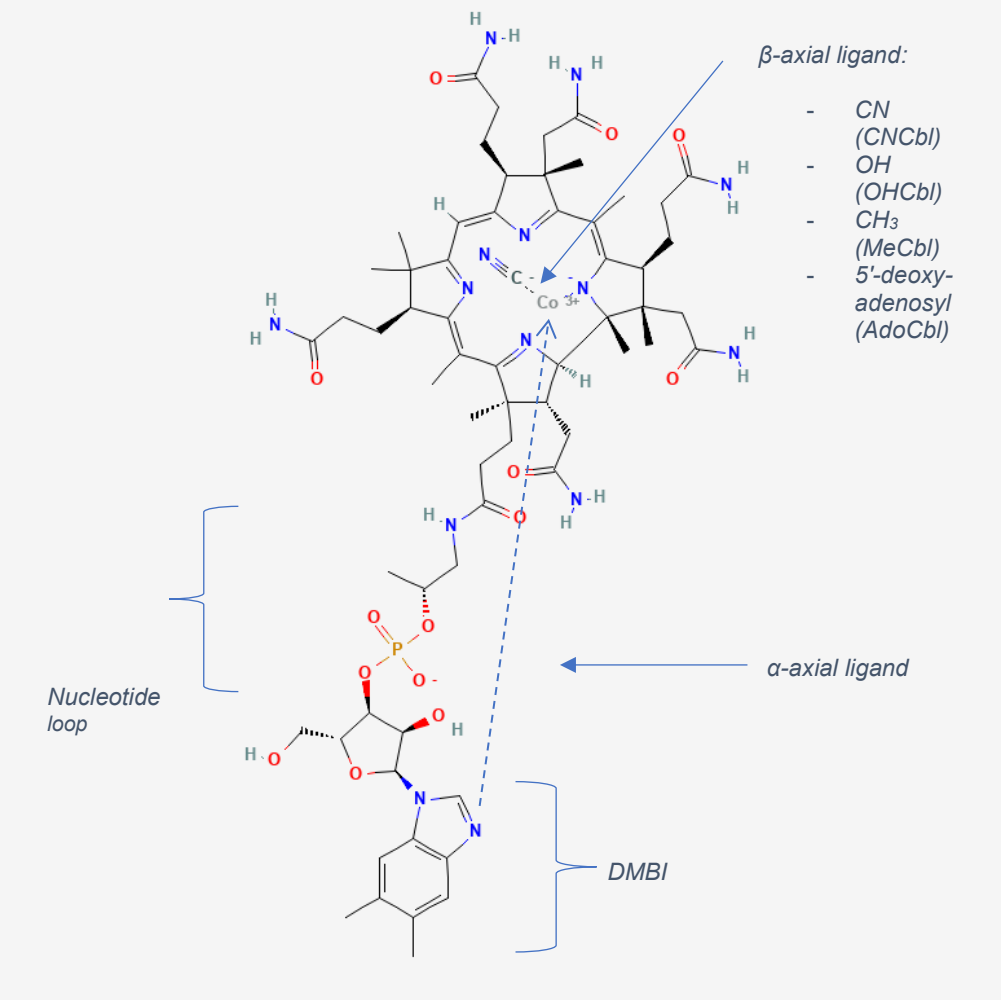

Figure 2 Molecular structure of vitamin B12.

(Adopted from PubChem, 2D structure image of CID 5311498 (vitamin B12), URL: https://pubchem.ncbi.nlm.nih.gov/compound/5311498#section=2D-Structure.)

2.2.2. Vitamin B12-dependent reactions in bacteria and archaea

Fermentation processes have been recognized as playing a crucial role energy production in many bacteria Thus, in the early stage of research, Roth and co-workers indicated that the importance of B12 biosynthesis was to support fermentation of small molecules and to generate both an oxidizable compound and an electron sink for use in balancing redox reactions (Roth et al., 1996). Additionally, in many anaerobic energy production processes, such as methanogenesis and amino acid fermentation, vitamin B12 is involved. This indicates that vitamin B12 is associated with most anaerobic metabolic processes (Martens et al., 2002).

The initial formation of vitamin B12 starts with the aminoacyl-tRNA molecule among almost all prokaryotes, except for the α-group of proteobacteria (Benner et al., 1989, Jahn et al., 1992, Martens et al., 2002). There are mainly four prominent reactions in bacteria and archaea which requires vitamin B12 (Martens et al., 2002):

1.) Acetyl-CoA synthesis; in acetogenic bacteria, the process of acetate formation requires methyl corrinoids to support the synthesis of acetyl-CoA from two molecules of CO_2 (Ragsdale, 1991, Stupperich, 1993).

2.) Methyl transfer in methane-producing bacteria.

3.) Ribonucleotide reductase, which is associated in DNA synthesis, one of the class which belong to the ribonucleotide reductase in adenosylcobalamin-dependent reductase, a good example is the vitamin B12-microbioglical assay test microorganism *Lactobacillus leichmanii* (Blakley, 1965, Blakley, Barker, 1964).

4.) fermentation processes in enteric bacteria; except *Escherichia coli*, among all other bacteria in the anaerobic fermentation of 1,2-propanediol, ethanolamine and glycerol, coenzyme B12 is required.

2.2.3. Microbial synthesis of vitamin B12

In nature, cobalamin biosynthesis pathway is in general categorised into two parts: a.) **aerobic**, with the participation of ***cob*** genes, mostly in bacteria belonging to the *Pseudomonas* genus and b.) **anaerobic**, with the participation of ***cbi*** genes, mostly in bacteria belonging to the *Bacillus* and *Salmonella* genus. On the other hand, bacteria from the *Propionibacterium* genus also have genes with prefix ***cbi*** and ***cob*** and it has been reported that all *Propionibacterium* strains which are capable to produce vitamin B12 show increased production under microaerophilic condition, especially under minimal oxygen conditions (Martens et al., 2002). The lower-ligand DMBI synthesis, however, requires oxygen participation (Martens et al., 2002, Piwowarek et al., 2018, Raux et al., 2000). The biosynthesis of DMBI also requires the participation of an enzymatic complex encoded by *bluB/cobT2*, which is a key to generate DMBI and attach to the corrin ring structure instead of adenine (Falentin et al., 2010). Most industrially produced vitamin B12 relies on microbial production instead of a chemical synthesis approach, since it is less labour intensive and cheaper (Piwowarek et al., 2018). Currently, genetically manipulating a *P. freudenreichii* strain has resulted in a significant enhancement of vitamin B12 production, whereas on the other side, naturally highly efficient strains have also been screened for, in combination with strategies to optimize the fermentation media (Piao et al., 2004, Piwowarek et al., 2018).

Previous studies have identified several key gene clusters and precursors involved in the biosynthesis of vitamin B12. For instance, the *cob* and *cbi* gene clusters are essential for the conversion of uroporphyrinogen III into cobalamin. These gene clusters encode enzymes responsible for various steps in the pathway, including the methylation, decarboxylation, and ring contraction processes that lead to the formation of the corrin ring, a core structure of vitamin B12. An overview of vitamin B12-producing bacterial strains and key gene clusters is provided below in Table 4.

Table 4 Vitamin B12 producing bacterial strains.

Species	Strain	Origin	VB12 Concentration	Substrate	Analytical Method	Gene	Gene Sequencing Method	Reference
L. reuteri	CRL 1098	Sourdough	50 µg/L		(RP)-HPLC-UV Vis	*cobA, cbiJ, cbiK, cysG/hemD*	-	(Taranto et al., 2003)
L. reuteri	CRL 1098	Sourdough	20 µg/L	Fermented soy milk	MBA* (Kelleher, Broin, 1991)	-		(Molina et al., 2012)
L. reuteri	CRL 1098	Sourdough	1.28 ± 0.26 µg/ml corrinoid (when $CoCl_2$ was added); 2.52 ± 0.20µg/ml corrinoid (when DMB and $CoCl_2$ was added)	Vitamin B_{12} Assay Medium	RP-HPLC	*cobS, cobT, cbiF, cbiS, cbiT, hemB, pduX*		(Torres et al., 2018)
L. reuteri	JCM 1112	Human feces	701±124 pg/ml (1,1 ng per mg of protein)		B12 assay, *P. freudenreichii* LMG 16412 as positive control	*pdu-cbi-cob-hem* gene cluster		(Morita et al., 2008)
L. reuteri	DSM 20016	Human feces	20.04 ± 4.17 µg/l	Vitamin B_{12} Assay Medium + 0.5% Glycerol	RP-UFLC-DAD			(Bharat Bhushan et al., 2016)
			58,36 ± 6,87 µg/L		MBA			
L. reuteri	ATCC 55730		15.640 ± 11,73 µg/L	Vitamin B_{12} Assay Medium	MBA HPLC-UV/VIS			(Mogna et al., 2011)
			18,030 ± 2,164 µg/L					
L. reuteri	DSM 17938		10.950 ± 7.446 µg/L					
			12.890 ± 1.289 µg/L					
L. reuteri	DSM 16143		2.790 ± 2.1204 µg/L					
			8.000 ± 1.04 µg/L					
L. reuteri	ZJ03		68.10 ± 1.28 ng/ml	Soy Joghurt	MBA	*cobT, cbiA*	Q-RT-PCR	(Gu et al., 2015)
			180.30 ± 5.87 ng/ml	Soy Joghurt +20 g/l Glucose +10 g/l Glycerol +1 g/l Fructose				

Table 4 Cont. Vitamin B12 producing bacterial strains.

Species	Strain	Origin	VB12 Concentration	Substrate	Analytical Method	Gene	Gene Sequencing Method	Reference
L. plantarum	-	Kanjika/Kanji	13 ng/g dry biomass	Modified vitamin B12 production medium acc. to (Quesada-Chanto et al., 1994)	ELISA	-	-	(Madhu et al., 2010)
L. plantarum	CN-225	Nukazuke (Japanese pickles)	1.8 ± 0.1 µg/L		MBA	*cob-pdu*	16S rRNA	(Masuda et al., 2012)
	CN-49		0.20 ± 0.03 µg/L					
L. plantarum	IB-1 GU797248	Idli-batter	8 µg/mL		MBA (indicator strain: *E. coli* NCIM 2068)	-	16S rRNA	(Iyer et al., 2013)
L. plantarum	BHM10(NCDC771)	Human milk	32.1 ± 4.78 µg/L	Vitamin B12 Assay Medium + 0.5% Glycerol	MBA	*(blub/)cobT, cbiB, cbiA, cbiP, cbiK*	PCR (amplicon)/ 16sRNA	(Bharat Bhushan et al., 2016)
			10.916 ± 1.55 µg/L		RP-UFLC-DAD			
	BCF20(NCDC772)	Child feces	70,13 ± 7,55 µg/L		MBA			
			23.904 ± 1.73 µg/L		RP-UFLC-DAD			
L. plantarum	LZ95	Infant feces	996 ± 37 µg/L (Intracellular*[a])	MRS broth	*[a] MBA *[b] SPE + RP- HPLC	no specific gene information regarding B12 production, PCR-based identification of strain specificity	16S rRNA	(Li et al., 2017)
			98 ± 15 µg/L (Extracellular*[b])					
	CY2	Fresh milk	565 ± 51 µg/L (Intracellular)					
			60 ± 9 µg/L (Extracellular)					
	CY3		400 ± 67 µg/L (Intracellular)					
			Nd (Extracellular)					
	ZJ008		19 ± 7 µg/L (Intracellular)					
			Nd (Extracellular)					
	ZJ316	Infant feces	160 ± 23 µg/L (Intracellular)					
			Nd (Extracellular)					
	ZJ5	Fermented mustard	41 ± 9 µg/L (Intracellular)					
			Nd (Extracellular)					

Table 4 Cont. Vitamin B12 producing bacterial strains.

Species	Strain	Origin	VB12 Concentration	Substrate	Analytical Method	Gene	Gene Sequencing Method	Reference
L. plantarum	V7	Infant feces	7.6 ± 0.3 µg/L (Intracellular) 41.3 ± 1.6 µg/L (Extracellular)	B12-free medium	UFLC	*cbiK*	16S-23S rRNA	(Kumari et al., 2021)
L. sakei	CN-28 CN-3	Nukazuke	0.27 ± 0.03 µg/L < 0.1 µg/L		MBA			(Masuda et al., 2012)
L. fermentum	CFR 2195	Child feces	29,45 ng/g of dry biomass					(Basavanna, Prapulla, 2013)
L. rossiae	DSM 15814	Sourdough				*pdu-cbi-cob-hem* cluster		(Angelis et al., 2014)
L. casei	LZ54	Infant feces	295 ± 42 µg/L(In.); ex. nd.					(Li et al., 2017)
L. coryniformis	CN-229	Nukazuke	2.0 ± 0.1 µg/L		MBA			(Masuda et al., 2012)
L. coryniformis	CRL 1001	Silage	1.34 ± 0.18 corrinoid µg/ml	Vitamin B_{12} Assay Medium + $CoCl_2$ (250 ng/ml)	RP-HPLC	*cobA, cobD, cobU, cobS, cobC, cbiA, cbiB, cbiC, cbiD, cbi E, cbiT, cbiF, cbiG, cbiH, cbiJ, cbiK, cbiL, cbiM, cbiN, cbiQ, cbiO, cbiP, hem A, hemC, hemB, hemL, hemD*		(Torres et al., 2018)
			2.98 ± 0.43 corrinoid µg/ml	Vitamin B_{12} Assay Medium + CoCl2 (250 ng/ml) +DMB (200 ng/ml)				
			3.45 ± 0.48 corrinoid µg/ml	Vitamin B_{12} Assay Medium + L-threonine (50 µg/ml)				

Table 4 Cont. Vitamin B12 producing bacterial strains.

Species	Strain	Origin	VB12 Concentration	Substrate	Analytical Method	Gene	Gene Sequencing Method	Reference
L. coryniformis	CRL 1001	-	CoCl2 addition: 0.15 ± 0.02 µg/mL; PseudoB12 4.57 ± 0.45 µg/mL; CoCl2+DMB addition: 0.54 ± 0.03 µg/mL ; PseudoB12 4.29 ± 0.18 µg/mL		LC/ESI-MS/MS	*cbiT, cbiS, pduX, pur*	-	(Torres et al., 2020)
P. megaterium[a]	DSM 319	Wild-type	200 mg/L		MBA using *S. enterica* AR3612	*cbiX/L, cobA*	sqRT-PCR	(Mohammed et al., 2014)
	DSM 509	Wild-type	0.08-0.26 µg/L		ELISA	*cbiXJCDET LFGA cysGA cbiY btuR hemAXCD BL sirA*		(Biedendieck et al., 2010)
P. freudenreichii subsp. shermanii[b]	ATCC 13673	-	0.6 mg/g cells		HPLC-DAD	-	-	(Assis et al., 2020)

*__MBA__ = Microbiological Assay; ***a*** = *Priestia.megaterium;* ***b*** = *Propionibacterium freudenreichii subsp. shermanii*

2.2.3.1. Biosynthesis of vitamin B12 in *Propionibacterium* genus

The biosynthesis of vitamin B12 in propionic acid bacteria, such as *Propionibacterium freudenreichii*, involves a complex interplay of gene clusters and biochemical precursors. These bacteria contain both *cob* and *cbi* gene clusters, indicating that successful cobalamin biosynthesis requires both anaerobic and aerobic conditions (Falentin et al., 2010, Hedayati et al., 2020). These gene clusters encode enzymes that catalyze the conversion of simple precursors into the intricate structure of cobalamin. Key precursors, as shown in Table 5, include uroporphyrinogen III, precorrin-2, cobyrinic acid, cobinamide, and adenosylcobinamide, all of which undergo various modifications, such as methylation, ring contraction, phosphorylation, and guanylylation, to form the final vitamin B12 molecule.

Originally isolated from Swiss-type cheese, *P. freudenreichii* is notably used in vitamin B12 production due to its ability to metabolize lactate into propionate, acetate, and CO_2 via the Wood-Werkman cycle, also known as the fermentative route of pyruvate to propionate. This cycle involves intermediates like succinyl-CoA and methylmalonyl-CoA. Methylmalonyl-CoA mutase plays a crucial role by catalyzing the conversion of methylmalonyl-CoA to succinyl-CoA, with adenosyl-cobalamin acting as a cofactor (Falentin et al., 2010, Takahashi-Iñiguez et al., 2012). The production of coenzyme B12 integrates into the bacterium's energy production cycle, contributing to its long-term survival strategy.

Beyond vitamin B12 production, the bacterium has gained attention for producing other functional ingredients, such as 5-aminolevulenic acid (Kiatpapan, Murooka, 2001), trehalose, and exo-polysaccharides (Cardoso et al., 2004, Deutsch et al., 2008). Additionally, as a Gram-positive bacterium with a qualified presumption of safety (QPS) listing, *P. freudenreichii* is cleared by EFSA for use in food and feed additives, food enzymes, food flavorings, and novel foods (EFSA BIOHAZ Panel et al., 2024).

In *P. freudenreichii*, active vitamin B12 biosynthesis involves DMBI production through the riboflavin pathway (Lawrence, Roth, 1995, Renz, 1970). The *cobA* gene, encoding uroporphyrinogen III methyltransferase, is crucial for converting uroporphyrinogen III to precorrin-2, marking a turning point in the biosynthesis of vitamin B12 and siroheme (Li et al., 2020, Sattler et al., 1995). Vitamin B12 biosynthesis regulation in this bacterium is controlled by a vitamin B12-riboswitch, *cbiMCbl*, located within the 5' untranslated region (5' UTR) of the *cobA* operon (Li et al., 2020). The concentrations of glucose, yeast extract, KH_2PO_4, and glycine in the culture medium significantly impact vitamin B12 production (Liu et al., 2021).

Furthermore, genome shuffling has enhanced vitamin B12 production in *P. freudenreichii / shermanii*, leading to the upregulation of enzymes critical to the pathway (Zhang et al., 2010). Comparative proteome analysis revealed that six upregulated proteins, including glutaminyl-tRNA synthetase, delta-aminolevulinic acid dehydratase, methionine synthase, riboflavin synthase, phosphofructokinase, and isocitrate dehydrogenase play a role in vitamin B12 biosynthesis (Zhang et al., 2010). Herman and Hopper's study identified key enzymes in the biosynthetic pathway, including cobyrinic acid a,c-diamide synthetase, a bifunctional enzyme, cobalamin 5-phosphate synthase, and adenosyl transferase (Herman and Hopper, 2003), indicating the importance of enzyme regulation for improved vitamin B12 production in *Propionibacterium* strains. Table 5 outlines several key gene clusters and their associated genes and precursors involved in vitamin B12 production.

Table 5 Key precursors and their functions in cobalamin biosynthesis (descriptions and enzyme classifications are adapted from Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (KEGG, 2023).

Key Precursors	Description
Uroporphyrinogen III	The initial precursor in the biosynthesis of vitamin B12, undergoing methylation to form precorrin-2.
Precorrin-2	Formed from uroporphyrinogen III by the action of uroporphyrinogen III methyltransferase (encoded by *cobA*). It undergoes further modifications to form precorrin-3.
Precorrin-3	An intermediate formed from precorrin-2 through the action of precorrin-2 dehydrogenase (encoded by *cobI*). It is further modified to form precorrin-4.
Precorrin-4	Formed from precorrin-3 by the action of precorrin-4 C11-methyltransferase (encoded by *cobJ*). It undergoes additional modifications to form precorrin-6A.
Precorrin-6A	Formed from precorrin-4 by the action of precorrin-6A synthase (encoded by *cobM*). It is reduced to precorrin-6B by precorrin-6A reductase (encoded by *cobF*).
Precorrin-6B	An intermediate that is further modified to form precorrin-8X.
Precorrin-8X	Formed from precorrin-6B and further modified by precorrin-8X methylmutase (encoded by *cobK*) to form hydrogenobyrinic acid.
Hydrogenobyrinic Acid	An intermediate that undergoes cobalt chelation to form cobyrinic acid.
Cobyrinic Acid	Formed from hydrogenobyrinic acid through the incorporation of a cobalt ion by cobalt chelatase (encoded by *cobL*). It is then converted into cobinamide.
Cobinamide	An intermediate formed from cobyrinic acid. It is further processed to form adenosylcobinamide.
Adenosylcobinamide	Formed from cobinamide through the addition of an adenosyl group. This intermediate is further phosphorylated and guanylylated to form adenosylcobinamide-GDP.
Adenosylcobinamide-GDP	A critical intermediate that undergoes the final steps of nucleotide loop assembly, including the attachment of the dimethylbenzimid
Adenosylcobinamide-GDP	A critical intermediate that undergoes the final steps of nucleotide loop assembly, including the attachment of the dimethylbenzimidazole moiety, to form the final cobalamin molecule.

2.2.3.2. Vitamin B12 biosynthesis in lactic acid bacteria (LAB)

Among the bacteria capable of synthesizing vitamin B12, lactic acid bacteria (LAB), and particularly *Lactobacillus reuteri*, have drawn considerable attention. This section of the literature review provides a detailed introduction to the vitamin B12 biosynthesis pathway in LAB, followed by an in-depth examination of the key gene clusters and biochemical precursors involved, as shown in Table 6.

Lactobacillus reuteri employs the anaerobic pathway for vitamin B12 biosynthesis (as depicted in Figure 3). This biosynthetic pathway initiates with the precursor uroporphyrinogen III, which is modified through processes such as methylation, ring contraction, and cobalt chelation to produce the final cobalamin molecule. The key players in this biosynthesis are the *cob* and *cbi* gene clusters. These clusters encode the enzymes essential for each step of the biosynthesis. The presence of oxygen-dependent enzymes, such as oxygenases, is a defining feature of the aerobic pathway in LAB and facilitates specific reactions not feasible under purely anaerobic conditions.

The biosynthesis of vitamin B12 in lactic acid bacteria involves a complex series of enzymatic reactions and intermediates, orchestrated by key gene clusters such as the *cob* and *cbi* clusters (Taranto et al., 2003, Torres et al., 2016). These gene clusters encode enzymes that catalyse the conversion of initial precursors like uroporphyrinogen III into the final cobalamin molecule (Warren et al., 2002). Understanding the roles of these gene clusters and their associated precursors is crucial for optimizing vitamin B12 production in lactic acid bacteria, which has significant implications for both industrial applications and nutritional supplementation (Balabanova et al., 2021, Huan Fang et al., 2017, LeBlanc et al., 2011).

Table 6 Gene Clusters and key genes involved in anaerobic cobalamin synthesis pathway, using L. reuteri *DSM 20016 as an example (KEGG, 2023).*

Key Genes	Function
cobA-hemD	Encodes uroporphyrinogen III methyltransferase, catalyzing two sequential methylation of uroporphyrinogen III to form precorrin-1 and precorrin-2.
CysG	Encodes precorrin-2 dehydrogenase, converting precorrin-2 to Sirohydrochlorin.
cbiK	Encodes sirohydrochlorin cobaltochelatase, forming early cobalt insertion
cbiL	Encodes cobalt-factor II C20-methyltransferase, involved in the methylation of cobalt-factor II.
cbiH	Encodes cobalt-factor III methyltransferase, catalyses the tetrapyrrole ring contracts as a result of methylation of C-17, also convert cobalt-precorrin-3 to cobalt-precorrin-4.
cbiF	Encodes precorrin-4 C11-methyltransferase, catalyses the methylation of C-11 in cobalt-precorrin-4 to form cobalt-precorrin-5A.
cbiG	Encodes cobalt-precorrin 5A hydrolase, catalyzes the hydrolysis of cobalt-precorrin-5A, which results in the contraction of the porphyrin-type tetrapyrrole ring. A vital step to form the core corrin ring structure.
cbiD	Encodes cobalt-precorrin-6A synthase, catalyzing the C-1 methylation of cobalt-precorrin-5B, forming cobalt-precorrin 6A.
cbiJ	Encodes cobalt-precorrin-6A reductase, catalyzing the reduction of cobalt-pre corrin-6A to cobalt-precorrin-6B. Preparing the corrin ring for subsequent methylation.
cbiT	Encodes cobalt-precorrin-6B (C15)-methyltransferase, catalyzes the methylation of cobalt-precorrin-6B and transforms it into cobalt-precorrin-7.
cbiE	Encodes cobalt-precorrin-7 (C5)-methyltransferase, catalyzing the methylation of cobalt-precorrin-7
cbiC	Encodes cobalt-precorrin-8 methylmutase, converting cobalt-precorrin-8 to cobyrinate by methyl rearrangement.
cbiA	Encodes cobyrinic acid a,c-diamide synthetase, catalyzing the synthesis of cobyrinate a,c-diamide from cobyrinate using either L-glutamine or ammonia as the nitrogen source.
cbiB	Encodes adenosylcobinamide-phosphate synthase, involved in the adenosylation of cobyrinic acid a,c-diamide.
cbiP/cobQ	Encodes adenosylcobyric acid synthase, converting adenosylcobyrinic acid a,c-diamide into adenosylcobyric acid.
cobA/cobO	Encodes uroporphyrinogen-III C-methyltransferase
cobN, cobS, cobT	Involved in the final steps of nucleotide loop assembly and attachment of the lower axial ligand, dimethylbenzimidazole.

