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NOVEL MICROALGAE FORMULATIONS COMPRISING LACTIC ACID BACTERIA FOR HUMAN CONSUMPTION

by

Mónica Costa Ribeiro

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfil the requirements of Master of Science degree in Applied Microbiology

by

Mónica Costa Ribeiro

Place: Escola Superior de Biotecnologia of the Universidade Católica Portuguesa

Supervisor: Professor Paula Teixeira

Co-Supervisor: Professor Rui Morais

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ABSTRACT

Hypocobalaminemia is a global health condition that has several health implications. Bacteria can synthetise *de novo* vitamin B12 whilst humans acquire it from dietary sources. To reverse vitamin B12 deficiency, microbiological alternatives have been developed to increase vitamin B12 content in food and research has focused on lactic acid bacteria (LAB) as potential cobalamin producers. *Chlorella vulgaris* is one of the most relevant eukaryotic green microalgae, taking a significant role in human nutrition. It was already reported that *C. vulgaris* can uptake exogenous cobalamin from the environment, thus being capable of being used as fortified food.

In the present work, selected strains of LAB, isolated from a *C. vulgaris* culture grown in a photobioreactor, were assessed for their cobalamin biosynthesis capacity. *Pediococcus pentosaceus* L51 presented the highest cobalamin production capacity (28.19 ± 2.27 pg mL^{-1}), followed by *Lactococcus lactis* E32 and *Lactobacillus brevis* G31 which produced, respectively, 6.18 ± 1.08 pg mL^{-1} and 5.47 ± 0.37 pg mL^{-1}. *Lactobacillus plantarum* G12 was not considered a cobalamin producer. Genes involved in cobalamin biosynthetic pathway were searched through comparative genomics. Four genes (*hemL*, *cbiT*, *cobD* and *cobC*) were identified in *Lac. lactis* E32, *L. brevis* G31 and *L. plantarum* G12 genome. Although *hemL* was not present in *P. pentosaceus* L51 genome, gene *cobA* was identified. Moreover, in this strain two genes associated with cobalamin-dependent propanediol utilization (*pduU* and *pduV*) were also identified.

Strains selected as potential cobalamin producers were lyophilized along with *C. vulgaris* culture. Addition of *P. pentosaceus* L51 grown in organic medium N formulation to *C. vulgaris* culture successfully increased vitamin B12 content in the mixed culture freeze-dried powder to a value of 522.97 ± 23.09 ng g^{-1}. Additionally, a vitamin B12 content of 10.22 ± 0.53 ng g^{-1} in a freeze-dried powder of *C. vulgaris* culture grown axenically in vitamin B12-free medium was determined. LAB survival during freeze and freeze-dried storage was studied. During frozen storage, *C. vulgaris* demonstrated a protective effect on the viability of LAB strains. However, the opposite effect was verified during storage of freeze-dried cultures. In both conditions, *P. pentosaceus* L51 has shown evidences to be more resistant than *Lac. lactis* E32 and *L. brevis* G31 to stress conditions.

Keywords: *Chlorella vulgaris*, Lactic Acid Bacteria, Probiotic, Vitamin B12

RESUMO

A hipocobalaminemia é uma condição comum a nível global que tem muitas consequências para a saúde. Apenas as bactérias têm capacidade de sintetizar vitamina B12 *de novo*, enquanto que os humanos têm de a adquirir da dieta. Para reverter a deficiência em vitamina B12 têm surgido alternativas microbiológicas e a investigação tem dado ênfase ao potencial das bactérias do ácido lático (BAL) como produtoras de cobalamina. *Chlorella vulgaris* é uma das microalgas eucarióticas mais relevantes, tendo um papel significativo na nutrição humana. A sua capacidade de adquirir cobalamina exógena do ambiente foi descrita, sendo por isso possível utilizar esta microalga em alimentos fortificados.

No presente trabalho, foram isoladas BAL a partir de uma cultura de *C. vulgaris* crescida em fotobioreatores e foi estudada a sua capacidade de biossíntese de cobalamina. *Pediococcus pentosaceus* L51 demonstrou ter a maior capacidade de produção de cobalamina (28,19 ± 2,27 pg mL^{-1}). *Lactococcus lactis* E32 e *Lactobacillus brevis* G31 produziram 6,18 ± 1,08 pg mL^{-1} e 5,47 ± 0,37 pg mL^{-1}, respetivamente, enquanto que *Lactobacillus plantarum* G12 não foi considerada produtora. Foi ainda pesquisada a presença de genes envolvidos na síntese de vitamina B12 através de uma análise genómica comparativa. Quatro genes envolvidos na via biossintética, (*hemL*, *cbiT*, *cobD* and *cobC*), foram identificados no genoma de *Lac. lactis* E32, *L. brevis* G31 and *L. plantarum* G12. Apesar do gene *hemL* não estar presente no genoma de *P. pentosaceus* L51, o gene *cobA* foi identificado. Além disso, dois genes envolvidos na utilização do propanodiol (*pduU* e *pduV*) também foram encontrados no genoma desta estirpe.