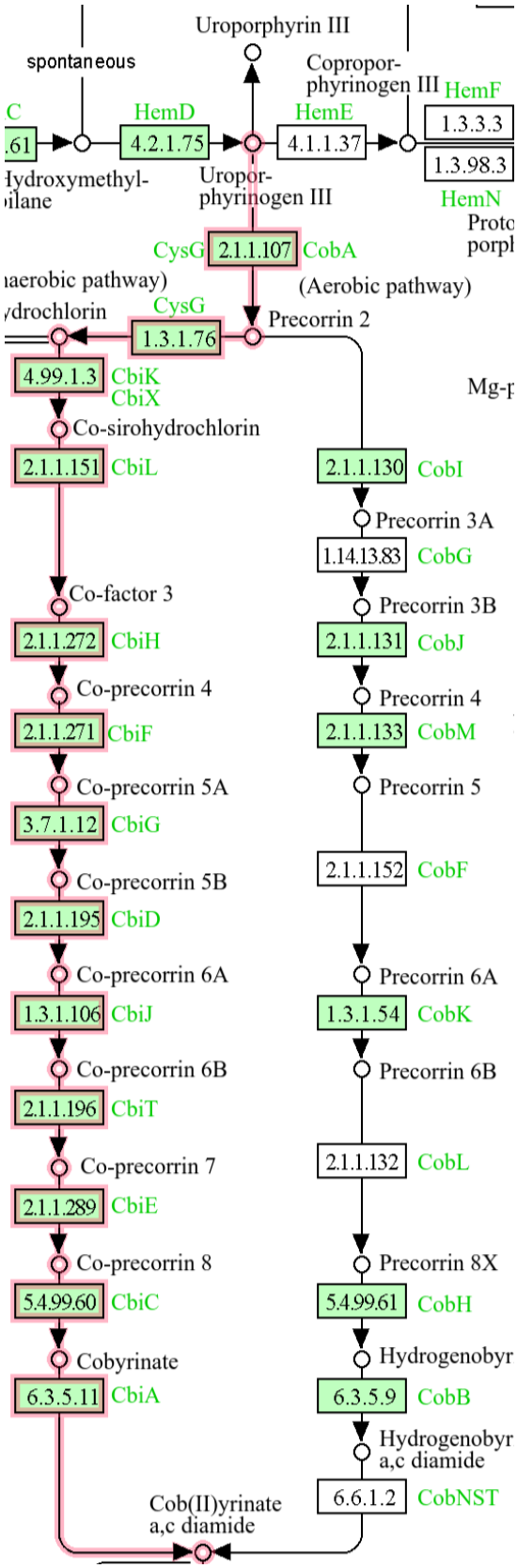

Figure 3 KEGG-Porphyrin metabolism from Lactobacillus reuteri subsp. reuteri *DSM 20016 (KEGG, 2023).*

(Red mark: Cobalamin biosynthesis, anaerobic).

2.2.4. Gene Expression

The biosynthesis of vitamin B12 involves a complex multi-enzyme pathway, and recent studies have focused on understanding the gene expression and regulation of this process in various organisms, particularly *Lactobacillus* species (Rodionov et al., 2003, Santos et al., 2008). The gene expression in vitamin B12 biosynthesis among *Lactobacillus*, particularly *L. reuteri*, has been studied extensively (Santos et al., 2011, Torres et al., 2020). For instance, the coenzyme B12 gene cluster of *L. reuteri* CRL1098 has the unique feature of clustering together the *cbi, cob,* and *hem* genes, consisting of 29 ORFs encoding the complete enzymatic machinery necessary for *de novo* biosynthesis (Santos et al., 2008). Transcriptional analysis showed it to be expressed as two tandem transcripts of approximately 22 and 4 kb, carrying *cobD* gene among others (Santos et al., 2008). Both transcripts appear to be similarly regulated and are induced in the late-exponential growth phase (Santos et al., 2008). Evidence for a regulatory mechanism of negative feedback inhibition by vitamin B12 itself was found (Vitreschak et al., 2003).
Furthermore, *L. reuteri* harbours the genes responsible for glycerol utilization and vitamin B12 synthesis within a genetic island. The findings reported in the study could be used to improve the production yield of vitamin B12, 1,3-propanediol, and reuterin, all industrially relevant compounds (Morita et al., 2008, Santos et al., 2011).

Transcriptional analysis of vitamin B12 production in *L. reuteri* has been reported in several studies (Santos et al., 2008, Santos et al., 2011, Saulnier et al., 2011, Spinler et al., 2014). One study used cDNA microarray experiments to analyse the transcriptional response of *L. reuteri* to a medium lacking cysteine, supporting the observed high vitamin B12 production (Santos et al., 2009). Another article reported the functional identification of a PocR-like transcription factor regulating glycerol utilization and vitamin B12 synthesis in *L. reuteri*, which could be used to improve the production yield of vitamin B12 (Santos et al., 2011). Additionally, *L. rossiae* has been identified as a metabolically versatile species within the genus *Lactobacillus* with the potential for de novo biosynthesis of vitamin B12 (Angelis et al., 2014). These studies provide insights into the genetic regulation and metabolic pathways involved in vitamin B12 production by *Lactobacillus* bacteria.

2.3. Vitamin B12 quantification and detection method

Traditional methods for B12 quantification include microbiological assays, one of the oldest methods for B12 quantification, which exploit the growth dependency of specific bacteria (e.g., *Lactobacillus leichmannii*) on cobalamin (Kelleher, Broin, 1991). These assays indicate how well the test organisms are growing in mediums supplemented with vitamin B12 standard solutions based on turbidimetric methods. While cost-effective and historically significant, they suffer from cross-reactivity with inactive B12 analogues and long incubation times, poor precision and relatively low specificity (Karmi et al., 2011, Kumar et al., 2010).

Advances in chromatography, particularly high-performance liquid chromatography (HPLC) coupled with UV or fluorescence detection, improved specificity by separating B12 from analogues and co-eluting substances. However, HPLCs often require extensive sample pre-treatment (e.g., cyanide derivatization) and lack sensitivity for trace-level analysis in biological fluids (Karmi et al., 2011).

The advent of chemiluminescence immunoassays (CLIA) and enzyme-linked immunosorbent assays (ELISA) revolutionized clinical diagnostics by offering high-throughput, automated platforms with enhanced sensitivity (pg/mL range). These methods rely on antibodies targeting intrinsic factor-B12 complexes, minimizing interference from analogues. Despite their widespread use in biological samples, their applications in food samples are still limited and inconsistencies in antibody specificity and matrix effects in serum/plasma remain challenges (Gherasim et al., 2013, Quadros, 2010).

Recent innovations have focussed on mass spectrometry (MS)-based techniques, such as liquid chromatography-tandem MS (LC-MS/MS), which provide unparalleled accuracy by distinguishing B12 isoforms (e.g., methylcobalamin, adenosylcobalamin) and quantifying these simultaneously. LC-MS/MS is now considered the gold standard for research applications, though its high cost and technical complexity limit routine clinical use (Hannibal et al., 2016).

There are currently only few existing official methods that have been developed for determination of vitamin B12 in food, the details are shown in Table 7.

Table 7 Existing official standard methods in vitamin B12 quantification.

Method	Scope	Sample extraction and purification	Analytical instrument	Reference
AOAC 952.20	Vitamin preparations	-	Microbiological Assay	(AOAC Official Method 952.20, 2023)
AOAC 986.23	Milk-based infant formula	-	Microbiological Assay	(AOAC Official Method 986.23, 2023)
AOAC 2011.08 (1st action)	Infant formular and adult nutritionals	Immunoaffinity extraction	HPLC, UV-Vis detector	(Giménez, 2014)
AOAC 2011.10 (Final action)	Infant formula and adult nutritionals	C8 or C18 SPE clean-up (column switching)	HPLC, UV-Vis detector	(Butler-Thompson et al., 2015)
AOAC 2011.16 (1st action)	Infant formula and adult nutritionals by Surface Plasmon Resonance (Test kits method)	Qflex Kit Vitamin B12 PI —Product Code BR 100677 (Biacore).	Biacore Q	(Vyas et al., 2012)
AOAC 2014.02 (Final action)	Infant Formula Adult/Pediatric Nutritional Formula	EASI-EXTRACT® VITAMIN B12 LGE (R-Biopharm AG; Product Code P88).	HPLC or UHPLC system, UV-VIS or PDA detector	(Giménez, Martin, 2018)
ISO 20634:2015 (equivalent to AOAC 2011.10)	Infant formula and adult nutritionals	SPE clean-up	HPLC-UV	(International Organization for Standardization, 2015)

2.3.1. Microbiological assay

Various microbiological assays have been developed for vitamin B12 quantification, mainly distinguished by the use of different test organisms such as *Escherichia coli* (Goldberg et al., 1957), *Euglena gracilis* or *Ochromonas malhamensis* and *Arthrobacter lochhead* (Goldberg et al., 1957, Stroiński, Schneider, 1987). Most of the abovementioned test organisms have been applied less due to their cross-reaction with many other inactive B12 analogues, such as vitamin B12 methylamide, vitamin B12 monoacids, pseudovitamin B12, which can lead to overestimation of vitamin B12 levels (Ford, 1959). The official AOAC reference method 952.20 utilises *Lactobacillus delbrueckii* subsp*. lactis* DSM 20355 as the test organisms, previously named *Lactobacillus leichmanii*, also known as ATCC 7830, which has been reported to be auxotrophic to cyanocobalamin. A free form of Cbl is essential for the growth of this strain in a nutrient medium (Watanabe, Bito, 2018a). In food and biological samples, vitamin B12 is frequently bound to enzymes and transport proteins (Fedosov et al., 1996). Therefore, the common extraction process often involves combining enzymatic digestion with α-amylase or proteinase (or both) and heat extraction with potassium cyanide (KCN). Boiling with KCN at pH 4.0–4.5 for 30 min is commonly used as a Cbl extraction method among bioassays, while Thin-Layer-Chromatography (TLC), HPLC, and LC-MS, were used for quantification of cyanocobalamin (Bito et al., 2016, Kelleher, Broin, 1991, Watanabe, Bito, 2018a).

2.3.2. Chromatographic methods

Chromatographic methods have been widely used in the separation and identification of vitamin B12, all chromatographic methods such as thin layer chromatography (TLC), gas-liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS) and HPLC systems are available for separation and determination of CNCbl and its related compounds in food materials. However, in recent studies on vitamin B12 detection method development, HPLC systems have been extensively used due to better precision and accuracy. Although specificity remained a challenge in trace analysis of vitamin B12, especially in non-fortified food. For instance, in some complex samples such as processed foods (3-20 ng/g), applying HPLC system with fluorescence detection is not recommended (Pakin et al., 2005).

The quantification of vitamin B12 in food using HPLC systems mostly refers to reverse-phase HPLC system (RP-HPLC) and can be coupled with UV-visible spectrophotometry or mass spectrometry (HPLC-MS), as well as other detectors such as fluorescence and electrochemical detection. Regarding to detection of vitamin B12 at a very low concentration in non-fortified food, HPLC-UV method suffers poor sensitivity and selectivity, meanwhile the method development via using ultra high-pressure liquid chromatography-mass spectrometry (UHPLC-MS) system has improved the precision and selectivity in determination of very low concentration of vitamin B12 in non-fortified food by using isotopically labelled internal standard (Wang et al., 2021).

Sample extraction and purification of vitamin B12 involves key steps such as method development and optimization, where specific sample preparation, including solvent extraction, enzymatic treatment, and solid-phase extraction, is employed to handle the low concentration and complex food matrices (Deptula et al., 2015, Gentili et al., 2008, van Wyk, Britz, 2010). Immunoaffinity columns can also be used for efficient purification of Vitamin B12 (Heudi et al., 2006).

Quantification involves internal standards, often isotopically labelled vitamin B12 analogues, and calibration curves with standard solutions (Kahoun et al., 2021, Kesava Raju et al., 2013). This method is applied across various food matrices, including dairy, meat, seafood, and fortified foods (Luo et al., 2006, Pakin et al., 2005). These methods can measure not only the total vitamin B12 content but also differentiate among the various cobalamin forms such as methylcobalamin, adenosylcobalamin, hydroxocobalamin, and cyanocobalamin (Wang et al., 2021, You, 2019). Despite challenges such as matrix effects and low concentrations, recent literature focuses on enhancing sensitivity, accuracy, and efficiency, underpinning the critical of HPLC-MS in providing reliable vitamin B12 data for nutrition labelling and dietary assessments (Wang et al., 2021).

2.3.3. Other analytical methods

Ridascreen®Fast Vitamin B12 (R-Biopharm AG, Darmstadt, Germany), a commercially available product that utilizes a direct competitive enzyme-linked immunosorbent assay (ELISA) for vitamin B12 determination. As described by the manufacturer, it is suitable for the quantitative determination of vitamin B12 in milk, milk powders, food for special medical purpose, grain and cereals, fortified flour, vitamin powders, vitamin mixtures, vitamin tablets, and vitamin juices. The competitive ELISA technique is renowned for its application in routine screening of fortified vitamin B12 (cobalamin), particularly in food analysis, supplement verification, and certain clinical practices (Nexo et al., 2002), due to its high sensitivity, speed and lower equipment needs (Kong et al., 2017, Yang et al., 2015).

This immunoassay format utilizes the competitive binding between free vitamin B12 in samples and immobilized B12-protein conjugates for limited antibody binding sites (Kong et al., 2017). The principle of the test is based on an antigen-antibody reaction. In this setup, free vitamin B12 from the sample competes with immobilized B12-protein conjugates to bind to specific anti-vitamin B12 antibodies.

In the commercial applications, such as the Ridascreen®Fast Vitamin B12 test kit, these antibodies are pre-coated onto microtiter wells (R-Biopharm AG, 2023). An enzyme-linked antigen (or labelled) is also used in the process. It can only attach to the capture antibody if the antibody site is not already occupied by an antigen from the standard or test samples. Any excess labelled or unlabelled antigens are washed away, and a substrate is added thereafter. The signal produced is inversely proportional to the antigen concentration in the sample: a higher concentration of antigen results in less labelled antigen binding to the capture antibody, thereby generating a weaker signal (Kohl, Ascoli, 2017). Table 8 presents the advantages and limitations of this method compared to the two other determination methods mentioned earlier.

Other methods, such as chemiluminescence Immunoassay (CLIA), which applies immunoassay principles with chemiluminescence detection, and radioimmunoassay (RIA), are often utilized in clinical studies for routine diagnostics, including screening and monitoring for vitamin B12 deficiency (Trad et al., 2025). However, these methods will not be discussed in detail in this study.

Table 8 Methodological comparisons of vitamin B12 determination in food analysis.

Method	Principle	Advantages	Limitations	Ref.
ELISA	Free B12 from sample competes with immobilized B12–protein conjugate for antibody	Sensitive to high vitamin B12 levels, easily adaptable to labs, and ideal for fortified food and supplement quantification.	Matrix interference, specificity requires a well-designed sample extraction, unsuitable for the analysis of native or non-fortified foods.	(Yang et al., 2015)
Microbiological assay	Growth response of B12-dependent bacterial strains	Highly sensitive and specific historically	Laborious and slow; now largely replaced in routine labs.	(Hughes, McNulty, 2018)
HPLC / LC-MS	Chromatographic separation of vitamers with UV/MS detection	High specificity and selectivity to vitamers	Cost, instrument complexity, sample prep - not ideal for high throughput screening	(Tsiminis et al., 2017)

3. MATERIALS AND METHODS

3.1. Bacterial strains and pre-screening of co-fermentation cultures

3.1.1. Microorganisms and culture media

This study utilized a total of 10 bacterial strains, including 9 strains belonging to the *Lactobacillus* species and one strain of *P. freudenreichii*, as listed in Table 9.The *Lactobacillus* strains were obtained from the culture collection of the Department of Microbiology and Biotechnology of the Max Rubner-Institut in Kiel, while *P. freudenreichii* was obtained from Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The probiotic strain *Lactobacillus plantarum 299* was obtained as freeze-dried powder from the company Probi AB from Sweden. This culture was stored at -20°C before rehydration and streaked out for purity before producing the stock culture for further usage.

Table 9 Bacterial strains utilized in this work.

No.	Strain name	***Lactobacillus* and *Propionibacterium* species[a]**	**Culture collection**
1	92071	*Limosilactobacillus reuteri subsp. kinnaridis*	MRI-MBT*, Kiel, Germany
2	92126	*Limosilatobacillus reuteri subsp. reuteri*	MRI-MBT*, Kiel, Germany
3	20016	*Limosilatobacillus reuteri subsp. reuteri*	DSMZ GmbH, Brauschweig, Germany
4	92281_i2	*Limosilactobacillus fermentum*	MRI-MBT*, Kiel, Germany
5	92072	*Limosilactobacillus fermentum*	MRI-MBT*, Kiel, Germany
6	92117_i3	*Lactiplantibacillus pentosus*	MRI-MBT*, Kiel, Germany
7	92117_i1	*Lactiplantibacillus pentosus*	MRI-MBT*, Kiel, Germany
8	92397	*Lactiplantibacillus plantarum*	MRI-MBT*, Kiel, Germany
9	299	*Lactiplantibacillus plantarum*	Probi AB, Lund, Sweden
10	20271	*Propionibacterium. freudenreichii* subsp. *freudenreichii*	DSMZ GmbH, Brauschweig, Germany

* MRI-MBT: Max Rubner-Institut für Mikrobiologie und Biotechnologie, Kiel, Germany

[a] The genera *Limosilactobacillus* and *Lactiplantibacillus* were previously known as *Lactobacillus* but were then reclassified in a taxonomical study.

For long-term storage of bacterial strains, 700 µL of an overnight culture in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke) was added to 300 µL of glycerol solution (80 %, AppliChem GmbH, Darmstadt), mixed and stored at -80 °C. As an alternative to glycerol preservation, Roti Store cryotubes (Carl Roth GmbH, Karlsruhe) were used according to the manufacturer's instructions and stored at -80 °C.

For growth and cultivation of *P. freudenreichii* strains, *Propionibacterium* broth (PPB), or *Propionibacterium* agar (PPA) was prepared according to the recipes provided in the DSMZ guidelines (2022). Detailed information about the specific media compositions and preparation protocols can be found in the Appendix 7.1. *Lactobacillus* strains were grown in MRS medium or vitamin B12 assay medium (VBAM) (Sigma-Aldrich, St. Louis, MO), depending on the specific study. Prior to experiments, the *Lactobacillus* strains were initiated by streaking from glycerol stocks onto MRS agar and cultured at 37°C, then 10mL overnight culture under microaerophilic conditions (CampyGen 2.5L (Oxoid, Basingstoke) in VBAM was prepared before actual incubation by inoculating a single colony from the MRS agar plate. For reactivating *P. freudenreichii* before experiments, the stock culture was streaked onto PPA and incubated at 30 °C for 4 days in anaerobic jars. Liquid precultures were then inoculated from well-separated colonies and grown for 3 days under a microaerobic atmosphere using CampyGen 2.5L (Oxoid, Basingstoke) in an anaerobic jar. Experimental cultures were inoculated at stationary growth phase with a bacterial count of 8.0 log - 9.0 log CFU/mL and grown for 3-7 days under a microaerobic atmosphere.

3.1.2. Growth phase identification of pre-culture for vitamin B12 production

Growth curves were performed to estimate the optimal duration and temperature for all the bacterial strains to enter the exponential (log) phase or the stationary phase, which are the phases at which the vitamin B12 biosynthesis was reported to be at maximum. Growth curve measurement was conducted for *P. freudenreichii* and all the *Lactobacillus* strains.

The culture medium (30mL of either PPB or VBAM) was inoculated with 0.3 mL of inoculum (1%) from the preculture as described in 3.1.1, as corresponded to the oat drink inoculation ratio during the fermentation. The incubation and measurements were conducted in duplicate in BioSan LV Personal Bioreactor RTS-1C (Riga, Latvia). Optical densities at 850 nm (OD850) were read for generating the growth curves. *P. freudenreichii* was incubated at 30°C without rotation, while *Lactobacillus* strains were tested at both 30°C and 37°C with a rotation speed set at 250 rpm. OD850 were read at 10-minute intervals. Total bacterial counts and pH-value were measured at the begin and the end of the incubation.

3.2. Single fermentation and co-fermentation in oat beverage for vitamin B12 production

3.2.1. Pre-culture preparation

Potential *Lactobacillus* candidates were selected as described in 3.1.2 for further fermentation experiments in oat-based matrix. The *Lactobacillus* cultures were prepared for both single-strain fermentation and co-fermentation with *P. freudenreichii* together in the commercial oat-drink. Pre-cultures were prepared before the actual inoculation into oat-drinks and were prepared following the procedure mentioned in 3.1.1. The *P. freudenreichii* strain was grown on PPA anaerobically for 3 days and then in PPB at 30°C for 48 hours. *Lactobacillus* strains were grown anaerobically on MRS agar at 37°C for 24 hours and then in VBAM at 37°C for 24 hours microaerophilic. The medium used for inoculation was then centrifuged at 13,300 rpm for 5 minutes (Biofuge pico, Heraeus Instruments, Hanau, Germany), followed by two washes with 0.85% sodium chloride solution. The concentration was adjusted to 5.0 log colony forming units (CFU)/mL for *Lactobacillus* and 7.0 log CFU/mL for *P. freudenreichii* at the time of inoculation.

3.2.2. Fermentation in commercial oat-drink and production of vitamin B12

For further studies, a commercial oat-based drink from *Oatly* oat drink organic (Oatly AB, Malmö, Sweden) was utilized. Overnight culture of *P. freudenreichii 20271* (see 3.1.1) was inoculated at the initial cell density with 7.0 log colony forming units (CFU)/mL and *L. reuteri* 92071 was at a concentration of 5.0 log CFU/mL.

Blank samples were prepared with 0.85% NaCl solution into Oat-drink (1%, v/v) in duplicate as a control. At time 0, 24, 48, 72 h, all samples including the blank samples were taken assessed for cell count and pH value and then stored at -28°C for later vitamin B12 quantification.

3.2.3. Cell counts enumeration and determination of pH changes

To estimate the number of viable cells, 1 mL of oat drink collected from each sampling point was serially diluted with 9 mL sterile saline (8.5 g/L NaCl) and 100 µL of appropriate dilutions were plated out onto the agar plates. PPA plates were used specifically for the enumeration of *P. freudenreichii* and these were incubated anaerobically (in anaerobic jars with Anaerogen, Oxoid, Basingstoke, UK) for 4 days and aerobically for 1 day at 30°C. This resulted in *P. freudenreichii* colonies becoming brownish and distinguishable from colonies of other microorganisms. MRS agar plates (Oxoid) were used for the enumeration of *Lactobacillus*. Total aerobic bacteria cell count was determined using PCA (plate count agar) plates, which were incubated at 30°C for 48 hours, whereas MRS plates were incubated at 37°C for the same duration.
2 mL samples were collected at the same sampling points as described in 3.2.2 from the same sample used for cell counts enumeration and were used for pH measurement with a digital pH-meter (Knick Elektronische Messgeräte GmbH & Co. KG, Berlin).

3.3. Identification and quantification of vitamin B12

3.3.1. Determination of corrinoids from food and biological samples by the microbiological assay (MBA)

Commercial test kits - VitaFast® Vitamin B12 kits were purchased from R-Biopharm AG (Darmstadt, Germany). Each test kit contains a microtiter plate with 96 wells which are coated with *Lactobacillus delbrueckii* subsp. *lactis* for vitamin B12 determination. Vitamin B12 assay medium (solid), cyanocobalamin standard (solid) and sterile water were included in the test kits for the assay. The sterile water was used to prepare the medium and dissolve the standard concentrate for the serial dilution of standard curve. Sample preparation was performed according to the manufacturer's instructions with some modifications, as the kit procedure was not exclusively designed for native vitamin B12 detection in cereal samples.
At first, a comparison of sample extraction efficiency of oat-based matrices was carried out, for extracting the free form of corrinoids. The incubation period and the efficiency of enzymatic treatment were considered as two important parameters in the comparison, which was carried out according to three different methods including the manufacture’s instruction as described in Table 10.

Table 10 Method comparison for effective sample extraction (modified according to (Gebhardt, 2022)).

	Method 1 (KYSIL, 2014)	**Method 2 (Chamlagain et al., 2018)**	**Method 3 (VITAFAST@vitamin B12, 2017)**
Pepsin	100 mg	-	-
Taka-diastase (from *Aspergillus oryzae*)	30 mg	20 mg	75 mg
Acetate Buffer	5 mL	5 mL	5 mL
Sample	5 mL	5 mL	5 mL
Incubation Temperature	37°C	37°C	37°C
Incubation Duration	180 min	40 min	60 min

Sample extraction was carried out by weighing pepsin and Taka-diastase, as described in Table 10, for each method. This was done in a 15 mL light-proof sterile centrifuge tube (VWR International LLC, Radnor, USA). Then, 5 mL of oat drink and 5 mL of acetic acid buffer (1% KCN, pH 4.5) were added and homogenized.

The enzyme digestion process was initiated by incubating the centrifuge tubes for 0.5h, 1h, 1.5h, 2h, and 3h in a shaking incubator (BS 4, B.Braun SE, Melsungen) at 120 rpm and 37°C. The extraction was then followed by heating the samples in a water bath at 95°C for 30 min, after which samples were rapidly cooled to below 30°C on ice, and then centrifuged at 4700 rpm for 25 min.

An additional microbiological purity control was performed with 1 mL of sample extract to ensure there was not any bacterial contamination after the extraction process. 1 mL sample extract was spread among three PCA plates and evenly distributed on the surface of the agar plate using a Drigalski spatula. The plates were incubated at 30 °C for 7 days, any visible colonies were examined after the incubation. Two replicates were made with each sample extract.

The test assay was performed by pipetting 150 µL of the vitamin B12 assay medium into each well and adding 150 µL of the prepared sample extracts and standards into assigned wells. Technical triplicates were performed for each batch. The stripes were covered with adhesive foil and incubated at 37°C in the dark for 48 h. After the incubation, adhesive foil was removed and optical density was measured using a microplate spectrophotometer (Epoch 2, Agilent Technologies, Inc., Santa Clara USA) at a wavelength of 630 nm.

3.3.1.1. Determination of vitamin B_{12} in oat beverage and other cereal beverages

Oat beverages from three different manufacturers, along with a rice beverage and a spelt beverage, were investigated. As suggested in the manufacture's instruction for total vitamin content (native and added vitamin B12) for the beverage product, cereals and baby food, a sample extraction dilution factor of 40 (1:40 final dilution) was applied.

The sample extraction process was followed by steps described in 3.3.1 with the following modifications: 300 mg Taka-diastase was applied and incubated for 1 h with the same condition mentioned previously, then the total extract was filled up exactly to 40 mL with extraction buffer. The following preparation for the test assay was identical as described in 3.3.1.

Spike recovery studies were conducted in oat beverages to validate matrix effect and calculate recovery rates. The cyanocobalamin provided in the test kits were used in the spiking experiment, which were added to the sample prior to the extracting. The expected level of vitamin B12 presented in natural enriched oat beverages through lactic acid bacteria fermentation is described in 3.3.1.2, which were used as target concentration in the spiking experiment. The medium and high level of spiked sample extract has been further diluted after sample extraction (as described in 3.3.1.2 a.) and b.), to meet the range of standard curve.

Following the manufacture's recommendation, a reagent blank with deionized water were carried out to ensure that no vitamin B12 was presented in the sample extraction or influenced by enzymes used (e.g., Taka-diastase).

3.3.1.2. Matrix effect and recovery studies

After the sample extraction procedure, theoretical concentration of vitamin B12 in the sample extract should be either low: 0.3 µg/L, medium: 2.5 µg/L or high: 5 µg/L. According to the standard curve concentration range provided by R-Biopharm AG, the medium- and higher- level of vitamin B12 containing sample extract was further diluted with sterile water from the test kit by the following steps:

a.) Dilution of medium-level vitamin-B12 sample extract (2.5 µg/L) → dilution factor 2(1:2) → 0.5 mL sample extract + 0.5 mL sterile water from test kit → final concentration (0.125 µg per 100 mL)

b.) Dilution of high-level vitamin-B12 sample extract (5.0 µg/L) → dilution factor 5(1:5) → 0.2 mL sample extract + 0.8 mL sterile water from test kit → final concentration (0.1 µg per 100 mL)

3.3.1.3. Data analysis

The assays were repeated in three independent experimental days with biological triplicates and technical triplicates on the microtiter plate to obtain a consistent result. The standard curve was generated by BioTek Gen5 software (Agilent Technologies, Inc., Santa Clara, USA). A 4-parameter nonlinear regression model was used to evaluate the reliability of the standard curve, as recommended from the manufacturer's instruction. Further statistical data analysis was performed as described in 3.5.

3.3.2. Method development and determination of cyanocobalamin and pseudocobalamin via UPLC-MS/MS

3.3.2.1. Experimental design and analytical platform

In this study, we aimed to identify biologically active vitamin B12 producers among *Propionibacterium* and *Lactobacillus* species using liquid chromatography-mass spectrometry (LC-MS). This platform was selected for its high sensitivity and specificity in differentiating bioactive cobalamins from structurally similar analogues (e.g., pseudo-B12), as demonstrated in preliminary screening of *Spirulina sp.* and *Chlorella sp.* supplements. To optimize extraction efficiency, two distinct sample preparation workflows—immunoaffinity chromatography (IAC) and solid-phase extraction (SPE)—were established and systematically compared for their ability to isolate free vitamin B12 from complex biological and food matrices, including a representative food matrix (straight-dough bread). Two extraction workflows were applied to all matrices (unfermented, fermented and food samples). Detailed protocols for each method are provided in subsequent sections.

3.3.2.2. Chemicals and Reagents

All solvents applied in this work were hyper-grade for LC-MS. Methanol, acetonitrile, water, formic acids, ammonia solution were obtained from Merck KGaA (Darmstadt, Germany). Acetic acid and ammonium formate were obtained from VWR international LLC (Darmstadt, Germany). Cyanocobalamin (≥98%), Taka-diastase (EC 232-588-1, *Aspergillus oryzae*), pepsin (EC 232-629-3, porcine gastric mucosa) and sodium cyanide were purchased from Merck KGaA (Darmstadt, Germany). A 32% sodium hydroxide solution was obtained from Carl Roth GmbH (Karlsruhe, Germany). The 1% sodium cyanide solution was prepared by dissolving 1 g sodium cyanide in 100 mL de-ionized water to stabilize labile cobalamin forms during extraction.

A 1 mg/mL cyanocobalamin stock solution was prepared with 50 mg cyanocobalamin standard (≥ 98% purity) dissolved in 50 mL solvent and aliquots were stored at -18°C until use.

Spirulina (*Arthrospira platensis*) and *Chlorella* (*C. vulgaris*) supplements (Pukka Herbs Ltd., Bristol, UK) were procured for method validation, given their documented variability in B12 analogues (Bito et al., 2016; Andrade, 2018).

Matrix-matched calibrants were generated using unfermented assay medium (Propionibacterium culture medium 91 (DSMZ, Germany)) to account for ion suppression effects (Ly et al., 2020), as well as a straight-dough bread (prepared in-house) were procured for method comparison between IAC and SPE.

3.3.2.3. Food matrix preparation

Straight-dough bread was selected as a model food matrix due to its relevance as a fortified food product and its moderate complexity in protein and carbohydrate content. The bread was prepared according to Edelmann et al. (2016) as follows:

The dough was prepared by mixing wheat flour, salt, and sugar in proportions detailed in Table 11. Fresh yeast was dissolved in 35°C tap water and combined with fat (e.g., butter or oil) before incorporation into the dry ingredients. For the experimental group, 80 mL of a *Propionibacterium freudenreichii* inoculum (10^9 CFU/mL, pre-cultured for 168 h, as described in 3.1.1) was added to the mixture. A control dough, excluding the bacterial inoculum, was prepared in parallel for comparative analysis. The dough was mechanically kneaded at medium speed for 5 min, followed by manual kneading to ensure homogeneity, and rested for 10 min at room temperature.

Pre-fermentation samples (20 g) were collected, frozen at -20°C, and reserved for baseline vitamin B12 quantification. Subsequently, the dough portions were proofed at 33–37°C for 90 min in a humidity-controlled proofer. Proofed doughs were baked at 180°C for 30 min, cooled to room temperature, and stored at -20°C until further analysis.

Table 11 Composition of ingredients for straight-dough baking.

Ingredient	Weight (g)
Wheat flour	400
Tap water	238
Fresh yeast	16
Sugar	12
Margarine	12
Salt	4

3.3.2.4. Enzymatic digestion and IAC extraction (Method I- Gold standard)

The extraction and purification processes were performed using EASI-EXTRACT® Vitamin B12 immunoaffinity columns (R-Biopharm Rhone Ltd, Glasgow, UK) following the manufacturer's guidelines with protocol adaptations for the matrices studied.

For bacterial medium samples, 15 mL of fermented *Propionibacterium freudenreichii* culture (10^9 CFU/mL) was homogenized via cell disruption using an IKA® T18 Ultraturrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany) to release intracellular corrinoids. Food matrices, such as unfermented or fermented oat beverage, were prepared by blending 15 mL of sample with 50 mL LC-MS grade water using the Ultraturrax (11,000 rpm, 3 min). To stabilize labile corrinoids, 100 µL of 1% (w/v) potassium cyanide (KCN) was added to convert all forms to cyanocobalamin (CNCbl). The same suspension was partially used for the HybridSPE method (see below).

Enzymatic digestion was performed to liberate bound vitamin B12 from the matrix. The homogenate was first adjusted to pH 2.5 with 0.1 M HCl, followed by the addition of 0.5 g pepsin and incubation at 37°C for 40 min to hydrolyse protein complexes. The pH was then raised to 4.5 using 1 M NaOH, and 0.5 g α-amylase was introduced to degrade carbohydrate binders during a second 40 min incubation at 37°C. Enzymatic activity was terminated by boiling the sample for 20 min, followed by rapid cooling on ice.

Post-digestion, the sample was divided into pre-weighed 50 mL amber centrifuge tubes (VWR International, Radnor, PA, USA) and centrifuged twice (3,500 rpm, 10 min). After the first centrifugation, the supernatant was transferred to a 250 mL amber glass bottle, while the residual pellet was re-suspended in 10 mL of 50 mM sodium acetate buffer (pH 4.5), vortexed, and centrifuged again. Supernatants from both steps were pooled for subsequent purification. For bread or dough samples, an additional filtration step was implemented to reduce particulate interference: supernatants were passed through glass fibre filters (MN 85/75 BF 90 mm; Macherey-Nagel GmbH & Co. KG, Düren, Germany) prior to column loading. Bacterial medium supernatants, however, were loaded directly onto the IAC column without filtration.

The immunoaffinity column was mounted onto a CHROMABOND SPE vacuum manifold (Macherey-Nagel GmbH & Co. KG, Düren, Germany). After draining the activation buffer, the pooled supernatant was loaded onto the column under a controlled vacuum (5 mbar), maintaining a flow rate of ≤2 mL/min to ensure optimal binding. Following loading, the column was washed with 10 mL of ultrapure water under rapid flow to remove non-specific contaminants. The column was then air-dried to eliminate residual moisture. Vitamin B12 was eluted at a slow flow rate (1 drop/sec) using 3.5 mL of 100% methanol, and the column was dried again under vacuum to recover residual eluate. The collected eluate volume was precisely measured via pipette gauging (to the nearest 0.1 mL), vortexed for homogeneity, and transferred to an amber chromatography vial for LC-MS analysis.

To mitigate photodegradation, all steps (including eluate handling) were conducted under light-protected conditions using amber glassware, and filtration devices were shielded with aluminium foil. Hazardous reagents, such as KCN, were handled exclusively within a chemical safety hood to ensure operator safety.

3.3.2.5. Vitamin B12 extraction via HybridSPE-phospholipid purification (HSPE) (Method II – In house developed SPE method)

Instead of an enzymatic digestion, a protein precipitation was applied directly to the sample suspension after cyanalytion. The protein precipitation was done by adding 1,0 mL acetonitrile containing 1% formic acid into 0.2 mL sample (5 ad.1) in a 2 mL safe-lock amber tube (Eppendorf AG, Hamburg, Germany). The mixture was vortexed for approximately 10s and centrifuged in an Eppendorf microcentrifuge (Model 5415R, Eppendorf AG, Hamburg, Germany) at 13,200 rpm for 5 min. 1.2 mL of supernatant was loaded onto the SPE column connected to the vacuum manifold, passing through the supernatant at 5 mbar. After loading, the column was air-dried until the liquid residue was removed from the column. Then the vitamin B_{12} was eluted with 200µL water/methanol/formic acid mixture [75:25:0,05(v/v/v)] containing 5% ammonia. The definite volume of the eluate was measured also by pipet gauging method in microliters. 100µL eluted sample was transferred to an insert prepared in an amber vial and then injected onto the LC-MS system.

3.3.2.6. Ultra-high performance liquid chromatography (UPLC) and mass spectrometry (MS) system

The LC-MS/MS system consisted of a Dionex UltiMate 3000 UHPLC coupled to a Bruker AmaZon SL ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (Bruker, Karlsruhe, Germany).
The UHPLC system was equipped with a Kinetex Core-Shell C18 column (2.1 × 50 mm, 1.7 µm) (Phenomenex, Aschaffenburg, Germany), connected to a pre-column (sub-2µm core-shell column with 2.1mm internal diameters) (Phenomenex, Aschaffenburg, Germany). The mobile phase consists of 0.05% formic acid and 23.47 mM ammonium formate in water (Solvent A) and methanol (Solvent B). Effective chromatographic separation was achieved with a multi-step gradient elution mode (14,5 min) as follows: (T_{min} / solution of B): 0- 5.0 min/ 10% B; 5.0 – 9.5 min/ 37.5% B; 9.5 – 13.0 min /100% B; 13.0 – 14.5 min/ 10% B. The flow rate was 0.2 mL/min, the column temperature was at 45°C and the injection volume was 2µL. The same conditions are applied to the standard as well.

The operation conditions for MS scan are in positive ion ESI mode, and the optimized setups are as follows: capillary voltage 4550 V, end plate offset 300 V, nebulizer(nitrogen) 20.0 psi, dry gas (nitrogen) 7.0 L/min, dry temperature 250°C. The scanning was carried out with an ultra-scan mode with a range of 100 – 1500 m/z. The data acquisition was applied with Bruker Compass Data Analysis software version 1.7.

3.3.3. Determination of biofortified cobalamin in oat drink

15 mL of fermented oat beverage sample was applied. The sample was prepared by following the method described in 3.2.2. In the co-fermentation set-up, a more precise quantitative method was applied which could detect cyanocobalamin and pseudo-cobalamin with immunoaffinity chromatography purification, since in the later phase of this study, an in-house synthesized cyanocobalamin internal standard (IS) was developed for a higher quantification accuracy. The IS was produced with strain *P. freudenreichii subsp.freudenreichii* DSM 20271 in a modified medium to simulate the production of a stable isotope of cyanocobalamin(CNCbl) following and modified from (Wang et al., 2021). The detailed methods are described in (Lenz et al., 2023a, Lenz et al., 2023b).

3.4. Genomic identification and molecular biological methods

3.4.1. Whole genome sequencing for vitamin B12 producers among *Lactobacillus* species (published manuscript (Gao et al., 2023)).

3.4.1.1. Isolation of genomic DNA from *Lactobacillus* species

All *Lactobacillus* strains were cultivated in MRS broth (VWR, Darmstadt) at 37°C for 24h and presumptively identified by 16S rRNA gene sequencing at Max Rubner-Institut in Kiel. The total genomic DNA was extracted using the peqGOLD bacterial DNA kit (VWR International, Radnor) according to the manufacturer's instructions. 1.5 mL of cell suspension from overnight culture was used for DNA extraction. For the cell lysis, instead of 10 minutes incubation time as indicated in the manufacturer's instruction, 1 hour incubation was performed on a Thermo-block (Eppendorf AG, Hamburg) with shaking at 240 rpm to ensure a complete cell lysis. DNA purity was assessed by spectroscopic measurements with NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA) at 260 nm, 230 nm and 280 nm (A260/280 and A260/230 ratios). For a long-term storage the eluted DNA extract was stored at -20°C until further application. DNA concentration for the library preparation was quantified by using Qubit™ dsDNA BR Assay kit (Thermo Fisher Scientific, Waltham) via a multimode microplate reader SPARK® (Tecan Trading AG, Männedorf). The average DNA fragment length distribution was assessed using the TapeStation system (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol.

3.4.1.2. Whole genome sequencing

The genomic DNA library was prepared using the TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The DNA of samples was mechanically sheared using the Covaris M220 (Covaris, Woburn), generating fragments with an average size of approximately 400bp. PhiX DNA was used as an internal control for the sample purification and library preparation procedure. The MiSeq reagent kit v2 was used for 2x 250-bp paired-end sequencing with the MiSeq platform, according to the manufacturer's instructions (Illumina, Munich, Germany). The raw sequencing data (generated reads are in FastQC files) were trimmed using Trimmomatic v. 0.32 (parameters: Phred33, sliding window:4:15, leading:3, minlen:45) (Bolger et al., 2014) and *de novo* assembled using SPAdes v. 3.13.2 (parameters: –careful, –k 21,33,55,77) (Bankevich et al., 2012). All contig sequences that were shorter than 500 bp or contaminated with PhiX sequence were removed using the BBDuk pipeline with default parameters (DOE Joint Genome Institute).

3.4.1.3. Species identification via Type (Strain) Genome Server (TYGS)

The assembled genome's FASTA file was analysed against type strains using the Type (Strain) Genome Server (TYGS), a web-based tool that employs 20215 type strains from deposited species (as of January 2024). The Type Strain Genome Server is a platform specifically designed for the comparison of microbial genomes, particularly type strains. If the digital DNA-DNA Hybridisation (dDDH) values exceed a certain threshold, typically around 70% for species delineation, the query genomes are assigned to the same species as the reference genomes.
Orthologous average nucleotide identity (ANI) and *in silico* DNA-DNA hybridization were calculated with the most closely related type strains (see Appendix 7.2) using the orthologous ANI tool (OAT) (v. 0.93.1) (Lee et al., 2016) and the Genome-to-Genome Distance calculator (GGDC) server from DSMZ with formula 2. Species level cut-off values were 96% for ANI and 70% for dDDH according to previous studies (Goris et al., 2007).

3.4.1.4. Identification of vitamin B12 biosynthesis genes and genome annotation

All contigs were annotated using the Bacterial and Viral Bioinformatic Resource Center (BV-BRC) v.3.28.9 tools, prokka (v. 1.12) and the NCBI Prokayotic Genome Annotation Pipeline (PGAP) v. 6.4 with default parameters. Draft genome features related to cobalamin, folate and riboflavin biosynthesis genes were investigated.

3.4.2. Relative gene expression to vitamin B12 biosynthesis via quantitative real-time PCR

3.4.2.1. RNA isolation of *Lactobacillus* strains

To study gene expression and understand key gene regulations in vitamin B12 biosynthesis among *Lactobacillus* strains, *Lactobacillus reuteri* subsp*. kinnaridis* 92071 was used, since it produces cobalamin instead of pseudo-vitamin B12 under the treatment conditions. RNA isolation was performed using RNase-free barrier tips (10 µL, 100 µL, 1000 µL), microtubes (200 µL and 1.5mL), molecular biology grade water, and nuclease-free digesting enzymes. All materials were treated with UV-light for 30 minutes prior to experiments to ensure their sterility.
The strain was revived by streaking frozen stocks at -80°C onto MRS agar plates and incubating them at 37°C for 24 hours. Single colonies were then inoculated into 10 mL of vitamin B12 assay medium and incubated overnight at 37°C. Growth curves were generated using a Spark® multimode microplate reader (Tecan Life Sciences, Männedorf, Switzerland) to determine the various growth phases, particularly the mid-exponential and stationary phases, to investigate the gene regulations involved in vitamin B12 biosynthesis. 500 µL of a grown bacterial culture was collected at different growth phases, including mid-exponential (16h) and stationary phases (25h).
1mL of RNAprotect™ Bacteria Reagent (Qiagen, Hilden, Germany) was added directly to the bacterial culture to preserve RNA integrity during the harvesting process. The mixture was vortexed well and incubated for 5 minutes at room temperature. The bacterial cells were then harvested by centrifugation at 5,000 × g for 10 minutes to pellet the cells, and the supernatant was discarded. The RNeasy® Mini Kit (Qiagen; Hilden, Germany) was used for RNA isolation according to the manufacturer's instructions with slight modifications, mechanical disruption combined with an enzymatic digestion were applied for an effective extraction of RNA as described below:

a. 20µL RNasy-free Protease K and lysozyme (25 mg/mL) added to the resuspended bacterial cells and incubated at a 37°C for 20 minutes to facilitate cell wall lysis.

b. Mechanical disruption was performed using a cell Disruptor Genie® (Scientific Industries, Inc.; New York, USA) at 3000 rpm. The bacterial suspension was transferred to tubes containing silica beads, the disruption was paused each two-minute cycle and samples were taken on ice for 30s to ensuring efficient cell disruption without excessive heat generation.

An on-column DNase digestion with an RNase-free DNase set (Qiagen GmbH, Hilden) was applied to each RNA isolation to eliminate any trace genomic DNA (gDNA) contamination in the total RNA sample. The DNase was aliquoted in a single-used portion and stored at -20°C before use. The final RNA was eluted using 30 µL RNase-free water and was stored at -80°C until further analysis. RNA isolation was performed in triplicate, each replicate consisted of an independent culture and RNA extraction process.

3.4.2.2. RNA quality control and integrity assessment

The quality and quantity of isolated RNA were assessed by measuring the absorption at 230 nm, 260 nm, and 280 nm using spectrophotometry (e.g., NanoDrop™ 2000) and/or fluorometric measuring methods (e.g., Qubit™ RNA HS Assay Kits) by Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples were tested for purity, concentration, and integrity to ensure they met the requirements for downstream applications. Only RNA samples with an RNA purity ratio (e.g., A260/280 ratio) of 1.8 or higher were labelled and used for transcriptome sequencing.
To assess RNA integrity, the RNA Integrity Number (RIN) was measured by capillary electrophoresis with an RNA ScreenTape and analysed with TapeSation Analysis Software 4.1 (Agilent Technologies, Inc. 2021).

3.4.2.3. Quantitative real-time reverse transcription PCR

Quantitative real-time PCR (RT-qPCR) was used to investigate the impact of key precursor additions on vitamin B12-related gene regulation. Gene expressions were measured in different growth phases (late exponential- and stationary- phase) and in the presence and absence of key precursors. Partial primers were used as indicated in previous studies, and the remaining primers were designed manually using Primer3 (Ye et al. 2012). The conditions of primers were set to have a melting temperature ™ between 59 and 61 °C. The primers were diluted to a working solution of 10mM and used in the PCR. Each targeted gene and corresponding primers were presented in Table 12. *16S* r*RNA* was taken as a reference gene for the gene expression study.

Table 12 Candidate genes and their Primers used for RT-qPCR.

Gene Name	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon size (bp)**	**Ref.**
16S rRNA	ACG TGC TAC AAT GGA CGG TA	ACT AGC GAT TCC GAC TTC GT	120	Torres et.al 2018
CobB	AGC TGG TGT TTC GAC TGC TT	AGT GTT CGT GCC AGC TTT CT	272	Current Study
CobQ_1	GGT GTT TTG CCA ATG AGC AGT	GGA GTT CCA AGT TCC TCT GCC	209	
CobQ_2	TTA GCT CTT TTC GGC GGT GG	CCC GGA ATC TTT TCG CCA TC	179	
CobS	AAG CAC ATG GCG AAT TAT TG	TTC GTG ACC TTT CGT CGA T	132	Torres et.al 2018
CobT	AAA CAG CAG CAG AAG TTG TTG GGC	CAT TGC TCC AGC CAT TGC ACC TAA	182	

The reverse transcriptions (RT) and cDNA synthesis were performed by applying iScript™ gDNA Clear cDNA Synthesis (Bio-Rad Laboratories. Inc, USA) according to the manufacturer's instructions. A second DNase digestion were applied to 14µL RNA template (with a final concentration at 450 ng RNA) from each sample was first digested with DNase to eliminate residual gDNA contamination presented in the RNA extractions, then converted into cDNA in 20 µL volume.

Synthesized cDNA was diluted in 1:3 ratio with RNase-free water, and 3 µL diluted cDNA were used as template for downstream real-time qPCR application with Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories. Inc, USA). 3 µL of nuclease-free water was used as the no-template control (NTC), both cDNA template and negative control reaction were prepared in triplicate. iTaq Universal SYBR@ Green Supermix was applied as a master mix fluorescent dye for the detection and amplification was carried out in 96-well plates. A detailed composition of cDNA template and used primer concentrations are shown in Table 13.
The conditions for the amplification of a two-step real-time qPCR application is shown in Table 14.

Table 13 Primer concentration and reagent set-up for real-time qPCR application.

Reagent	Final Concentration	Volume
cDNA Template		3 µL
iTaq Universal SYBR@ Green Supermix (2x)	1x	7.5 µL
Primer Forward (10µM)	500 nM	0.75 µL
Primer Reverse (10µM)	500 nM	0.75 µL
Nuclease-Free H2O		3 µL
Total		15 µL

Table 14 Two-step real-time qPCR conditions for the amplification.

	Temperature	Time	Cycles
Polymerase Activation and initial denaturation	95°C	30 s	1x
Denaturation	95°C	30 s	
Annealing	60°C	60 s	40x
Melt Curve Analysis	65-95°C	0.5°C increments at 2-5 sec/step	

The verification was done by applying a reverse transcription reaction without the reverse transcriptase and the same amount of RNA sample was used in a parallel preparation to the reverse transcription assay. A no-RT control reaction with iScript No-RT Control Supermix (Bio-Rad Laboratories. Inc, USA) was applied for the control of gDNA contamination in RNA. *C*q values are defined as acceptable if they were next to the end of the cycling or away from *C*q values generated for the cDNA samples.
The reverse transcription products were quantified by Nanodrop 2000 and then used in the gene-specific real-time quantitative PCR assay.

Specificity and product detection were checked by performing a temperature-gradient melt curve analysis after amplification in each run. The relative quantification method (2−ΔCt) was used to determine the expression levels of the target genes normalized to the 16S rRNA housekeeping gene of *L. reuteri* using primers mentioned above (Table 12). Comparisons were made between the different growth phases and in the vitamin B12 assay medium with the presence and absence of precursors.

3.4.3. Transcriptomic response into differentially expressed genes (DEGs) via whole transcriptome sequencing

The RNA samples used for whole transcriptome sequencing were prepared identically as mentioned in 3.4.2.1, followed with an RNA purity control with fluorometric methods. Only RNA samples with a RIN of 8.0 or higher, were labelled and used for transcriptome sequencing. gDNA contamination was also accessed and gDNA removal was performed before library preparation.

3.4.3.1. Library preparation and transcriptomic analysis

To perform transcriptomic analysis, the isolated RNA samples were further processed using the Illumina Stranded Total RNA Prep Kit with Ribo-Zero Plus (Illumina, San Diego, CA). The rRNA depletion was performed via Ribo-Zero treatment. The reagent kit was using NextSeq 1000/2000 P2 Reagents (300 Cycles) v3. Strain-specific oligo probe pools of *Lactobacillus* strains were applied. The Illumina-indexed adaptors were ligated after cDNA synthesis, end repair, A-base addition, and ligation according to Illumina's protocol.
The quality and quantity of the constructed libraries were assessed using a D1000 Assay Kit with the Agilent TapeStation system (Agilent Technologies, Santa Clara, CA). Libraries meeting the quality criteria were selected for sequencing. Paired-end libraries were sequenced on the NextSeq 2000 sequencer (Illumina, San Diego, CA, USA) at Deutsche Institut für Lebensmittel-technik e.V. (DIL) in Quakenbrück, Germany.
To ensure the reliability of the results, two independent biological replicates of RNA from each strain were sequenced.

3.4.3.2. RNA-Seq Data Analysis

The raw sequencing data obtained from the Illumina platform were stored in FASTAQ format. The raw reads were aligned with reference genome of *L. reuteri* 92071 (GenBank accession no. JAQOWD000000000.1). The alignment and mapping to the reference were performed with Geneious Prime® (2024.0.7).

Differential gene expression comparisons were also conducted using Geneious Prime® (2024.0.7). Cells grown in non-treatment VBAM were compared to those grown in the same medium supplemented with precursors. Additionally, the cells grown in both mid-exponential (16h) and stationary phase (25h) were compared under both conditions. Specifically, in total four comparison groups (untreated 16h vs. 25h, treated with precursors 16h vs. 25h, untreated 16h vs. treated 16h, untreated 25h vs. treated 25h) and biological duplicate of each sample were chosen to determine the differential gene expressions (DEGs) in the RNA-seq data. Genes regarding to cobalamin biosynthesis were considered significantly differentially expressed with an absolute value of Log2 fold change (|log2FC|) greater than 0.5 (1.4-fold) and p value < 0.05.

Principal component analysis (PCA) clustering plots were used to visualize the distributions and clustering of the data, and volcano plots were used to visualize the differential expression results.

3.5. Statistical Analysis

The data evaluation, visualization and statistical analysis for all the data presented in this study (except of the transcriptomic data) were generated using GraphPad Prism (Version 10.3.0) (GraphPad Software, 2023). The statistical differences were analysed by using variance (ANOVA) or Student's t-test, to determine significant differences in gene expression between samples. The significance level was set at $p < 0.05$.

Transcriptomic data analysis and visualization was carried out with Geneious Prime® (2024.0.7).

4. RESULTS

4.1. Pre-screening of *Lactobacillus* candidates for vitamin B_{12} production

All *Lactobacillus* strains were inoculated into VBAM to observe their ability to grow without the presence of any trace level of vitamin B12, all tested *Lactobacillus* were able to grow in the test medium. All strains were therefore also used for vitamin B12 determinations via IAC-HPLC/MS-MS or SPE extraction method.

The growth curves of vitamin B12 producing candidates were generated and the points where the bacterial growth entered the mid-exponential and the stationary phase were then defined as sampling points for further vitamin B12 analysis. The growth curve of each vitamin B12 producing candidate strains is shown in Figure 4.

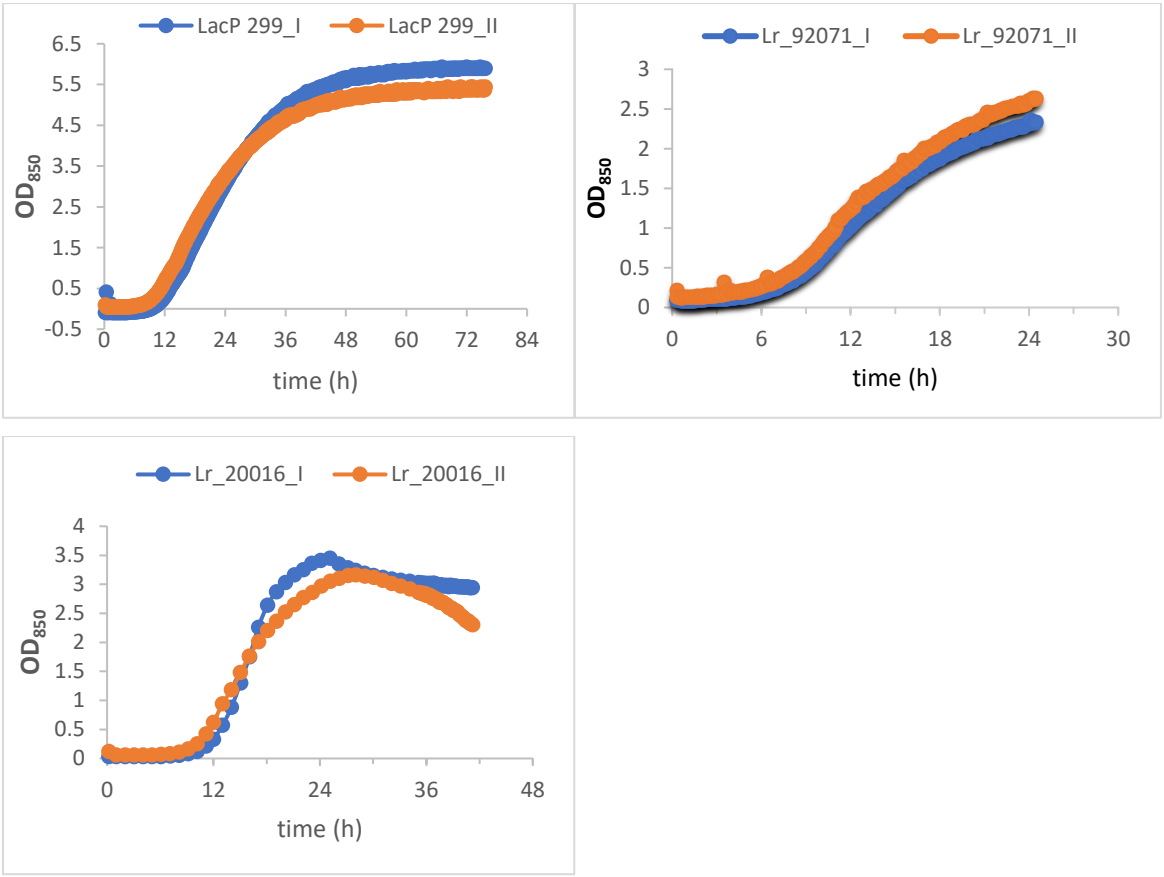

Figure 4 Growth curves of L. plantarum *299,* L. reuteri *DSM 20016, and* L. reuteri *MRI 92071 in VBAM, determined in duplicate (LacP 299_I/II, Lr_20016_I/II, Lr_92071_I/II) for 72, 25, and 42 hours, respectively.*

In each of the growth curves there were two independent biological replicates from the stock culture. *L. plantarum* 299 as incubated at 30°C and *L. reuteri* strains was incubated at 37°C. Lag, exponential and stationary growth phase were recognized through visualization of the optical density plotted against time. From the growth curve of the *L. plantarum* 299 strain, the lag phase ended at about 10±1h, whereas mid-exponential phase was at about 16h and 18h, the stationary phase began at about 35h and 42h. For the *L. reuteri* strain, the mid-exponential phase was at about 16h and after 24h both strains were entering into the stationary growth phase. Therefore, samples were taken at 16h and 48h for further vitamin B12 quantification.

4.2. Oat-based beverage fermentation with *Lactobacillus* and *Propionibacterium* strains

4.2.1. Microbial counts of single fermentation and co-fermentation in oat-based beverages

The growth potential of each strain of lactic acid bacteria and propionic acid bacteria, from the Max Rubner-Institut Department of Microbiology and Biotechnology own culture collection, as well as from commercial suppliers, was assessed in an oat-based beverage. Samples were collected at specific time points to generate growth curves and enumerate bacteria. These same samples were subsequently analysed for vitamin B12 concentration at the corresponding time points. The bacterial enumeration results of monoculture fermentation in oat beverages for screening vitamin B12 production is shown in Table 15. In the control oat-based beverage, no LAB could be detected throughout the fermentation. The initial inoculation density of both *L. plantarum* strains was at 5.9 log CFU/mL. Both *Lactobacillus* strains showed growth in the oat matrices, reaching the stationary phase with cell densities of 8.9±0.1 and 9.2±0.3 log CFU/mL after 24 hours of incubation, respectively, and remained stable thereafter.

Table 15 Cell enumeration of Lactobacillus *monoculture grows in oat-based beverage.*

Time (hours)	0	24	48	72
LAB	Log CFU/mL			
Control*	n.d*	n.d*	n.d*	n.d*
L. plantarum 299	5.9±0.1	8.9±0.1	9.0±0.1	8.8±0.2
L. plantarum MBT 92397	5.8±0.1	9.2±0.3	9.0±0.1	8.8±0.2

*Control=oat beverage without inoculum; n.d = not detected.

L. reuteri 92071 as a vitamin B12 producing candidate from previous screening results, was assessed for its growth in monoculture fermentation as well as in co-culture fermentation with *P. freudenreichii* DSM 20271 in the oat-based beverage. To ensure that there were no microbiological contaminants, all the control samples were examined by spreading plate method on PCA and Standard-I agar plates for background microbiota. There was no visible microbiological growth observed in the oat-based beverage before the inoculation. Throughout the fermentation process, in the control sample, neither LAB nor PAB could be detected (data not shown in figure). As presented in Figure 5, the initial viable cell counts of *L. reuteri* in the monoculture fermentation after inoculation was 5.60 ± 0.02 log CFU/mL and the strain reached a maximum viable cell count of 7.70 ± 0.03 log CFU/mL) after 24 hours. In the monoculture fermentation of *P. freudenreichii,* the initial viable cell count was 7.72 ± 0.05 log CFU/ mL and this strain was able to reach a maximum count of 8.67± 0.03 log CFU/mL after 72 hours.

In the co-culture fermentation, the initial viable cell count was 5.50 ± 0.05 log CFU/mL for *L. reuteri* and 7.56 ± 0.10 log CFU/mL for *P. freudenreichii*. The viable cell counts of *L. reuteri* increased to 7.24± 0.06 log CFU/mL and that of *P. freudenreichii* to 8.80 ± 0.15 log CFU/mL at 24 hours. The cell density of both strains remained stable from 24 hours to 72 hours.

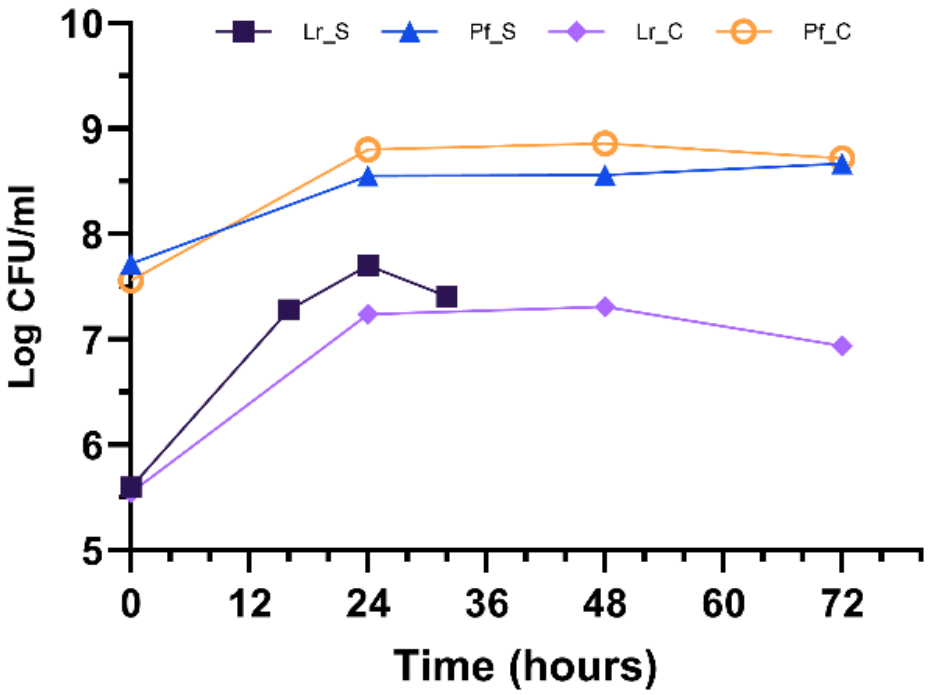

Figure 5 Viable cell counts (log10 CFU/mL) of L. reuteri *MRI 92071 and* P. freudenreichii *DSM 20271 single (Lr_S, Pf_S) and co-inoculated (Lr_C, Pf_C) in oat-based beverage at 30°C during the fermentation process.*

4.2.2. Acidification of the oat-based beverages

The acidification of *Lactobacillus* monoculture grown in oat-based beverage is presented in Figure 6. The initial pH of the oat beverage was approximately 6.5. The control sample (without inoculation) maintained a consistent pH at 6.5 ±0.2 throughout the fermentation.

When fermented with either of the *L. plantarum* strains, up to 24 hours incubation, the pH decreased from 6.5 to 3.8 or 3.9 for each strain, respectively. From 24 to 48 hours, the pH decreased by only a further 0.5 for each strain and only little further decrease in pH occurred from 48 to 72 hours (only 0.1 for each strain).

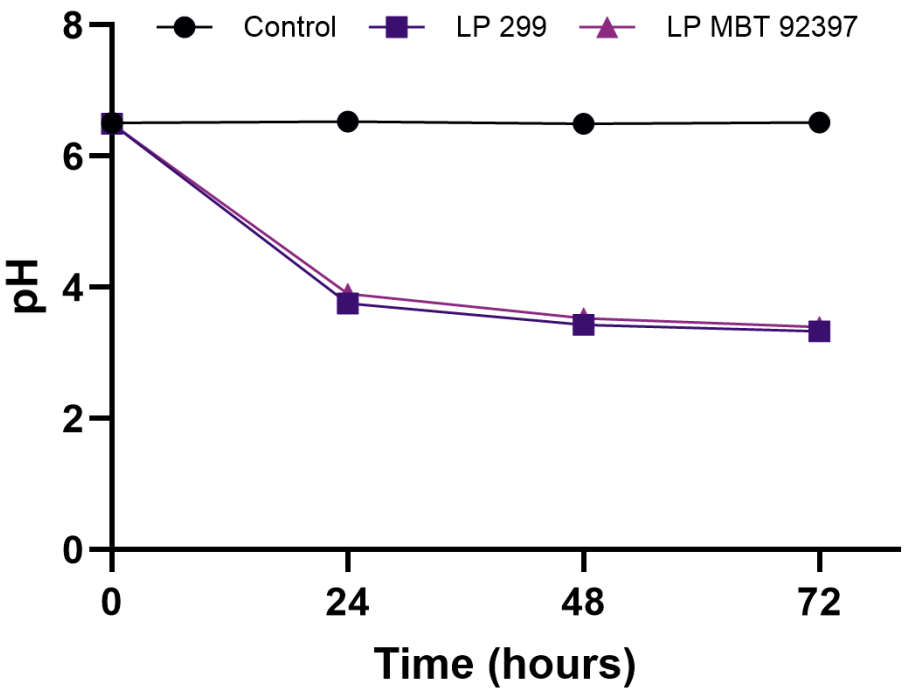

Figure 6 pH changes of L. plantarum *299 (LP 299) and* L. plantarum MBT 92397 *(LP MBT 92397) in oat beverage fermentation.*

In the experiment focusing on the bio-enrichment of vitamin B12 through fermentation, the data shown in Figure 7, revealed distinct acidification patterns for two microbial cultures in an oat-based beverage.

When *L. reuteri* 92071 was used as a monoculture, the pH value of the medium decreased from its initial level (approximately 6.4) to 5.1 after 24 hours, eventually reaching a final pH of 4.6 at the end of the fermentation process. Conversely, the *P. freudenreichii* DSM 20271 monoculture showed a pH decrease to 5.9 within the first 24 hours. This pH level then remained stable at 5.9 for the duration of the fermentation period of 72 hours.
In the co-culture fermentation process, where both *P. freudenreichii* DSM 20271 and *L. reuteri* 92071 were introduced into the oat-based beverage, the pH reached approximately 5.3 after 24 hours. By 48 hours, the pH had decreased to 5.0, and by the end of the fermentation period at 72 hours, the final pH was 4.9.

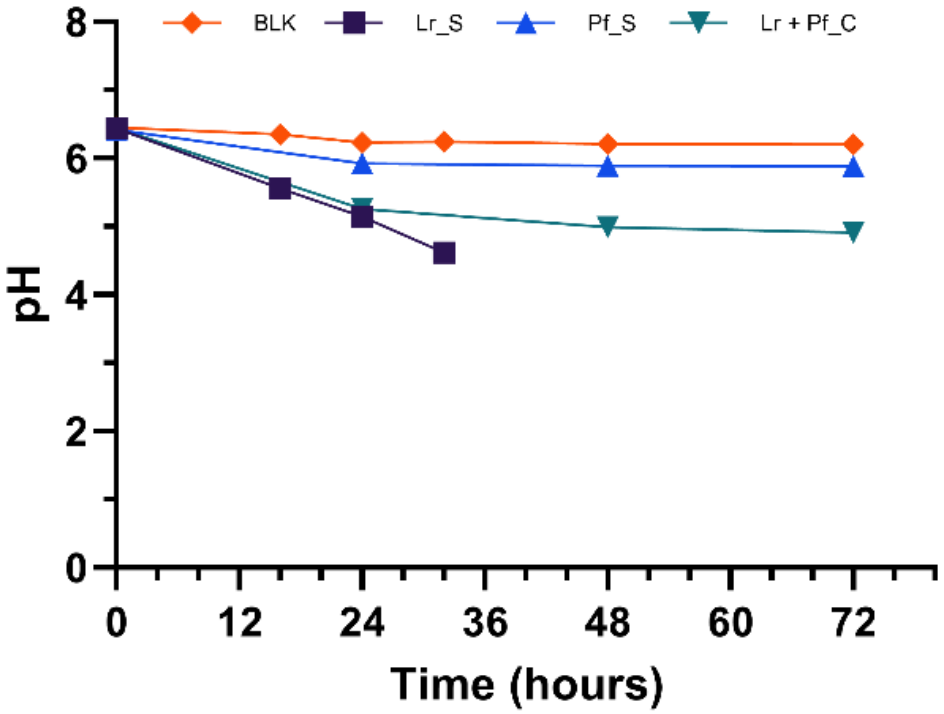

Figure 7 pH changes of blank oat-based beverage (BLK), monoculture fermentation of L. reuteri *92071 (Lr_S) and* P. freudenreichii *DSM 20271 (Pf_S), and co-culture of both strains (Lr+Pr_C) in oat-based beverage. All the values are the mean of three biological replicates.*

4.3. Qualitative and quantitative determination of total corrinoids and cyanocobalamin in medium and food matrix

4.3.1. Preliminary test to determine the optimal incubation period and enzymatic treatment

To determine the optimal incubation duration for sample extraction from oat-based food matrices, a preliminary test was conducted by comparing incubation times ranging from 30 to 180 minutes. As demonstrated in Table 16, the optical density decreased with an extension of the incubation period. The optical density was highest at 0.159 ± 0.01 with a 30-minute incubation and decreased to 0.113 ± 0.01 with a 180-minute incubation. Notably, decreases in optical density were observed between the 30- and 60-minute incubation periods, as well as between the 120- and 180-minute incubation periods. These decreases indicate periods of enzymatic activity affecting sample turbidity. The 60-minute incubation period was identified as potentially the most suitable for optimal enzymatic digestion under the conditions tested. Therefore, the combination of pepsin at 100 mg and Taka-diastase at 30 mg with a 60-minute incubation period was selected for application in sample extraction.

Table 16 Variation of incubation time on the efficiency of sample extraction from oat-based beverage.

Sample ID	Enzyme Concentration (mg/mL)		Incubation Time (minutes)	Optical Density (630nm)
	Pepsin (mg)	Taka-diastase (mg)		
S1	100	30	30	0.159±0.01
S2	100	30	60	0.139±0.01
S3	100	30	90	0.135±0.00
S4	100	30	120	0.130±0.00
S5	100	30	180	0.113±0.01

Furthermore, vitamin B12 concentrations were measured to evaluate the amount of free vitamin B12 extracted during the sample preparation process. However, the calculated concentrations based on the OD value at 630 nm (OD630) could not be accurately determined, as they either exceeded the detectable range of the test kit's standard series (calculated to be 1.875 ng/mL), or were below the lower limit of quantitation (LLOQ), which is 0.225 ng/mL.

Consequently, OD630 was used as a comparative parameter, as vitamin B12 concentrations were calculated based on these OD630 values. As shown in Table 17, the combination of pepsin and Taka-diastase treatments yielded the highest OD630 value of 0.927±0.06, whereas the treatment with only Taka-diastase resulted in the lowest OD630 value of 0.017±0.01.

Table 17 Comparisons of different enzymatic digestions for sample preparation.

Sample ID	Enzyme Concentration (mg/mL)		Incubation Time (minutes)	Microbial Growth (OD 630nm)	Vitamin B12 Concentration (ng/mL)
	Pepsin (mg)	Taka-diastase (mg)			
S1	100	30	60	0.927±0.06	>1.875
S2	100		60	0.919±0.03	>1.875
S3		30	60	0.017±0.01	<0.225

4.3.1.1. Verification and recovery tests in oat-based matrix

The sterility control of the sample extract showed that there was no growth of visible bacterial colonies.

Vitamin B12 standard from the test-kit was used to generate the standard curve, as shown in Figure 8, the concentrations ranging from 0.03 µg – 0.18 µg/100 mL with the coefficient of determination (R^2 =0.998).

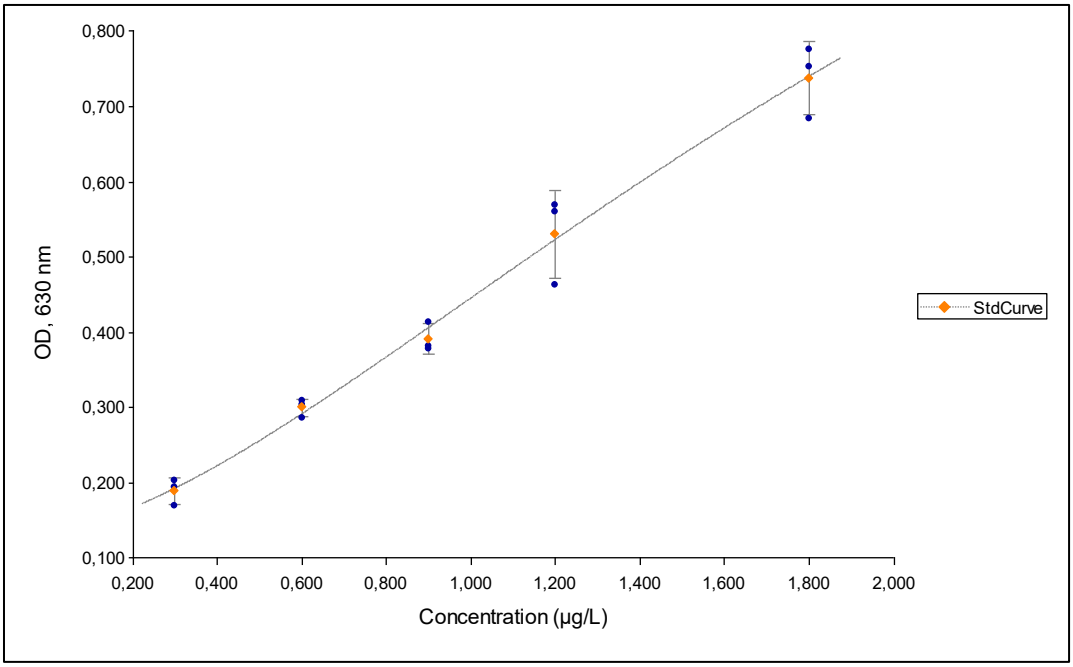

Figure 8 Standard calibration curve of vitamin B12 using standard solution (0,03 µg - 0,18 µg/100 mL) from VitaFast® kits, the OD value of the blank medium is subtracted from the OD value of the standard solution.

The measured absorbance at 630nm for each standard solution is indicated below in Table 18, corresponding absorbance at 630nm subtracted from the standard blank is indicated as Blank 630.

Table 18 Standard solution and corresponding optical density at 630nm.

Std. Conc.(µg/L)	**Abs.(630nm)**	**Blank 630**
0.0	0.106	0.000
0.3	0.200	0.094
0.6	0.300	0.194
0.9	0.391	0.286
1.2	0.531	0.425
1.8	0.719	0.613

As shown in Table 19, vitamin B12 concentration of spiked oat beverages were calculated based on the standard solution and corresponding OD at 630 nm as mentioned in Table 18, based on the range of standard curve, the lower limit of quantification (LOQ) was 0.225 µg/L and higher limit of quantification was 1.875 µg/L. The spiked oat beverage at a low level (0.3 µg/L) showed a calculated result after sample extraction of 1.054±0.1 µg/L. The recovery rate was 252%, the relative standard deviation was at about 14%. The other spiked oat beverages at a medium and high level (2.5 µg/L and 5 µg/L) were both exceeded standard curve range and above the calculated vitamin B12 concentration of 1.875 µg/L.

Table 19 Spiked recovery tests in oat beverages.

Sample	**Abs.(630nm)**	**Blank 630**	**Cal. vitamin B12 Conc.* Mean ±SD (µg/L), (RSD, %)**
Oat Blank	0.121	0.011	<0.225
Oat Low	0.510	0.400	1.054±0.1(13.9)
Oat Medium	1.156	1.046	>1.875
Oat High	1.250	1.139	>1.875

*Calculated vitamin B12 concentration

Furthermore, to identify minimal interfering substrates and to establish the optimal measurement range for vitamin B12 quantification in an oat-based matrix, various dilution levels were tested. An inter- and intra-assay comparison was conducted to verify the sensitivity and accuracy of vitamin B12 quantification.

As shown in Table 20, average concentration of vitamin B12 in oat-based sample matrices with various dilution ratio and other parameters, such as standard deviation and coefficient of variation, are presented. Calculated vitamin B12 content in the reagent blank, which contained no oat-based sample matrices, for Day 1 ranged from 0.26 to 1.92 µg per 100 mL across the triplicates, with an average concentration of 1.20g per 100 mL. For Day 2, the calculated vitamin B12 content ranged from 0.24 to 0.60 µg per 100 mL, with an average concentration of 0.26 µg per 100 mL. Within the same day, relative standard deviations (%RSD) among analytical triplicates reached or exceeded 20%.

Similar to the reagent blank, the calculated vitamin B12 content in diluted oat-based sample matrices, prepared with a 1:20 dilution ratio and treated with Taka-diastase, also exhibited variability among analytical triplicates, with relative standard deviations (%RSD) reaching or exceeding 20%.

When the 1:20 diluted sample was extracted without enzymatic digestion, the average vitamin B12 level was higher than that of the reagent blank on Day 2, which had a vitamin B12 level of 0.26 µg per 100 mL and a reliable %RSD of 19.44. The vitamin B12 levels in this sample ranged from 0.53 µg to 2.43 µg per 100 mL.

Table 20 Vitamin B12 quantification in different dilution levels of oat-based sample matrices.

Sample ID*	**Average Conc. ±SD (µg/ 100mL), % (RSD, %)**	
	Day 1	Day 2
RB (n=3)	1.20±0.65 (54,19)	0.26±0.16 (19.44)
Dil 20 (n=3)	1.25±0.30 (23.73)	1.56±0.71 (45.83)
Dil 40 (n=3)	0.68±0.04 (6.12)	0.72±0.13 (18.17)
Dil 20 (w/o Taka-diastase)	0.53±0.01 (2.21)	0.56±0.02 (3.13)

*RB= Reagent Blank; Dil 20= Dilution factor 20; Dil 40 =Dilution factor 40; Dil 20 (w/o Taka-diastase) = Dilution factor 20 without Taka-diastase

4.3.1.2. Verification of matrix-effect from oat and other cereal-based beverages

The measured vitamin B12 concentrations across different types of commercially available cereal-based beverages are presented in Table 21. Based on the standard curve shown above, the calculated mean vitamin B12 concentration ranged from 0.58 µg to 1.46 µg per 100 mL among triplicate samples. Additionally, the mean concentration ranged from 0.69 µg to 1.30 µg per 100 mL between two experimental days. The percent coefficient of variation (%CV) for the intra-assay shown below indicated a Between-Run repeatability (intermediate precision) of the test kit for quantification of vitamin B12 in oat and other cereal-based matrices.

Table 21 Native vitamin B12 contents determined by microbiological assay (MBA) test kit (VitaFast®B12) among various cereal-based beverages on two independent days with triplicates in each day. The results are expressed as µg/100mL for the mean value.

Sample	Intra-assay						Inter-assay		
	Mean		SD		%CV		Mean	SD	%CV
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2			
RBW*	0.08	0.12	0.00	0.07	2.4	54.8	0.10	0.03	0.3
Oatly (Oat)	1.46	1.15	0.43	0.28	29.7	24.5	1.30	0.22	0.2
Alnatura (Oat)	0.81	0.58	0.23	0.07	28.5	12.6	0.69	0.16	0.2
Kölln (Oat)	1.60	1.45	0.11	0.20	7.1	13.9	1.53	0.10	0.1
Alnatura (Rice)	1.09	0.85	0.57	0.28	52.4	33.1	0.97	0.17	0.2
Allos (Spelt)	1.13	1.24	0.03	0.12	2.5	10.0	1.19	0.08	0.1

*RBW=reagent blank

Among all the cereal-based beverages tested, as indicated in Figure 9, the vitamin B12 contents determined in oat beverage from Oatly did not significant differentiate from any other brand, however, the oat beverage from Alnatura was significant different than the product from Kölln. Though the oat beverage from Kölln did not show a significant difference compared to rice or spelt beverage.

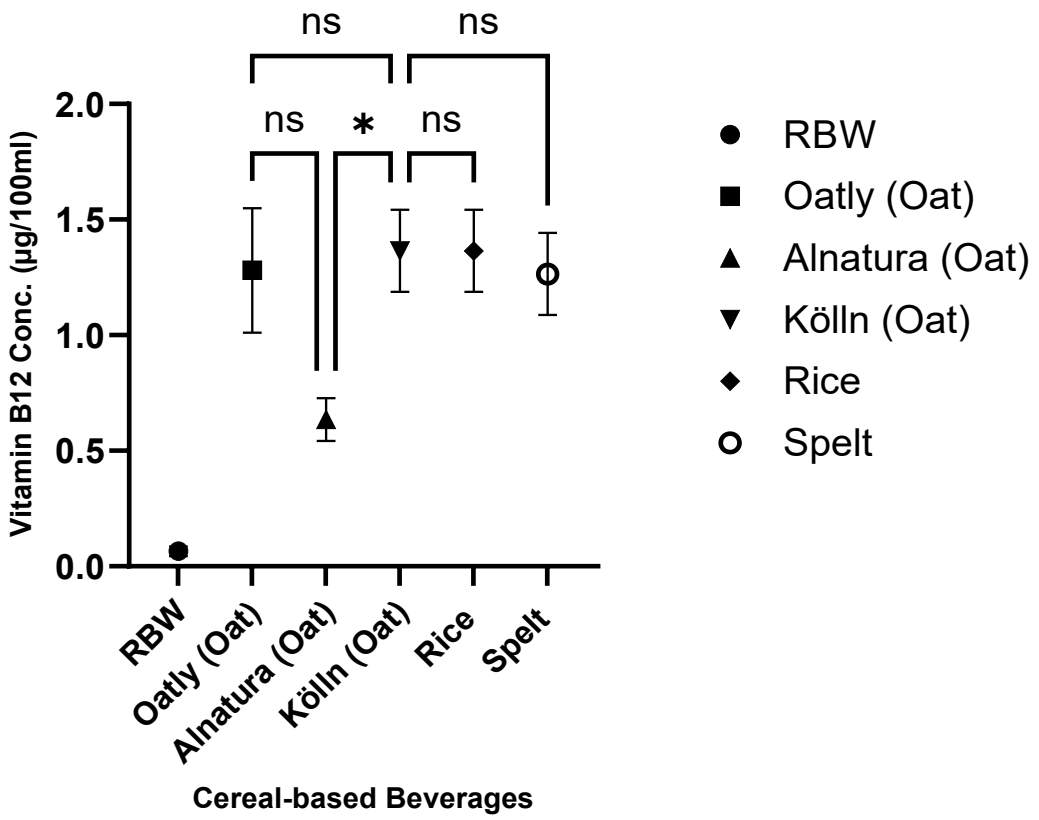

*Figure 9 Vitamin B12 concentration of three different oat beverage products and other cereal-based dairy alternative beverage. *RBW=reagent blank*

4.3.2. Quantification of cyanocobalamin and pseudo-cobalamin via LC-MS/MS

4.3.2.1. UHPLC-MS optimisation for vitamin B12 analysis

Various active forms of vitamin B12 were identified using an ion-trap mass spectrometer, as depicted in Figure 10. A stable retention time for cyanocobalamin (RT = 7.0 min) was achieved via a buffered mobile phase system. An external calibration curve was freshly prepared daily, covering a range from 1.0 µg/L to 250 µg/L with excellent linearity on average (R^2 = 0.9995). The instrument's lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were 0.001 ng/injection and 0.003 ng/injection, respectively, for a 2 µL injection, calculated as three times the baseline noise.

The compound cyanocobalamin (CNCbl) exhibited a retention time of 7.0 minutes, which remained consistent throughout the analyses. However, fluctuations in environmental temperature resulted in a retention time of 6.8 ± 0.4 minutes for CNCbl. The complete high-performance liquid chromatography (HPLC) analysis had a total run time of 14.5 minutes. The electrospray ionization tandem mass spectrometry (ESI-MS/MS) spectrum of CNCbl displayed a peak at m/z 678.28 corresponding to $[M+2H]^{2+}$, with prominent product ions observed at m/z 997.30 corresponding to $[M+H\text{-}DMBI\text{-}sugar\text{-}PO_3]^{+}$, m/z 635.70 corresponding to $[M\text{-}CoCN+H]^{2+}$, and m/z 359.10 corresponding to $[DMBI+Sugar+PO_3+H]^{+}$. In contrast, the spectrum for OHCbl revealed a peak at m/z 664.70 corresponding to $[M+H\text{-}OH]^{2+}$, sharing similar fragmentation patterns with CNCbl. In the case of AdoCbl, the spectrum was doubly charged and showed a mass of m/z 790, and the following precursor ion was at m/z 527 $[M+3H]^{3+}$.

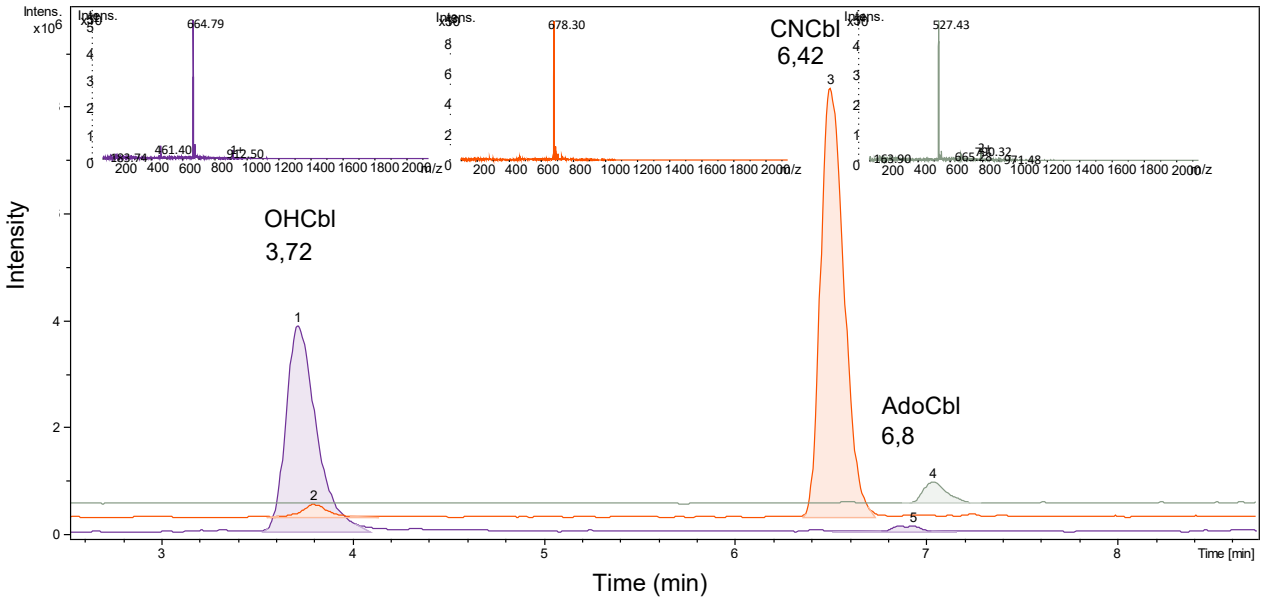

Figure 10 UHPLC chromatogram and ion fragmentation patterns for CNCbl (m/z 678.30), OHCbl (m/z 664.79), and AdoCbl (m/z 527.43)

4.3.2.2. Vitamin B12 quantification via immunoaffinity chromatography (IAC) (Method I-Gold standard)

To evaluate the extraction efficiency and reliability of the immunoaffinity chromatography (IAC) method, recovery experiments were conducted at two cyanocobalamin (CNCbl) concentrations representative of naturally fortified food matrices. The precision of the method was assessed through within-run repeatability (intra-day variability) and intermediate precision (inter-day variability), with results summarized in Table 22.

At the lower spiked concentration (2 µg/L) in the unfermented culture medium, mean recovery across duplicate analyses was approximately 97%, with a relative standard deviation (RSD) of 14%, indicating robust inter-day consistency. However, intra-day variability revealed a divergence in recovery rates: values of 88% and 87% were observed on Days 1 and 2, rising to 117% on Day 3.

In contrast, the higher spiked concentration (20 µg/L) exhibited markedly reduced recovery (64%) and reduced variability (RSD = 6.8%).

In spiked B12-extraction buffer, at both concentration the recovery was approximately 86% with an RSD < 20%, the recovery was in between both concentrations in the unfermented medium matrix.

Table 22 Recovery of cyanocobalamin in spiked unfermented matrix with immunoaffinity clean-up and LC-MS quantification. (n=2, for each day)

Sample	Spiked amount (µg/L)	Recovery, % (RSD, %)			
		Day 1	Day2	Day3	Mean
Unfermented medium	0	N. D	N. D	N. D	N.D.
	2	88.2 (8.8)	86.6 (5.9)	116.8 (1.8)	97.2 (14.3)
	20	58.1 (8.4)	66.6 (24.5)	67.9(7.9)	64.2 (6.8)
Extraction buffer	2	75.5 (0.008)	-	99.5 (21.9)	87.5 (13.7)
	20	-	101.73 (15.8)	69.3 (13.5)	85.5 (19.0)

*N. D= Not detected

A matrix-matching calibration was also conducted, six concentrations of CNCbl standard solution were spiked in the unfermented medium (from 5.4 to 60 µg/L) and three concentrations were spiked in the fermented medium, as shown in Table 23, the average recovery rated was about 51%, whereas four concentrations of CNCbl standard solution was spiked in the fermented medium (from 0 – 10.2 µg/L) and the average recovery rate was about 32%. At a lower concentration of 1µg/L, no spiked CNCbl standard was being detected.

The linearity of CNCbl in medium matrix was $R^2 = 0.996$ and solvent was $R^2 = 0.998$.

Table 23 Recovery rate of IAC extraction method in matrix-matching calibration of CNCbl standard in both unfermented and fermented medium (n=2).

Sample	Spiked amount (µg/L)	Recovery, % (RSD, %)
Unfermented medium	5.4	56.9 (91.9)
	7.8	58.9 (48.5)
	10.2	44.2 (5.3)
	15.0	53.5 (37.7)
	30.0	48.9 (28)
	60.0	48.7 (29.7)
Fermented medium (LAB)	0.0	N. D
	1.0	N. D
	5.4	31.3 (23.5)
	10.2	32.5 (3.3)

*N. D= Not detected

4.3.2.3. Vitamin B12 determination via HybridSPE-Phospholipid purification (HSPE) (Method II – In house developed SPE method)

Additionally, the necessity of applying enzymatic digestion as an extraction process was also evaluated. As illustrated in Table 24, the concentration of CNCbl measured with the SPE method with and without enzymatic digestion obtained a similar result of 13 µg/g. Meanwhile, the measured concentration via IAC purification method was at about 12 µg/g, which was comparable to SPE method. A one-way analysis of variance (ANOVA) was conducted to evaluate differences in vitamin B12 quantification between the immunoaffinity chromatography (IAC) and solid-phase extraction (SPE) methods, as well as the effect of conducting an enzymatic digestion procedure prior to SPE compared to omitting this procedure. The analysis yielded a *p*-value of 0.83 ($p > 0.05$), indicating no statistically significant differences on the quantification of vitamin B12 using either IAC or SPE. Furthermore, the results obtained after enzymatic treatment prior to SPE clean-up were not significantly different from those without enzymatic treatment.

Table 24 Determination of vitamin B12 levels in B12 enriched bread by addition of fermented P. freudenreichii *DSM 20271 suspensions by both IAC clean-up and SPE method (N=2).*

	IAC (µg/g)	SPE_E(µg/g) *a	SPE_NE(µg/g) *b
Bread	12.3(±0.3)	13.7(±0.04)	13.6(±1.1)

Values are means ± SD.

*a enzymatic digestion before SPE

*b non-enzymatic digestion before SPE

4.3.2.4. Identification of vitamin B12 contents in other biological samples

The previously mentioned HSPE extraction method was also applied to determine vitamin B12 in biological samples such as the fermented medium of *P. freudenreichii* strain and *L. plantarum* strains. As shown in Table 25, vitamin B12 levels in the fermented samples of *L. plantarum* strains arranged from 1.0 to 2.0 µg/L, with the lowest concentration among all the samples at 1.1 µg/L with 200µL sample volume. In contrast, *P. freudenreichii* (60.0 µ/L) could produce 60 times more than *L. plantarum* strains.

Table 25 Vitamin B12 contents determined by HSPE extraction in the fermented medium of P. freudenreichii *and* L. plantarum *strains (N=1)*

Strains	CNCbl (µg/L)
P. freudenreichii subsp*. shermanii* DSM 20271	60.0
L. plantarum Nr. 3.3.3*	1.1
L. plantarum Nr. 19.2*	2.4

*****Originated from strain collection of food technology department at university of applied sciences Fulda.

4.3.2.5. Selectivity for the active vitamin B_{12} and pseudo- vitamin B_{12}

In the current study, the selectivity for determining active B12 and pseudo-vitamin B12 was demonstrated. The selectivity and specificity of the method were studied by comparing the HPLC chromatograms of a CNCbl standard, sample extracts from *Spirulina* sp. and *Chlorella* sp. supplements, and a fermented sample from the *L. reuteri* DSM 20016 strain. Potassium cyanide was added during sample preparation to convert all active forms of B12 coenzymes to the stable form CNCbl.

The retention time of the CNCbl was at 7.25 min and the eluted peaks of *Spirulina sp.* supplement were at 7.0 min. which eluted slightly earlier than CNCbl, and is suggested to be pseudo- vitamin B12 with adenine. The mass spectrum of CNCbl is observed at m/z 678.28 $[M+2H]^{2+}$, while the pseudo-vitamin B12 appears at m/z 672.77 $[M+2H]^{2+}$. In the pseudo-vitamin B12, the nucleotide moiety undergoes fragmentation to produce a fragment with m/z 348.07 $[Adenine+Sugar+PO3+H]^{+}$, as shown in Figure 11.

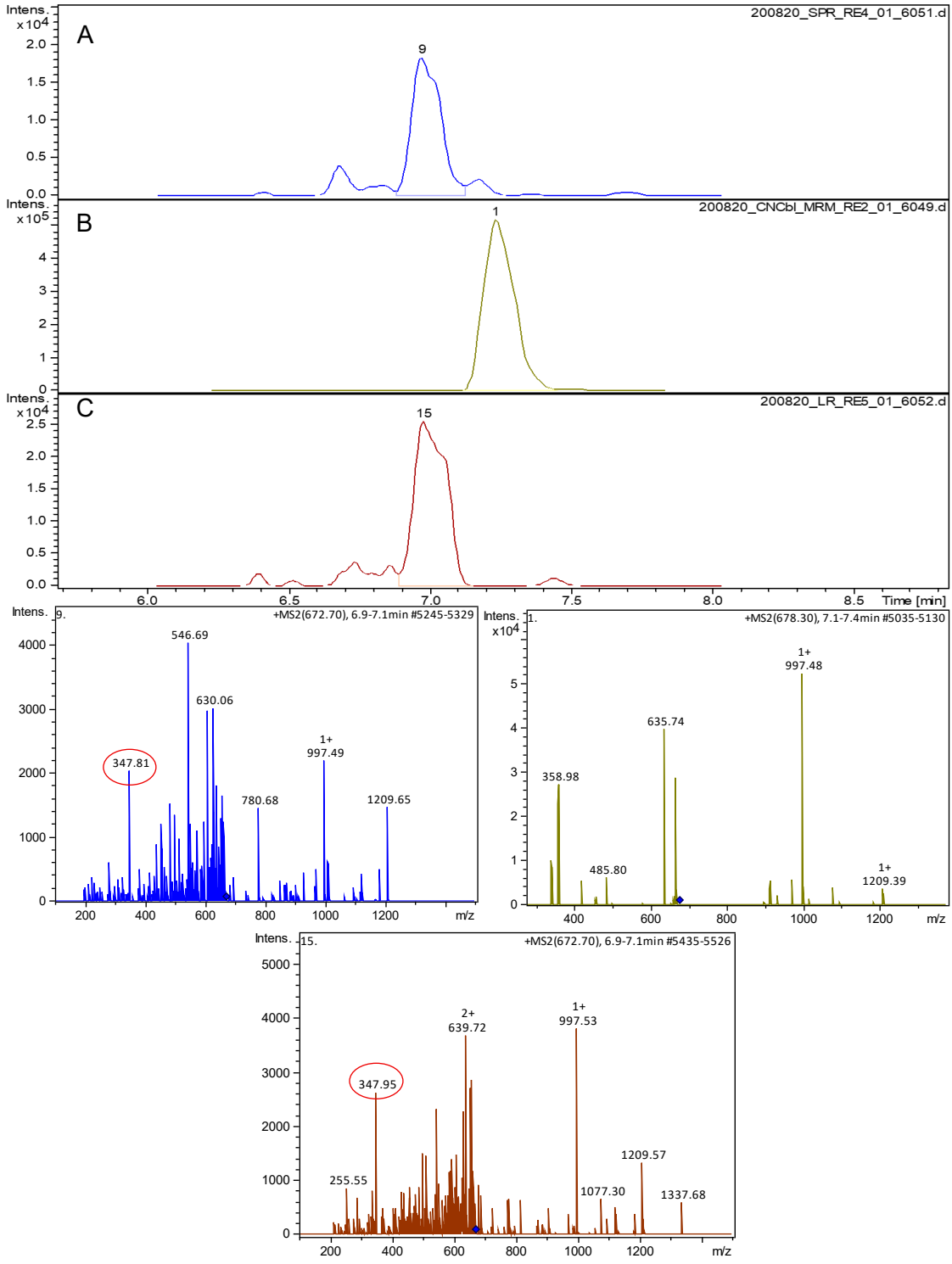

Figure 11 Chromatogram and mass spectrum of pseudo- vitamin B12 from L. reuteri *DSM 20016 and* Spirulina sp. *extract in compared to CNCbl standard. A: pseudovitamin B12 (*Spirulina sp.*); B: CNCbl standard; C: pseudovitamin B12 in* L. reuteri *DSM 20016 cell extract. The mass spectrum is displayed in the same colour as chromatogram. blue: pseudovitamin B12 (*Spirulina *sample); green: CNCbl standard; red: pseudovitamin B12 (*L. reuteri *DSM 20016)*

4.4. Determination of vitamin B12 production in single-fermentation and co-fermentation in oat-based beverages

4.4.1. Vitamin B12 production by the *L. reuteri* 92071 strain and co-fermentation with the *P. freudenreichii* DSM 20271 strain

The production of vitamin B12 was quantitatively analysed at each time point where the viable cell counts were monitored, up until 72 hours of fermentation in co-fermentation setups. In single-strain fermentation setups with *Lactobacillus* strains, the vitamin B12 levels throughout the fermentation process ranged between 0.10±0.02 – 0.23±0.01 µg/L. Among the *Lactobacillus* strains, the vitamin B12 production was on average approximately 0.16 µg/L. Notably, the co-fermentation setups demonstrated an increased vitamin B12 production, with concentrations reaching 10.04 ± 0.42 µg/L.

Initial analysis of the unfermented control (0 h) oat beverage confirmed baseline vitamin B12 levels below the method's detection limit. Upon inoculation, *L. reuteri*-inoculated samples exhibited cyanocobalamin concentrations (0.12 ± 0.01 µg/L), suggesting negligible initial extracellular B12 contribution from the inoculum. In contrast, *P. freudenreichii*-inoculated samples displayed a modestly elevated cyanocobalamin level (0.76 ± 0.01 µg/L) at 0 hour, exceeding that of both the control and *L. reuteri* samples. This discrepancy may reflect trace intracellular vitamin B12 in the pre-cultured inoculum.

As illustrated in Figure 12, co-culture fermentation of *P. freudenreichii* and *L. reuteri* in the oat beverage yielded a rapid increase in vitamin B12, achieving peak concentrations of 10.04 ± 0.42 µg/L by 24 h. These levels remained comparable up to 48 h (10.02 ± 0.38 µg/L), indicating a stabilization phase in cobalamin biosynthesis. However, a gradual decline to 8.14 ± 0.00 µg/L was observed by 72 h, suggesting potential late-phase metabolic shifts or degradation dynamics in the co-culture system. The vitamin B12 production from the monoculture fermentation with *P. freudenreichii* remained stable between 24 and 48 hours, ultimately reaching a maximal yield of 9.12 ± 0.45 µg/L at 72 hours.

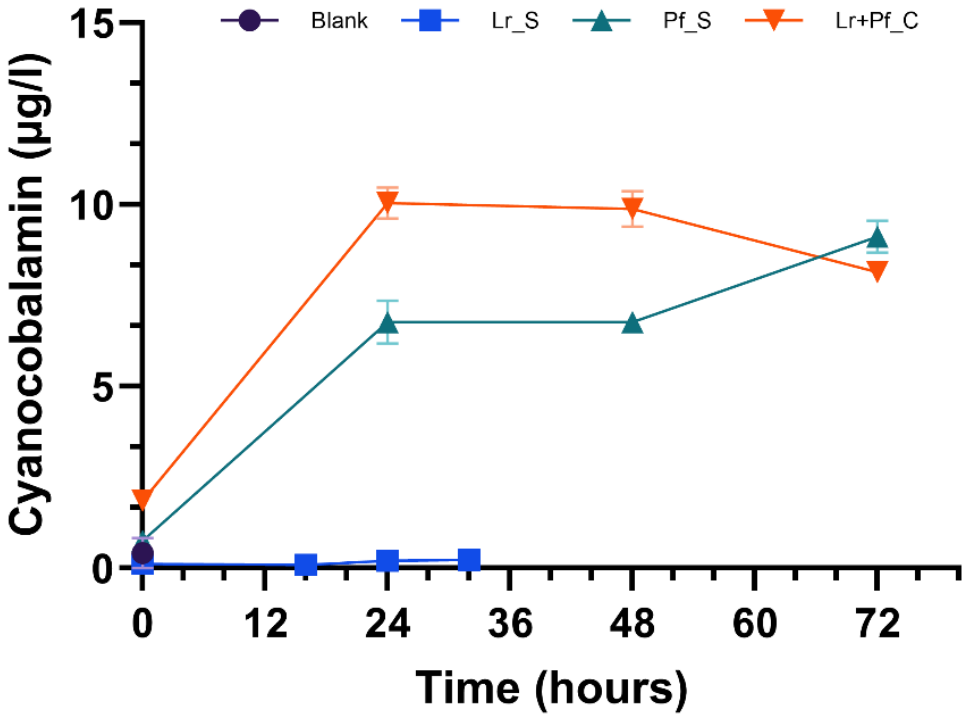

Figure 12 cyanocobalamin concentration of monoculture fermentation of L. reuteri *92071*, P. freudenreichii *DSM 20271 and co-culture fermentation of both strains in oat-based beverage.*

4.4.2. Pre-screening of *L. plantarum* strains for vitamin B12 production in an oat-based beverage

In the pre-screening studies, *Lactobacillus* strains such as *L. plantarum* 299 and *L. plantarum* MBT 92397 were first examined in VBAM via HSPE extraction and measured through LC-MS/MS to validate the analytical accuracy. Between two candidates, only *L. plantarum* 299 showed a minimum level of vitamin B12 production at 48 h with 1.14 µg/L, whereas no vitamin B12 was detected in the vitamin B12 assay medium inoculated with *L. plantarum* MBT 92397. Thus, *L. plantarum* 299 was further applied in an oat-based beverage fermentation. The amounts of vitamin B12 produced by each strain in vitamin B12 assay medium is shown in Table 26.

Table 26 Vitamin B12 quantification of Lactobacillus *strains in vitamin B12 assay medium (VBAM) via HSPE exaction and HPLC-MS/MS.*

Fermentation Time (h)	0	24	48	72
Sample	Cal. Cyanocobalamin (µg/L)			
VBAM - BLK	n.d.	n.d.	n.d.	n.d.
VBAM - LP 299	0.07	0.16	1.14	n.d.
VBAM - LP MBT 92397	n.d.	n.d.	n.d.	n.d.

n.d.= not detected

In studies conducted on vitamin B12 production in oat-based beverages with the addition of precursors using the selected Lactobacillus candidate strains, no increase in vitamin B12 production was observed, as shown in Table 27. Each sample set was analysed in triplicate for quantifying cyanocobalamin. However, the quantified concentrations showed inconsistencies across the triplicates. Therefore, using a statistical mean to represent the final concentration was deemed inaccurate. Instead, along with the mean value (as shown in Table 27), the minimum and maximum concentrations were used to assess the levels of vitamin B12 present in each sample.

The mean vitamin B12 concentration in oat-based beverage fermented with *L. plantarum* 299 was 0.37 µg/L, while the concentration in the non-inoculated (blank) oat-based beverage was 0.28 µg/L. The increase in concentration from the mean value was 0.09 µg/L. The maximum concentrations in both samples were similar, with values of 0.48 µg/L and 0.42 µg/L, respectively. In the sample set in which glycerine was added as a precursor, the mean vitamin B12 concentration in the fermented sample was 0.24 µg/L, compared to 0.08 µg/L in the non-inoculated blank. The increase in concentration was 0.16 µg/L. However, the maximum concentrations remained similar, with values of 0.38 µg/L and 0.35 µg/L.

For the sample set with riboflavin supplied as a precursor, there was no increase in the mean concentration between the fermented and unfermented (blank) samples. Additionally, the difference in maximum and minimum concentrations between the two samples was small. Since all increases in mean vitamin B12 concentration are below the limit of quantification (LOQ) for SPE quantification method, no observable increase in vitamin B12 content could be detected during the fermentation process, regardless of precursor addition.

Table 27 Vitamin B12 production of L. plantarum *299 in oat beverage with addition of glycerol, riboflavin and nicotinamide.*

	Cal. Cyanocobalamin (µg/L)			
Sample ID*	Minimum	Maximum	Mean	Increased Value
Oat -Blk	0.14	0.42	0.28	
O_Lp 299	0.25	0.48	0.37	0.09 ↑
O-Gly-Blk	-0.20	0.35	0.08	
O-Gly-Lp 299	0.09	0.38	0.24	0.16 ↑
O-RN-Blk	-0.15	-0.06	-0.11	
O-RN-Lp 299	-0.17	-0.05	-0.11	0.00 →

***Oat -Blk** = Oat beverage blank; **O_Lp 299** = Oat beverage inoculated with *L. plantarum* 299; **O-Gly-Blk** = Oat beverage blank added with Glycerol (Gly); **O-Gly-Lp 299** = same sample matrices inoculated with *L. plantarum* 299; **O-RN-Blk** = Oat beverage blank added with Riboflavin (RN); **O-RN-Lp 299** = same sample matrices inoculated with *L. plantarum* 299

4.5. Identification of vitamin B12 biosynthesis genes and related gene regulations

4.5.1. Whole genome sequencing for selecting vitamin B12 candidates among *Lactobacillus* strains (published manuscript (Gao et al., 2023)

In total, 7 *Lactobacillus* strains from the culture collection of Max-Rubner-Institute (Kiel, Germany) were sequenced and the metabolic characteristics and potential for vitamin B12 biosynthesis were further investigated via whole-genome sequencing technology. Three of the seven candidate stains were found to possess the genes for potential vitamin B12 biosynthesis, and the sequencing results are shown in Table 28.
The N_{50} values were between 24,975 and 242,008 bp, and the genome coverage ranged from 46- to 78-fold. The three draft genome sizes ranged from 1.80 Mbp to 3.18 Mbp, with GC contents between 38.52 and 52.19 mol%.

All three strains show a potential for B vitamin biosynthesis, particularly *L. reuteri* 92071*,* which contains the most complete genes involved in vitamin B12 biosynthesis. The genome sequencing resulted in 148 contigs, covering a total length of 2,201,984 bp, with a mol% G+C content of 38.52 and an N_{50} of 32,962 bp. Annotation of the draft genome predicted also other B vitamin biosynthesis genes such as folate and riboflavin among those three *Lactobacillus* strains. For other B vitamin biosynthesis, *L. plantarum* 92117-i3 possessed the most genes involved in folate and riboflavin biosynthesis when compared to the other two strains.

Table 28 De novo assembly of three lactic acid bacteria strains and draft genome features including cobalamin, folate and riboflavin biosynthesis genes.

Strain	***L. plantarum* 92117-i3**	***L. reuteri* 92071**	***L. fermentum* 92072**
No. of contigs	34	148	149
N_{50} (bp)	242,008	32,962	24,975
GC content (mol%)	44.62	38.52	52.19
Total length (bp)	3,180,242	2,201,984	1,806,298
Genome coverage (x)	48	46	78
No. of CDSs[a]	2,956	2,244	1,812
No. of tRNAs	53	62	52
No. of rRNAs	5	5	4
Pseudo genes	35	48	104
ANI[b] (similarity %)	*L. plantarum* (99.13%)	*L. reuteri* (98.47%)	*L. fermentum* (97.33%)
DDH[b] (formula 2)	*L. plantarum* (93.3%)	*L. reuteri* (88.2%)	*L. fermentum* (78.0%)
Cobalamin biosynthesis gene(s)	*cobB, cobQ*	*cobB, cobC, cobD_1, cobD_2, cobQ_1, cobQ_2, cobS, cobT, cobU,*	*cobB, cobQ*
	cbiO	*cbiA, cbiC, cbiD, cbiE, cbiF, cbiG, cbiH, cbiJ, cbiK, cbiL, cbiM, cbiN, cbiO, cbiQ, cbiT*	ND
	hemA, hemH	*hemA_1, hemA_2, hemB, hemC, hemL, hemH*	ND
Folate biosynthesis gene(s)	*folA, folB, folC, folD, folE, folK, folP, folT_1, folT_2*	*folB, folD, folE, folP,folT*	*folB, folC, folD,folE, folP*
Riboflavin biosynthesis gene(s)	*ribBA, ribD, ribE, ribF_1, ribF_2, ribH,*	*ribB, ribF*	*ribB, ribF*
Total number of paired reads	621,803	418,913	593,872
GenBank accession no.	JAQOWJ010000000	JAQOWD010000000	JAQOWE000000000
SRX/SRR/SRP/SRA/ENA accession no.	SAMN32886983	SAMN32886977	SAMN32886978

ND: Not Detected

4.5.2. Relative gene expression with RT-qPCR assay

The quantity and quality of the RNA extracted from both vitamin B12 assay medium, and precursors added assay medium are shown in Table 29. High quality RNA was obtained from bacteria samples with A260/A280 ratios ranging from 2.13 to 2.18.

Table 29 Concentration and purity of extracted RNA from bacterial samples.

Sample ID*	Nucleic Acid	Unit	260/280	260/230
VBAM_I_16h	141	ng/µL	2.18	1.77
VBAM_II_16h	141.6	ng/µL	2.14	2.14
CoF_I_16h	136.9	ng/µL	2.18	2.20
CoF_II_16h	120	ng/µL	2.14	1.82
VBAM_I_25h	131.3	ng/µL	2.13	1.98
VBAM_II_25h	148.4	ng/µL	2.14	2.20
CoF_I_25h	175.9	ng/µL	2.14	2.19
CoF_II_25h	182	ng/µL	2.14	2.09

*VBAM= vitamin B12 assay medium; CoF= vitamin B12 assay medium with co-factors;
I, II= two independent biological replicates; 16h= sampling point at 16 hours, 25h= sampling point at 25 hours.

RNA extracts from *L. reuteri* strain 92071 growing in vitamin B12-assay-medium were taken as a control and those growing in VBAM supplemented with precursors were compared with the control to investigate the regulation of vitamin B12 gene expression. All extracted RNA was of the required quality for RT-PCR applications. In addition, the integrity of the total RNA was assessed by 1.5% agarose gel prior to the RT-qPCR experiment.16S and 23S ribosomal RNA bands were found and no degraded RNA was identified (data not shown).

The *16s rRNA* gene was used as reference/housekeeping gene in the current study to normalize data, since the other candidate reference genes such as *gyrA* and *gmk1* in the previous study of primer validation did not show a good specificity (data not shown). *cobQ1*, *cobQ2, cobS* and *cobT* genes were used to assess the relative vitamin B12 biosynthesis gene expression in the different growth phases of the bacteria as shown in Figure 13.

cobS (adenosylcobinamide-GDP ribazoletransferase) and *cobQ1* (cobyric acid synthase, adenosylcobyric acid synthase) showed an up-regulation at 25h growth in vitamin B12 assay medium without the addition of precursors.

Relative to gene expression at 16 hours in VBAM, *cobQ2* and *cobQ1* were up-regulated at 25 hours. *CobT* exhibited slight up-regulation in the first biological replicate but demonstrated down-regulation in the second biological replicate. At 16 hours in VBAM with added precursors, *cobS, cobQ1*, and *cobQ2* showed significant up-regulation. However, in the second biological replicate, no changes in regulation were observed.

Since the gene regulation did not show any agreement with each other in two biological replicates, an absolute assessment of gene regulation in such case is not possible. However, all the genes were shown to be up-regulated in samples that were incubated for the 25 hours in VBAM compared to incubation in VBAM with addition of co-factors at both 16 hours and 25 hours.

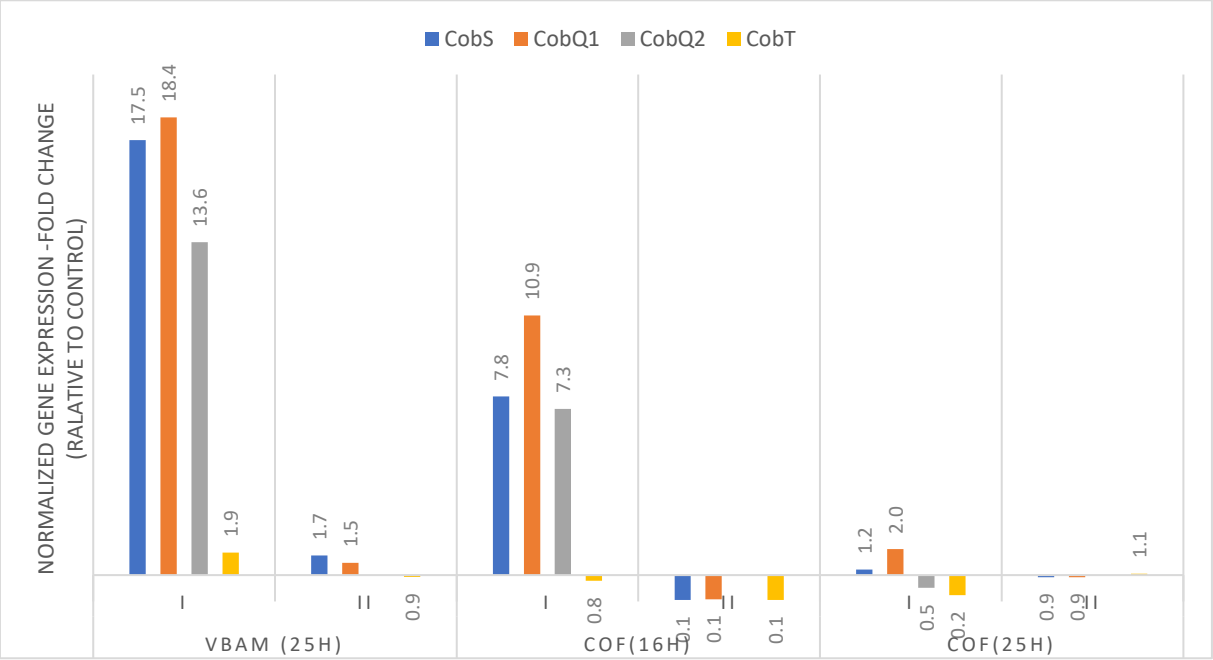

Figure 13 Normalized gene expression changes of cobS, cobQ1, cobQ2 *and* cobT *genes, compared to control sample in VBAM at 16 hours of incubation.*

4.5.3. Differential Gene Expressions (DGEs) via transcriptomic analysis

A whole genome transcriptome analysis was conducted to verify the gene expression regarding vitamin B12 metabolism in the presence of vitamin B12 key precursors. The primary goal was to obtain a good understanding on the growth-associated and precursor-associated changes in the mRNA expression levels of *L. reuteri* 20271 to get an in-depth insight into the vitamin B12 production in this bacterium. RPKM (Reads Per Kilobase Million), FPKM (Fragments Per Kilobase Million) and TPM (Transcripts Per Kilobase Million) values were calculated for each annotated transcript on the reference sequence of a contig.

The plot below in Figure 14 show data obtained with strain *L. reuteri* MRI 92071 cultured in vitamin B12 assay medium in the presence or absence of precursors. The precursors included DBMI, T-threonine and $CoCl_2$. In this case, samples with biological duplicates were compared in different growth phase (16h, 25h) according to the presence (blue) or absence (orange) of the precursors in the culture medium. In the PCA plot, each point represents a sample, indicating the different experimental groups (e.g., treated vs. control). The first principal component (PC1) accounts for 45.07% of the variance, while the second principal component (PC2) accounts for 37.32% of the variance.

The PCA plot reveals a clear separation between the treated and control groups along PC1, especially the control groups with 25 hours incubation. Indicating that the primary source of variation in the gene expression data is associated with growth conditions even compared in the same type of medium. This separation suggests that the gene expression at stationary phase significantly different compared to the gene expression at mid-exponential phase.

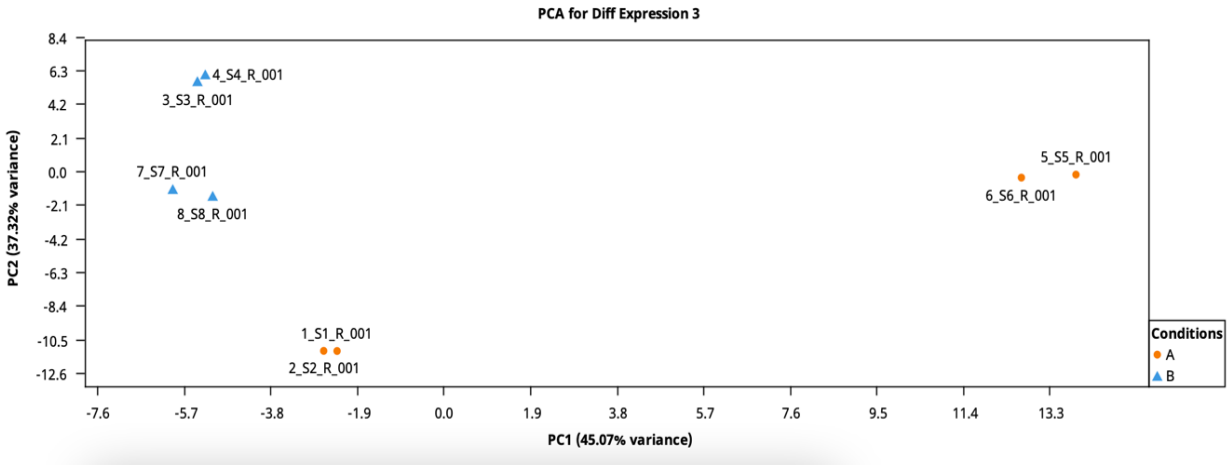

Figure 14 Assessing inter- and intra- group variability. (A) Principal component (PC) analysis plot displaying all 8 samples along PC1 and PC2, which describe 45.07% and 37.32% of the variability, respectively, within the expression data set. PC analysis was applied to normalized (reads per kilobases of transcript per 1 million mapped reads) and log-transformed count data.

At the 25-hour growth stage in VBAM medium, 373 genes up-regulated and 206 genes were down-regulated. Among these, 16 genes involved in the cobalamin biosynthesis pathway were up-regulated, whereas 2 genes, including the cobalamin ECF transporter, were down-regulated.

In contrast, when precursors were added to the VBAM medium, 93 genes were up-regulated, and 140 genes were down-regulated. Specifically, 2 genes associated with cobalamin biosynthesis were down-regulated under these conditions.

To understand the impact of precursors on the differentially expressed genes (DEGs) involved in the cobalamin biosynthesis pathway, a comparison was made between DEGs in *L. reuteri* MRI 92071 grown in VBAM medium and VBAM medium supplemented with precursors at each growth phase at each growth phase (16h and 25h).

During the mid-exponential growth phase (16h), 335 genes were found to be up-regulated in the VBAM medium with precursors, whereas 248 genes were down-regulated. Notably, 2 genes related to cobalamin biosynthesis, including a cobalamin ECF transporter were up-regulated, with no genes observed to be down-regulated. Most genes exhibited no significant changes. In the early stationary growth phase (25h), 210 genes were up-regulated, including 1 gene related to cobalamin synthesis. Conversely, 296 genes were down-regulated, with 16 genes linked to cobalamin synthesis, including cobalamin transporters, also being down-regulated.

To observe and compare the level of DEGs, as shown in the heatmap below (Table 30), all the key genes that participated in the metabolic pathway for vitamin B12 biosynthesis were listed. Although at 25 h of incubation, there were no significant vitamin B12 detected and only minimum quantity of total corrinoids were detected, at 25 h most key genes were up regulated in the vitamin B12 assay medium. In comparison to the control sample, when other intermediaries such as DMB, CoCl2 and L-threonine were added to the vitamin B12 assay medium, significant amount of vitamin B12 were detected, however, there were no significant change in terms of gene expressions were observed at 25 h of growth.

On the other hand, at the same incubation hours, to observe the effect to adding precursors on the vitamin B12 biosynthesis, the expression of key genes was compared between the B12 assay medium and medium added with precursors. Most of the key genes involved in the B12 biosynthesis were not significant differentially expressed in the medium with precursors, except that Cob(I)alamin adenosyltransferase (EC 2.5.1.17) showed an up-regulation at both 16 h as well as 25h of incubation. Respectively, at 25 h of incubation, most of the genes showed a down-regulation in the medium with precursors compared to the medium without.

In the current study, *cobT* was also listed in the comparison to determine the effect on gene expression during the addition of DMB in the medium. Interestingly, a slight downregulation was observed at 25 h in the B12 assay medium with precursors, whereas there was no other significant change between 16 h and 25 h of growth in B12 assay medium, as well as compared to B12 assay medium without precursors at 25h of growth, no changes were observed. As in the current study, an absolute value of Differential Expression Log2 Ratio greater and equal to 0.5 has been chosen to define differentially expressed genes. The level of differential expression is relative minimum. Since L-threonine was added as precursors in the medium, L-threonine 3-O-phosphate decarboxylase (*cobD*) encoding gene was also investigated for the DEGs among different growth phases. In the medium with precursors added, *cobD* showed similar degree of down-regulation as *cobT* at 25 h compared to 16 h growth. As well as a slight downregulation was observed at 25h in the medium with precursors compared in the medium without. This indicated, that in most cases in B12 assay medium supplemented with precursors, the expression of *cobD* at 25h was downregulated. On the contrary, when the bacterium was grown in the medium without precursors, the gene was up regulated.

Table 30 Heatmap of differential expressions based on Log2 ratio of key genes associated with vitamin B12 biosynthesis.

Protein function	Gene Name	A/B	C/D	A/C	B/D
		Differential Expression Log2 Ratio			
Adenosylcobinamide kinase (EC 2.7.1.156) / Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)	*cobU/ cobP*	nDE	-1.1	0.8	-1.3
Adenosylcobinamide-phosphate synthase (EC 6.3.1.10)	*cobD/ cbiB/ cobC*	1.2	nDE	nDE	-1.6
ATP:Cob(I)alamin adenosyltransferase (EC 2.5.1.17)		nDE	nDE	2.4	0.7
ATP:Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	*cobA/ BtuR/c cobO*	1.8	nDE	0.8	-0.7
ATP:Cob(I)alamin adenosyltransferase (EC 2.5.1.17), propanediol utilization					
Cobalamin synthase (EC 2.7.8.26)	*cobS*	nDE	-1.3	nDE	-0.8
Cobalt ECF transporter, additional substrate-binding protein CbiN	*cbiN*	1.6	nDE	nDE	-1.8
Cobalt ECF transporter, substrate-binding protein *cbiM*	*cbiM*	1.5	-0.5	nDE	-1.8
Cobalt-precorrin 5A hydrolase (EC 3.7.1.12)	*cbiG*	1.5	nDE	nDE	-1.8
Cobalt-precorrin-2 C(20)-methyltransferase (EC 2.1.1.151)	*cbiL*	1.6	nDE	nDE	-1.9
Cobalt-precorrin-3 C(17)-methyltransferase (EC 2.1.1.272)	*cbiH*	1.6	nDE	nDE	-1.8
Cobalt-precorrin-4 C(11)-methyltransferase (EC 2.1.1.271)	*cbiF*	1.5	nDE	nDE	-1.7
Cobalt-precorrin-5B (C1)-methyltransferase (EC 2.1.1.195)	*cbiD*	1.2	nDE	nDE	-1.6
Cobalt-precorrin-6B C15-methyltransferase [decarboxylating] (EC 2.1.1.196)	*cbiT*	1.4	nDE	nDE	-1.8
Cobalt-precorrin-7 (C5)-methyltransferase (EC 2.1.1.289)	*cbiE*	0.8			-1.1
Cobalt-precorrin-8 methylmutase (EC 5.4.99.60)	*cbiC*	1.5	nDE	nDE	-1.8
Cobalt-zinc-cadmium resistance protein		-1.2	nDE	-0.9	0.7
Cobalt/zinc/cadmium resistance protein *CzD*	*czD*	-0.5	nDE	nDE	0.6
Cobyric acid synthase (EC 6.3.5.10)	*cobQ*	1.1	-0.5	nDE	-1.4
Cobyrinic acid a,c-diamide synthetase (EC 6.3.5.11)	*cbiA*	1.4	nDE	nDE	-1.8
Putative amidotransferase similar to cobyric acid synthase		1.1	nDE	nDE	-1.0
Sirohydrochlorin cobaltochelatase CbiK (EC 4.99.1.3) @ Sirohydrochlorin ferrochelatase activity of c*biK* (EC 4.99.1.4)	*cbiK/C ysG*	1.6	nDE	nDE	-1.8
Substrate-specific component *cbrT* of predicted cobalamin ECF transporter		0.9	nDE	1.1	nDE
Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (EC:2.4.2.21)	*cobT*	nDE	-0.8	nDE	nDE
L-threonine 3-O-phosphate decarboxylase (EC 4.1.1.81)	*cobD*	1.3	-0.7	nDE	-1.6

**A) strain grown in VBAM at 16 hours. B) strain grown in VBAM at 25 hours. C.) strain grown in VBAM medium with DMB+CoCl2+L-threonine at 16 hours. D.) strain grown in VBAM medium with DMB+CoCl2+L-threonine at 25 hours.

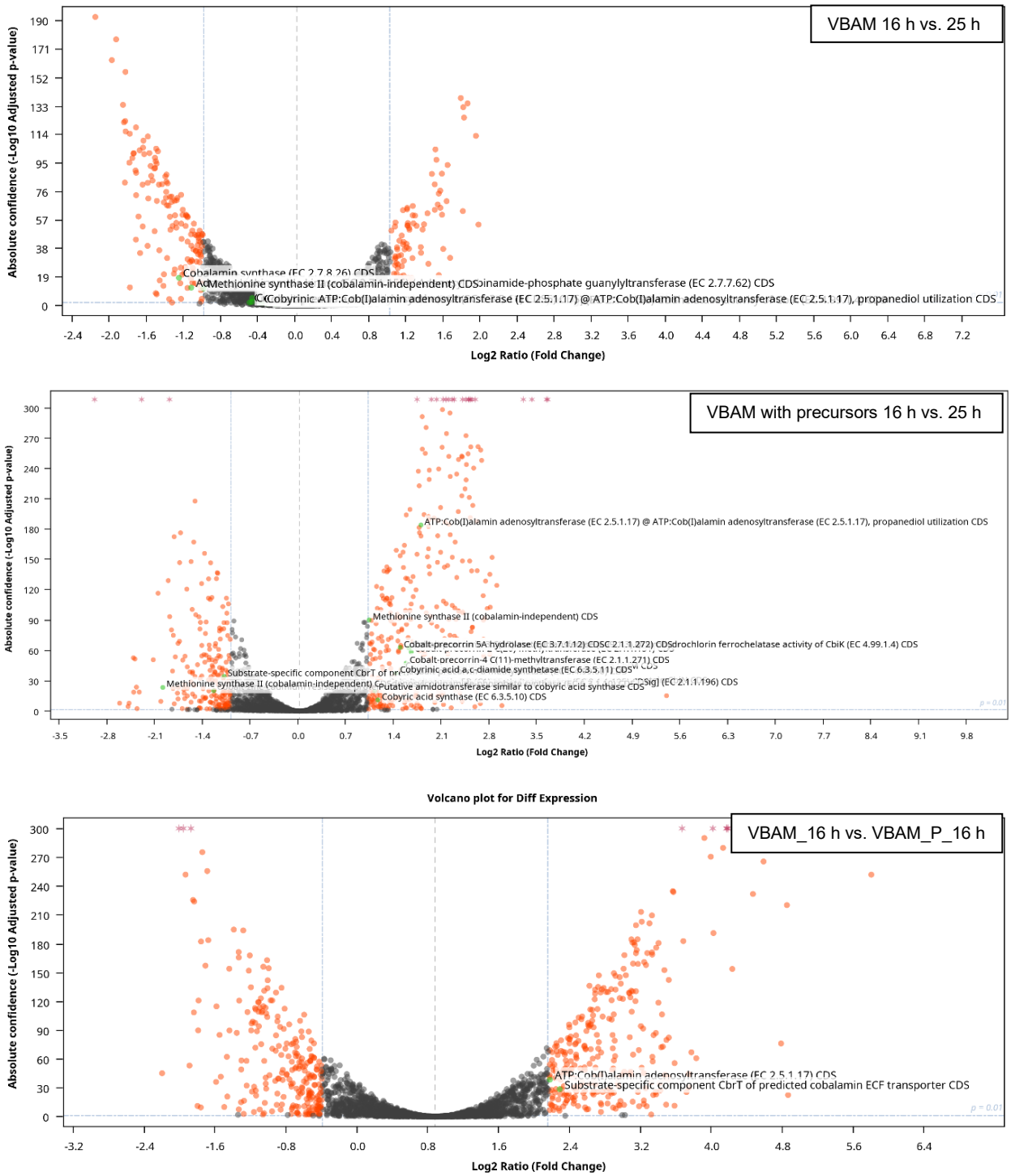

Figure 15 Volcano plots of differentially expressed genes under different growth phase for each condition. Genes with differences in expression are shown in orange colour, on the right side are genes up-regulated, on the left side are genes down-regulated, and genes with no significant differential expression are shown in grey, cobalamin-synthesis related genes are shown in green.

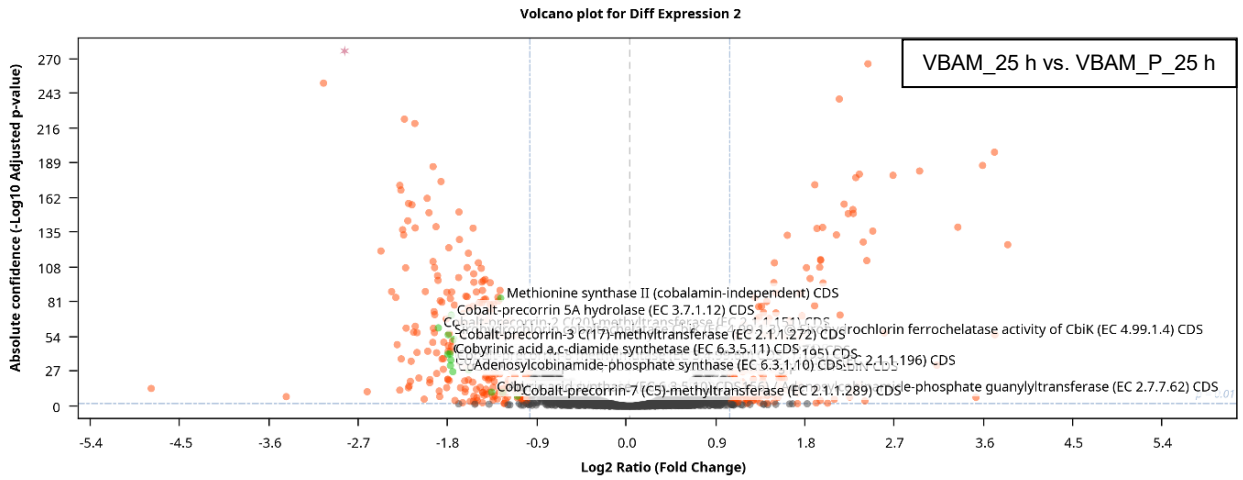

Figure 16 (Continued.) Volcano plots of differentially expressed genes under different growth phase for each condition. Genes with differences in expression are shown in orange colour, on the right side are genes up-regulated, on the left side are genes down-regulated, and genes with no significant differential expression are shown in grey, cobalamin-synthesis related genes are shown in green

5. DISCUSSION

5.1. Vitamin B12 quantification methods

5.1.1. Quantification via microbiological assay (MBA)

In food analysis, the MBA with test organism *L. delbrueckii* is often used as a routine test for vitamin B12, mainly due to its low cost. However, the microbiological assay was found to be limited to the quantification of total corrinoids in this study. It was also not possible to distinguish between biologically active and inactive vitamin B12, or to quantify very low levels of native or bio-fortified vitamin B12 (Table 19). To determine low vitamin B12 quantities in unfortified or bio-fortified products, a more precise analytical method, therefore, needed to be developed.

The VitaFast® Vitamin B12 (cyanocobalamin) microtiter plate test was claimed according to the manufacturer to be suitable for the quantitative determination of total vitamin B12 (added and natural vitamin B12) in milk products, cereals and baby food (ifp Institut für Produktqualität GmbH, 2017). The principle of the test kit is that the microtiter plate provided by this assay kit is pre-coated with *Lactobacillus delbrueckii spp. lactis (leichmannii),* and the growth of *L. delbrueckii* is dependent on the supply of vitamin B12 from the sample extract or the diluted extract. Such pre-coated plates could be advantageous for a quick and easy-to-use assay instead of the traditional methods, which require a pre-incubation of test organism for up to 24h. However, prior to the test assay, a sample pretreatment is required to be carried out. Considering that perhaps only trace level of vitamin B12 contents would present in non-fortified cereal beverages (e.g., oat), or in natural fortified cereal beverages via fermentation with *Lactobacillus* species, an effective and efficient sample extraction process would be needed to be developed.

For an efficient sample extraction, from the results we obtained (as described in Table 16), a 60-minute incubation was determined to be both effective and moderate for the enzymatic treatment during the sample extraction process for downstream applications. This was also in agreement with the product instruction of the VitaFast® kit, when 300 mg Taka-diastase was solely applied for the extraction.

On the other hand, the manufacturer's instructions specified the use of Taka-diastase (*Aspergillus oryzae*) as the sole enzyme for sample pretreatment. In contrast, other similar studies employed pepsin or amylase to digest proteins and carbohydrates in the sample matrix. Consequently, these enzymes were tested for their extraction efficiency. In this study, it was found in the oat-based matrices, a combination of both Taka-diastase and pepsin were more efficient at extracting free-vitamin B12.

As the results of this study showed in Table 20, increased levels of vitamin B12 were observed in samples prepared without enzymatic digestion, along with notable variability among analytical triplicates. This suggests the possibility of interferences in the sample extract. Further investigation is needed to determine whether the pre-coated indicator microorganism grows solely in the presence of vitamin B12. When the sample was treated solely with Taka-diastase, insufficient microbial growth was observed in the mixture of the sample extract of oat beverage and vitamin B12 medium mixture. This suggests that treatment with Taka-diastase alone is inadequate for digesting the sample matrix. Given that no significant differences were detected between using only pepsin and combined enzymatic treatments (Table 17), and that vitamin B12 concentrations exceeded the standard levels, further investigation was conducted. This investigation aimed to determine whether the increased vitamin B12 levels were due to its presence in pepsin, which is derived from porcine gastric mucosa.

In the recovery study, despite adding vitamin B12 at a very low level (0.3 µg/L), the final detection of vitamin B12 in the matrix was at more than three times higher than the expected concentration, with a recovery rate of 252% and RSD of 14%. Recovery rates are generally expected to be within the range of 70-120% (Imamoglu, Olgun, 2016, Konatu et al., 2017, Konatu, Jardim, 2018). RSDs or repeatability/reproducibility are typically expected to be $\leq$20% (Konatu, Jardim, 2018). Since the reproducibility of the test results is within the range, instrumental or methodological errors, or any contamination during the experimental process should be excluded. Additionally, a similar situation was found regarding the medium and higher levels of spiked samples. This indicated that other interfering components in the sample could stimulate the growth of the indicator strain that was pre-coated onto the microtiter plate. Another hypothesis is that the original oat beverage might have been contaminated with vitamin B12 during the manufacturing process, given that only trace amounts of vitamin B12 were detected in the blank matrix. Consequently, further verification was conducted on other organic, non-fortified cereal-based beverages.

To ensure measurement efficiency and accuracy, the sterility of the sample extract was always checked before the assay to assure that there was no false-positive result. This test kit was emphasized to be suitable for detecting vitamin B12 among cereal matrices according to the manufacture's specifications. However, when quantifying corrinoids among other cereal matrices such as rice drink and spelt drink, we obtained a false-positive results in this study. As previously indicated, neither native oats nor organically labelled cereal-based drinks should contain supplemented vitamins. Upon verification across various cereal-based beverages, including oat-based beverages from different manufacturers, it was found that the test kit (VitaFast B12) was not suitable for determining the native vitamin B12 content in these matrices. This is because the test results did not accurately reflect the actual concentration of vitamin B12 present in the native food matrices (as indicated on the nutritional labelling of the products, no vitamin B12 contents were shown). The growth of the indicator strain in the sample extract suggests that the pre-coated indicator strain may not be exclusively specific to the vitamin B12 containing assay medium. It appears to be stimulated by other potential substrates found in cereal-based matrices, which were not fully removed during the sample extraction process, resulting in a lingering matrix effect. As Matrix interference is a known issue in the determination of vitamin B12 in food products, including those containing cereals (Amritkar et al., 2020, Marley et al., 2009).

5.1.2. Quantification of cyanocobalamin and pseudo-cobalamin via LC-MS/MS

The immunoaffinity chromatography-liquid chromatography-mass spectrometry (IAC-LC-MS) method remains the gold standard for detecting both biologically active and inactive vitamin B12 forms, offering unparalleled selectivity that circumvents the limitations of microbiological assays. Its reliance on antibody-specific binding to cyanocobalamin (CNCbl) ensures precise extraction of free vitamin B12 from complex matrices. However, the high cost and labour-intensive nature of IAC necessitate exploration of alternatives, such as solid-phase extraction (SPE), for routine screening applications.

In the evaluation of extraction efficiency and reliability of the immunoaffinity chromatography (IAC), at the lower spiked concentration, the method demonstrated strong inter-day reproducibility, with mean recovery values aligning closely with theoretical expectations and a relative standard deviation reflecting acceptable analytical variability (Table 22). However, intra-day assessments revealed discernible shifts in recovery rates, with initial days showing marginally lower values compared to a subsequent moderate elevation (Table 22). These variations, while within an analytically feasible range, may point to subtle environmental or procedural factors—such as ambient temperature fluctuations, minor inconsistencies in mobile phase preparation, or gradual changes in chromatographic column efficiency—that could disproportionately influence method performance at trace-level concentrations (Kruve et al., 2015). The findings underscore the importance of stringent environmental controls and instrument monitoring to mitigate variability in low-concentration analyses, particularly over extended analytical sequences.

At elevated spiked concentrations, a pronounced reduction in recovery efficiency was observed, accompanied by reduced analytical variability (Table 22). This inverse correlation between analyte concentration and recovery may reflect saturation dynamics within the immunoaffinity capture phase, where binding site limitations or competitive matrix interactions could impede target-selective retention. Such behaviours aligns with documented challenges in immunoaffinity-based methods applied to complex matrices, where high analyte levels may exacerbate non-specific binding or disrupt equilibrium conditions during ligand-target association (Kruve et al., 2008). These findings underscore the necessity of optimizing immunoaffinity protocols for target concentration ranges and highlight the critical role of matrix-matched calibration or sample cleanup strategies to mitigate competitive interference in low-concentration analyses.

To stabilize labile cobalamins, potassium cyanide (KCN) was employed to convert all forms to CNCbl—a critical step validated by (Marley et al., 2009), who documented a 30% loss of hydroxocobalamin without stabilization. Despite this precaution, in the matrix-matching calibration experiments, IAC recovery rates exhibited significant matrix dependence: in unfermented medium yielded moderate recoveries (51.8%), while in fermented medium showed pronounced analyte loss (32%) (Table 23). Fermented matrices further exacerbated this trend, likely due to microbial metabolites amplifying matrix effects such as ion suppression or enhancement (Kruve et al., 2008).

Matrix effects, quantified via a standard-addition method (Kittlaus et al., 2011), revealed a suppression of -33% in microbiological samples, exceeding the ±20% threshold recommended by SANTE/11945/2015. The pronounced matrix effect observed in this study (-33%) underscores the limitations of the IAC method for quantitative vitamin B12 analysis without an internal standard, particularly in complex microbiological and food matrices. Ion suppression and co-eluting interferences, exacerbated by low endogenous B12 levels in plant-based or microbially enriched samples, preclude reliable quantification without corrective measures. However, the method demonstrated sufficient specificity and reproducibility to serve as an effective screening tool for preliminary identification of potential vitamin B12 producers. To enhance precision for quantitative applications, matrix-matching calibration (Ly et al., 2020)—is essential to account for suppression effects inherent to complex samples or developing an isotope-labelled internal standards—to enhance quantification accuracy in trace-level analyses (Wang et al., 2021).

In this study, SPE was evaluated as a cost-effective alternative for corrinoid extraction from biological and food matrices. Notably, when analysing B12-supplemented food samples, results from SPE and immunoaffinity chromatography (IAC) clean-up methods showed no significant differences (Table 24). This parity confirms that the enzymatic treatment was not necessary before employing the current SPE method and extraction protocols employed in this study effectively liberate protein-bound B12 from complex matrices, ensuring accurate detection regardless of the clean-up method. Such consistency is critical for validating SPE's utility in detecting bioactive B12, even in challenging matrices. This is particularly valuable in high-throughput microbial strain screening, where cost and labour efficiency are critical.

The practical value of SPE as a screening tool is further illustrated by its ability to differentiate bioactive cyanocobalamin (CNCbl) from non-bioactive analogues. For instance, SPE analysis of *Spirulina* (*Arthrospira platensis*) supplements revealed no detectable bioactive B12 (Figure 11), consistent with literature reports of its pseudo-cobalamin content (Bito et al., 2016; Watanabe et al., 2002; Watanabe et al., 1999a). In contrast, trace amounts of bioactive B12 (~0.1 µg/100g) were identified in *Chlorella* (*C. vulgaris*), likely derived from B12-synthesizing bacteria in its cultivation environment (Andrade, 2018). These findings align with chromatographic profiles of *L. reuteri* DSM 20016 (Figure 11), a known pseudo-B12 producer, which exhibited retention times identical to Spirulina's pseudo-cobalamin peak (Santos et al., 2007). This congruence highlights SPE's capability to prevent overestimation of B12 content—a common pitfall in nutritional analyses of algae-based supplements.

A significant challenge in quantifying pseudo-B12 lies in the absence of commercial standards. To address this, peak area comparisons with CNCbl standards (Deptula et al., 2017) offer a pragmatic workaround, enabling semi-quantitative estimation of analogue concentrations. While this approach cannot fully replace IAC for regulatory-grade analyses, it positions SPE as a viable preliminary tool for distinguishing bioactive and inactive corrinoids. For example, the SPE method successfully discriminated CNCbl in *Chlorella* from pseudo-B12 in *Spirulina*, corroborating earlier findings that *Chlorella*-derived B12 exhibits greater bioavailability (Watanabe et al., 2002). Furthermore, the retention time alignment between *Spirulina'*s pseudo-B12 and *L. reuteri* DSM 20016 provides a reference framework for identifying inactive analogues in microbial cultures.

In conclusion, while the SPE method is not inherently quantitative due to matrix-derived limitations, its specificity, cost efficiency, low sampling volume and adaptability make it indispensable for initial screening of B12 producers and fortified products. However, for a better precision especially in a low-concentration vitamin B12 quantification, such as bio-fortified plant-based food matrices, coupled with matrix-matched calibration and validation is needed prior to the quantification. Therefore, IAC-LC-MS was chosen as method of choice in this study.

5.2. Vitamin B12 production among *Lactobacillus* strains

5.2.1. Growth of *Lactobacillus* strains in vitamin B12 free medium and vitamin B12 production

At first, the growth phase of each *Lactobacillus* strains was being observed to identify the mid-exponential phase and early stationary phase, since those two phases are associated with maximum vitamin B12 production in other B12 producers such as *P. freudenreichii* (Deptula et al., 2017, Marwaha et al., 1983). The observations from the growth phase of each strain revealed that while variations existed among independent biological replicates, the variation during the mid-exponential phase was relatively smaller compared to the stationary phase. This suggests that individual stock cultures may enter the stationary phase at different time points, leading to inconsistencies in growth patterns. To address this, it is recommended that each individual pre-inoculum be screened for its growth curve before being used in further studies, whether for fermentation purposes or gene expression analyses. This screening ensures that the pre-inoculum is in an identical growth phase at the beginning of the experiment, thereby minimizing variability and enhancing the reproducibility of results. Such standardization is crucial for obtaining reliable and consistent data in studies requiring precise control overgrowth conditions.

Two *Lactobacillus* species, *L. reuteri* and *L. plantarum*, were tested *in vitro* for their ability to produce vitamin B12. According to our previous literature reviews (as described in 2.2.2), these strains are among the most promising candidates for vitamin B12 production. Across different references (see Table 4), concentration of vitamin B12 produced by *L. plantarum* species ranged from 0.20 ± 0.03 µg/L to 996 ± 37 µg/L depending on the quantification methods. In the work of Bhushan et al. ((Bhushan et al., 2017), B12 concentration in the vitamin B12 assay medium was found to be as high as 102.5 ± 11 µg/L, quantified using a microbiological assay. Meanwhile, other studies have shown that different strains of *L. plantarum* can have B12 concentrations as low as 1.8 ± 0.1 µg/L when quantified with the same method. These findings indicate that, even within the same species and quantified with the same method, vitamin B12 production can vary significantly between strains.

In our current study, *L. plantarum* 299 demonstrated minimal production of vitamin B12, with concentrations of 1.14 µg/L at 48 hours in vitamin B12 assay medium and from 0.25 to 0.48 µg/L in an oat beverage. In a separate study by Thompson et al. (Thompson et al., 2020), which used the same strain incubated in cauliflower and white bean-based matrices, the B12 concentration was found to be 0.048 ± 0.013 µg/100 g. Although direct comparisons are challenging due to differences in dry-mass calculations between the two studies, it can be observed that this strain may only produce trace amounts of vitamin B12. Moreover, in the study from Thompson et al. (Thompson et al., 2020) , vitamin B12 quantification was performed using a microbiological assay, which cannot distinguish between pseudo-vitamin B12 and biologically active vitamin B12. In contrast, our study utilized HPLC-MS/MS for quantification, measuring vitamin B12 as cyanocobalamin alone, resulting in lower observed levels of vitamin B12. Furthermore, some samples from the same batch culture could not be identified for vitamin B12 production because the concentration of cyanocobalamin was near the lower limit of detection. Therefore, it can be inferred that this strain cannot produce sufficient levels of biologically active vitamin B12.

On the other side, the quantification method could also result in a significant difference in the measured concentration of vitamin B12. For instance, in the work of Kumari et al. (Kumari et al., 2021), the intracellular B12 concentrations of various *L. plantarum* strains, measured in a B12-free medium, ranged from 7.6 to 24 µg/L using UFLC and 32 to 97 µg/L using the MBA method. These strains (see Table 4, p.11) produced much higher amounts of B12 compared to the strains utilized in this study, although B12 production is known to be strain-dependent. However, neither the UFLC nor the MBA detection method was able to identify the presence of pseudo-B12 forms, leaving uncertainties regarding the nature of the corrinoids produced. Specifically, it remains unclear whether these corrinoids are biologically active, raising questions about their functional relevance and potential applications. Based on previous research (as described in 2.2.2, Table 4), most *L. plantarum* strains reported to produce B12 have been analysed using either the MBA or HPLC method, which lack the specificity to distinguish between different forms of corrinoids. As a result, it remains unclear whether other strains within the species can produce biologically active B12. In contrast, this study utilized the LC-MS/MS method to identify the production of pseudo-B12, revealing that the strains examined here do not possess the capacity to produce biologically active B12.

This finding is significant, as these strains were able to grow well in the vitamin B12 assay medium, which is commonly used as an initial screening tool for identifying potential B12-producing candidates. This highlights the limitations of relying solely on growth in assay media and applying microbiological methods as a criterion for B12 production. It suggests that employing more specific analytical methods to confirm the presence of biologically active B12 is necessary.

5.2.2. Growth in single and co-fermentation in oat beverage and vitamin B12 production

5.2.2.1. The growth and acidification of LAB and PAB strains in oat-based beverage

Both *L. plantarum* strains tested in this study demonstrated a rapid growth in oat-based substrates from an initial cell density of ca. 5.9 log CFU/mL and increased to ca. 9.0 log CFU/mL after 24 hours of fermentation (Table 15), it remained unchanged thereafter. This agrees with the acidification pattern as well, the pH dropped rapidly from initial pH at 6.5 to 3.8 at 24 hours and remained in a minimum changed thereafter (see in Figure 6). This suggests that the fermentation or metabolic activity of these strains in oat-based substrates was completed after 24 hours.

Compared to the rapid growth and acidification of the *L. plantarum* strain in oat-based substrates, *L. reuteri* 92071 exhibited a slower growth pattern, characterized by a gradual increase in viable cell counts (from 5.5 log CFU/mL to 7.7 log CFU/mL), peaking at 24 hours post-inoculation before stabilizing (Figure 5). Although the initial cell density of the inoculum was similar to that of *L. plantarum*, both strains reached their maximum cell density at 24 hours. However, the maximum cell density of the *L. reuteri* strain was 2 log CFU/mL lower than that of *L. plantarum*.

A similar pattern could be observed in the acidification during the fermentation, in accordance with the rapid bacterial growth the pH decreased gradually from the initial level at 6.4 to 5.1 at 24 hours, reaching 4.6 at 32 hours by the end of the fermentation. By comparison the *L. plantarum* strain decreased the pH to 3.9 at 24 hours. Although this pH range ensures the microbiological safety of the fermented substrates, vitamin B12 production cannot continue below pH 5.0, since the cell death eventually resulted when the pH value is below 4 for LAB and 5 for PAB (Garrity et al., 2010, Hettinga, Reinbold, 1972). Perhaps this explained that one of the reasons that in the pre-screening study for vitamin B12 candidates, no vitamin B12 was detected in the sample inoculated with *L. plantarum* after 24 hours of incubation.

Moreover, in this study, all the B12 produced in the food matrix was converted to CNCbl for quantification. CNCbl is considered as the most stable form among all the biologically active cobalamins, therefore often used in pharmaceutical and supplement preparation (Lie et al., 2020). The maximum stability of CNCbl was at pH 6.0 at 4°C and highest degradation extent at pH 2.0, where temperature also showed influence. At the same pH range (e.g. pH 6.0) the same storage time, the retention of B12 was 92%, 85% and 81% at 4°C, 25°C and 37°C, respectively (Bajaj, Singhal, 2020). Maintaining a pH within the optimal stability range (approximately 4.0 to 7.0) is essential to prevent degradation of the vitamin during production. Operating below pH 5.0 may destabilize existing B12 in the substrates, leading to reduced yields. This is especially important when vitamin B12, produced via fermentation in various food matrices, was identified in its active forms, such as MeCbl, AdoCbl or OHCbl. These forms exhibit a comparable stability profile regarding pH range to that of CNCbl. However, they were much more likely to be degraded than CNCbl regarding photodegradation (Temova Rakuša et al., 2022).

The study of Hugenschmidt et al. (Hugenschmidt et al., 2011) reported a higher yield of vitamin B12 when the inoculation level of PAB was at 7 log CFU/mL, and a LAB-PAB ratio of 1-1000 gave a maximum vitamin B12 concentration. In the current study the initial inoculum level of PAB was set at about 7 log CFU/mL, and 5 log CFU/mL for LAB in the co-culture fermentation. In mono-culture fermentation *P. freudenreichii* DSM 20271 grew similar as reported in most studies (Chamlagain et al., 2016, Hugenschmidt et al., 2010) and reached a maximum cell density of 8.67± 0.03 log CFU/mL at 72 hours, where it entered the stationary phase (Figure 5). In contrast, *P. freudenreichii* strain grew more rapidly in the co-culture fermentation compared to the mono-culture fermentation, reaching the stationary phase at 24 hours with a cell density of 8.8 log CFU/mL. The interaction between the two bacterial species resulted in a synergistic effect particularly for *P. freudenreichii*, significantly accelerated the growth. This enhancement suggests that co-fermentation with *L. reuteri* 92071 provides a more conducive environment for bacterial proliferation of *P. freudenreichii*. This finding is consistent with the hypothesis posited by the other study (Hugenschmidt et al., 2011), which suggests that a synergistic mechanism may be involved in the co-culture of *P. freudenreichi* with *Lactobacillus* strains.

The resulting pH change was intermediate between the changes observed in the respective monocultures. The pH decreased more rapidly than in the *P. freudenreichii* monoculture fermentation, yet not as swiftly as in the *L. reuteri* monoculture. At 24 hours, the pH stabilized at approximately 5.3, maintaining a conducive environment for *P. freudenreichii* to synthesize vitamin B12. Thereafter, the pH decline occurred gradually, yet acidification was still observable until the end of fermentation, with the final pH reaching 4.9. Although there was no viable cell growth being observed for both LAB and PAB strains.

5.2.2.2. Vitamin B12 production with precursor supplementation and active vitamin B12 production in oat-based matrices via co-fermentation by *P. freudenreichii* and *L. reuteri*

The fermentation of oat beverage with strain *L. plantarum* 299 did not yield measurable increases in vitamin B12 production, even with glycerol and riboflavin supplementation. This contrasts with its previously demonstrated cobalamin biosynthetic capacity in vitamin B12 assay media, highlighting the critical influence of matrix-specific factors on microbial metabolic activity. The absence of a commercial cyanocobalamin internal standard during analysis introduced methodological constraints, as quantification relied on relative mass spectrometry peak areas. This approach occasionally produced anomalous negative values, likely due to matrix interference or baseline variability, complicating precise interpretation of low-concentration measurements. Hence, on the other hand, it showed the disadvantage of SPE screening method on biological samples with its matrix-effect. Notably, both fermented and unfermented samples exhibited lower calculated cyanocobalamin levels compared to the control blank, suggesting that riboflavin supplementation failed to stimulate B12 biosynthesis under these conditions. Parallel experiments with glycerol as an alternative precursor similarly showed no enhancement, reinforcing the conclusion that these additives did not overcome metabolic or environmental limitations in the oat matrix. In contrast to previous studies where *P. freudenreichii* was used to investigate the positive effects of supplying precursors such as riboflavin and niacinamide for the natural synthesis of DMBI (the lower ligand of vitamin B12) (Chamlagain et al., 2016, Hugenschmidt et al., 2011), riboflavin supplementation did not stimulate vitamin B12 production in *L. plantarum*. This discrepancy could be attributed to two potential factors: (1) the oat-based matrix may not have been a suitable environment for *L. plantarum* to produce vitamin B12, or (2) *L. plantarum* may lack the genetic machinery required to utilize riboflavin as a precursor for synthesizing the lower ligand necessary for vitamin B12 production.

The fermentation dynamics observed between monoculture and co-culture systems revealed distinct temporal patterns in vitamin B12 biosynthesis within the oat beverage matrix. Cobalamin production was increased dramatically in the co-fermentation set-ups (Figure 12). Although the synergistic interaction between *P. freudenreichii* and *L. reuteri* was not clearly reflected in terms of viable cell counts—with no significant differences observed between monoculture and co-culture fermentations—the co-fermentation setups demonstrated a significant elevation in cobalamin synthesis and a shortened fermentation period, reaching to the maximum production of B12 after 24 hours of fermentation anaerobically.

Then the co-culture condition demonstrated an early-phase production peak followed by stabilization and subsequent decline, contrasting with the linear accumulation observed in monoculture. This divergence suggests microbial interactions in the co-culture system—potentially due to competition for rate-limiting substrates, the question remains unclear, whether this decline was caused by consumption of B12 by *L. reuteri* strain or natural degradation, since at 72 hours no further growth of either *P. freudereichii* or *L. reuteri* was observed in the substrate. Therefore, further studies on an extended period of co-culture fermentation and degradation of vitamin B12 are required to clarify this question.

5.3. Gene regulation and expression in the corrinoid biosynthesis pathway

In the work from Santos et al. (Branco Dos Santos, 2008), the *rpsO* gene was applied as a control for the expression analysis due to its location which was downstream from the vitamin B12 gene cluster. This gene was verified in the RT-PCR assay in the current study, however, it was not utilized as a control in the study conducted by Torres et al. (2018). Additional data is required to ascertain the suitability of this gene as a control for expression analysis. Further studies should focus on determining whether *16s rRNA* gene or *rpsO* is more appropriate for gene expression studies specifically related to vitamin B12 biosynthesis, particularly in the context of relative quantification of gene expression via RT-qPCR assay.

In this study, the enzyme nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (*cobT*) was investigated due to it being crucial in the biosynthesis of vitamin B12 in bacteria. *CobT* is responsible for activating the lower ligand by attaching either dimethylbenzimidazole (DMB) or adenine to the corrin-ring structure of cobalamin (Claas et al., 2010, Deptula et al., 2015) . This attachment determines the form of cobalamin produced, influenced by the intermediates generated in previous steps or the growth environment. In this study, the differentially expressed genes (DEGs) related to *cobT* were compared across all experimental conditions to evaluate the gene regulation involved in the attachment of dimethylbenzimidazole (DMB) as a precursor to the corrin-ring structure and to assess whether *cobT* has an affinity for DMB on the lower ligand. However, no significant regulation was observed between the exponential and late-exponential phases, while a slight downregulation was observed in the stationary phase (Table 30).

This finding agrees with the work of Torres et al. (2018). *L. reuteri* grown in vitamin B12 free medium with $CoCl_2$ was applied in that study as control group and the gene expressions were compared among groups with adding other precursors such as DMB and L-threonine or ALA, where also no significant change was observed in the regulation between lag, exponential and late-exponential phase. ALA and DMBI have been suggested as a requirement for increased B12 production in other studies (Mohammed et al., 2014), since ALA was also one of the primary precursors participating in uroporphyrinogen III in the beginning of vitamin B12 biosynthesis.

In contrast, another study by Santos (Branco Dos Santos, 2008) observed gene up-regulation in the late-exponential phase in a vitamin B12-free medium without any supplementation. Indeed, the induction of B12 biosynthesis genes was stronger in the B12-free medium compared to a B12-free medium supplemented with exogenous cyanocobalamin, which served as a control group. This same induction was also stronger than in cells grown in a B12-free medium supplemented with a combination of precursors such as dimethylbenzimidazole (DMB), cobalt chloride (CoCl2), and L-threonine.

Additionally, this study also observed significant up-regulation of genes related to vitamin B12 biosynthesis in the late-log/early stationary phase (25h) compared to the mid-exponential phase (16h), which aligns with the findings of study from Santos (Branco Dos Santos, 2008). Moreover, in the B12-free medium supplemented with the three combined precursors, there were no noticeable differences in gene expression related to B12 biosynthesis. This result is consistent with the findings of Torres et al. (Torres et al., 2018), who reported no change in the regulation of key genes when other precursors were added, compared to a vitamin B12-free medium containing solely $CoCl_2$. Our results suggest that adding DMB and L-threonine did not impact the regulation of key gene expression involved in B12 biosynthesis.

5.4. Conclusions and outlook

This dissertation has demonstrated the significant potential of fermentation as a sustainable and effective strategy for the *in situ* biofortification of plant-based dairy alternatives with vitamin B12. Applying co-fermentation strategy utilizing *P. freudenreichii* DSM 20271 and *L. reuteri* 92071 in oat-based beverage has shown sufficient production of vitamin B12 (ca. 10.0 µg/L). In Europe, as the recommended daily allowance (RDA) is 4.0 µg/d published by the German Nutrition Society (DGE) (Richter et al., 2016, Ströhle et al., 2019). Approximately 400 mL fermented oat-based beverage would be possible to provide an adequate daily intake for healthy adults. Therefore, this bio-enriched beverage can help prevent vitamin B12 deficiencies among vegans and vegetarians. Furthermore, the results of co-fermenting *P. freudenreichii* DSM 20271 and *L. reuteri* 92071 strain in this study demonstrated a reduced period to achieve sufficient levels of vitamin B12 than a monoculture fermentation with *P. freudenreichii* DSM 20271 alone.

This biofortification process would be particularly interesting for the food industry from an energy efficiency perspective. Moreover, it offers a sustainable solution and strengthens the competitiveness of the organic food sector, given that Regulation (EU) 2018/848 on organic food restricts the use of chemically synthesised additives.

While this study has demonstrated successful enhancement of vitamin B12 in plant-based dairy using co-fermentation strategy, the yield of vitamin B12 from the co-fermentation process can still be improved, as consuming, for example, 400 mL of fermented plant-based beverages represents a relatively large quantity for an individual. $CoCl_2$, δ-aminolevulinic acid (ALA), and 5,6-dimethylbenzimidazole (DMB), has been shown to increase B12 yields in other studies (Chamlagain et al., 2016, Torres et al., 2016). In the current study, these precursors were supplemented to the *L. reuteri* 92071 strain, resulting in an increase in vitamin B12 production. However, since DMB and $CoCl_2$ are not permitted for addition in food processing, it is necessary to screen food raw materials that contain high amounts of cobalt. Additionally, riboflavin and niacinamide, which acts as precursors for synthesizing DMB, should be examined *in situ* to assess whether they enhance the yield of vitamin B12.

Looking ahead, future research should aim to gain a more detailed understanding of the stability of vitamin B12 during storage within food matrices. In this study, a decrease of vitamin B12 (8.14 µg/L) was observed in the co-fermented oat-based beverage at 72 hours. Monitoring the stability of vitamin B12 over a longer post-fermentation period is recommended, with particular attention to the stability of its biologically active cobalamin forms. Investigations into shelf-life stability under different storage and packaging conditions are also required to ensure that a sufficient vitamin B12 contents are preserved until consumption.

On the other hand, the pH-value is an important factor to maintain the stability of vitamin B12. In the co-fermentation set-up, not only was vitamin B12 maintained at its maximum level at 24 hours, but the pH was also retained at 5.1, which is the minimum condition for vitamin B12 stability. However, it is important to assess whether these conditions ensure microbial safety. Further experiments, such as challenge tests food pathogens, should be conducted.

Equally important will be studies addressing the bio-accessibility and bioavailability of vitamin B12 in such fermented plant-based matrices, using for example *in vitro* digestion models to get insights about its effectiveness in preventing deficiencies.

Lastly, from a product development perspective, sensory and consumer perception research will be needed to ensure that nutritional improvements do not come at the expense of product quality or acceptance. Particularly, as *Propionibacterium*-fermented products that consumers may find undesirable and is often described as "rancid" or "cheesy." This is due to the production of volatile compounds such as short-chain fatty acids (e.g., propionic acid) and other metabolites during fermentation (Luo et al., 2021). The co-fermentation set-up might mitigate attributes and enhance the sensory profile. However, more detailed and accurate consumer research should be conducted to enable a widespread industrial adoption.

By addressing these areas, along with the knowledge obtained from this study, *in situ* fortification of vitamin B12 via fermentation in plant-based dairy alternatives can serve as valuable functional food, contributing to a more sustainable and nutritionally adequate approach within the food industry.

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8. APPENDICES

8.1. Nutrient medium composition of *Propionibacterium* broth and agar

91: Propionibacterium Agar (PPA)

Trypton (Pepton aus Casein)	VWR Chemicals Leuven, Belgium
Yeast extract	Sigma-Aldrich Steinheim, Germany
Sodium lactate 50 % ph.eur.	VWR Chemicals Leuven, Belgium
Agar	EDM Millipore (Affiliate of Merck) Darmstadt, Germany

Microorganisms DSMZ

91: PROPIONIBACTERIUM AGAR

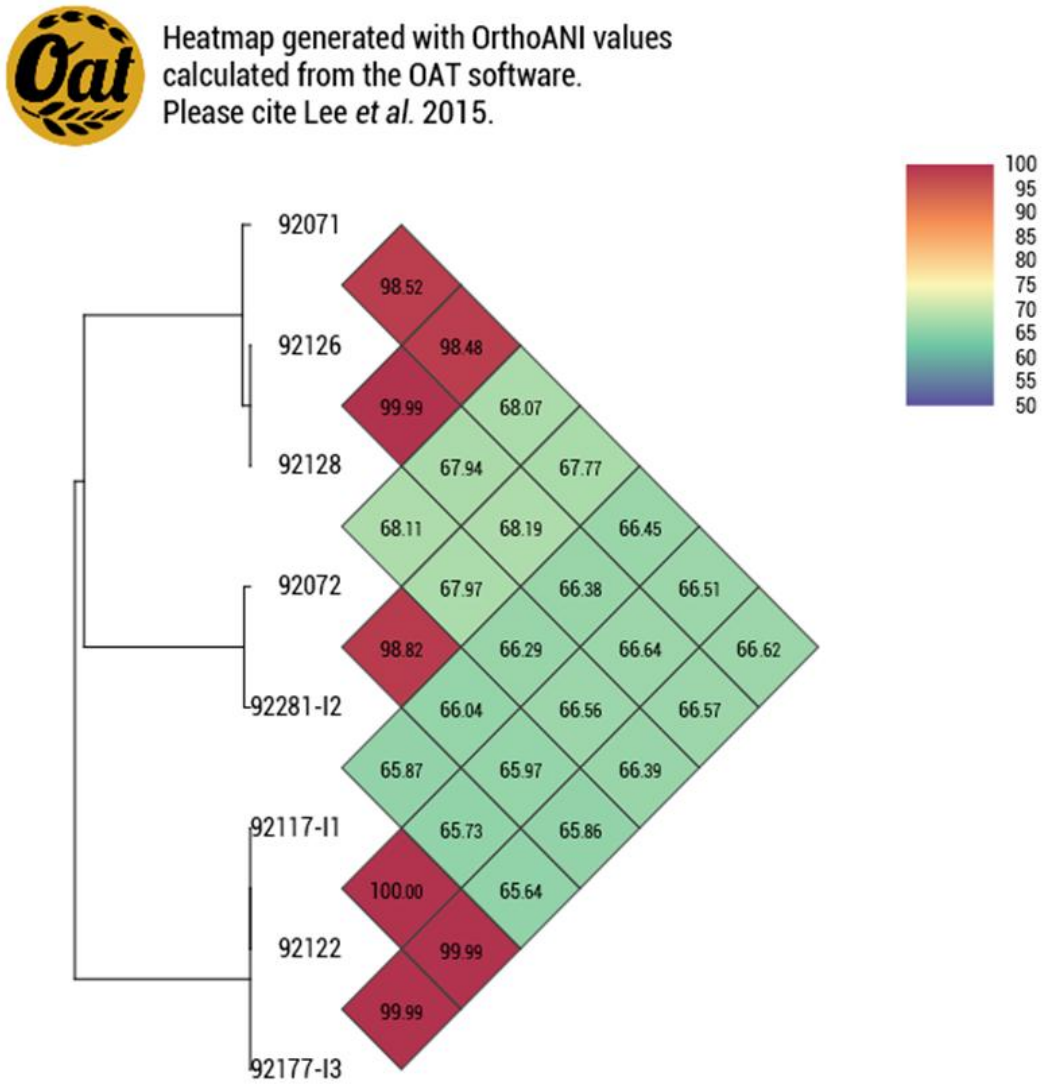
Final pH: 7.0 - 7.2
Final volume: 1000 ml

Casein peptone (tryptic digest)	10.00	g
Yeast extract	5.00	g
Na-lactate	10.00	g
Agar	15.00	g
Distilled water	1000.00	ml

Adjust pH to 7.0 - 7.2. For anaerobic handling of the liquid medium (without agar), prepare and distribute into Hungate tubes by use of the Hungate technique.

Propionibacterium Broth was produced by following the identical recipe mentioned above without the addition of agar.

8.2. Calculation of Orthologous Average Nucleotide Identity (ANI) and *In Silico* DNA-DNA Hybridization with closely related type strains (Lee et al., 2016)



Codes	Strains*
92071	*L. reuteri* 92071
92126	*L. reuteri* DSM 20016 from MRI strain collection
92128	*L. reuteri* DSM 20016 (DSMZ)
92072	*L. fermentum* 92072
92281-I2	*L. fermentum* 92281-I2
92117-I1	*L. plantarum* 92117-I1
92122	*L. plantarum* 92122
92177-I3	*L. plantarum* 92177-I3

*All the strains were from Max Rubner-Institut für Mikrobiologie und Biotechnologie (Kiel, Germany) strain collection, except *L. reuteri* DSM 20016 were obtained originally from Leibniz Institut DSMZ.