Numa segunda fase, as estirpes escolhidas como potenciais produtoras de cobalamina foram liofilizadas juntamente com uma cultura de *C. vulgaris*. A adição de cultura de *P. pentosaceus* L51, cultivada numa formulação N de meio orgânico, aumentou o teor de vitamina B12 na cultura mista liofilizada para um valor de 522,97 ± 23,09 ng g^{-1}. Além disso, foi determinado um conteúdo de vitamina B12 de 10,22 ± 0,53 ng g^{-1} numa cultura axénica liofilizada de *C. vulgaris,* cultivada num meio sem vitamina B12. Durante o armazenamento em congelação, *C. vulgaris* potenciou a viabilidade das BAL, enquanto que o efeito oposto foi verificado durante o armazenamento na forma de pó liofilizado. Nas duas condições, *P. pentosaceus* L51 mostrou ser mais resistente a condições de stresse do que *Lac. lactis* E32 e *L. brevis* G31.

Palavras-Chave: *Chlorella vulgaris*, Bactérias do Ácido Lático, Probiótico, Vitamina B12

CONTENTS

LIST OF ABBREVIATIONS

ALA	Aminolevulinic Acid
AV	Average Requirement
BBM	Bold Basal Medium
DBPC	Double Blind Randomized Placebo-Controlled
DMBI	1,3-dimethyl-2-phenyl-2,3-dihydro-1H-benzoimidazole
DRV	Dietary Reference Values
EAR	Estimated Average Requirement
FAO	Food and Agriculture Organization of the United Nations
FBCM	Food-Bound Cobalamin Malabsorption
GRAS	Generally Recognized As Safe
GMP	Guanosine 5'-monophosphate
HPLC	High Performance Liquid Chromatography
IOM	Food and Nutrition Board of the Institute of Medicine
LAB	Lactic Acid Bacteria
LOD	Limit Of Detection
MDR1	Multidrug Resistance Protein 1
MMA	Methylmalonic Acid
MRM	Multiple Reaction Monitoring
MRS	de Man Rogosa and Sharp
MSMS	Tandem Mass Spectrometry
MUFA	Monounsaturated Fatty Acids
OM	Organic Medium
PBR	Photobioreactor
PCR	Polymerase Chain Reaction
PUFA	Polyunsaturated Fatty Acids
RDA	Recommended Dietary Allowance
RNI	Recommended Nutrient Intake
SCFA	Short Chain Fatty Acids
SFA	Saturated Fatty Acids
SIR	Selected Ion Recording
WHO	World Health Organization

1. INTRODUCTION

1.1. Microalgae

Microalgae belong to a very diverse group of organisms that can live in numerous habitats and survive to challenging temperatures, salinities, pH values and light intensities. They can be classified as cyanobacteria (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), dinoflagellates (Dinophyceae) and 'pico-plankton' (Prasinophyceae and Eustigmatophyceae) and more than 40000 species have already been identified (Hu et al., 2008).

This group of microorganisms was proven to be useful not only for industrial purposes but also for clinical and environmental applications. Microalgae can be used for biofuels production, wastewater treatment and CO_2 mitigation, biofertilization, human nutrition, animal feeding, cosmetic industry and in high-value compounds, pigments and stable isotope production. Furthermore, they can synthetize antimicrobial, antiviral, antibacterial and anticancer drugs. *Arthrospira* (Spirulina), *Chaetoceros*, *Chlorella*, *Dunaliella*, and *Isochrysis* are the most widely used species (Rizwan et al., 2018).

The commercialization of microalgae started in Japan, in the beginning of the 60's, with large-scale cultivation of *Chlorella*. In the early 70's in Mexico, *Arthrospira*, known as *Spirulina,* began to be cultivated in large scale. In the 80's, *Dunaliella salina* cultivation to produce β-carotene in Australia was established as the third major microalgae industry. The facilities in Australia were followed by commercial plants in the USA and Israel. More recently, plants for *Haematococcus pluvialis* production, a source of astaxanthin, have been established in the USA and in India (Milledge, 2011, Spolaore et al., 2006, Barkia et al., 2019). After 30 years of a developing industry, by 2006, 5000 t of dry matter of microalgae biomass were produced per year, which translates in a turnover of approximately US$ 1.25×10^9 per year (Spolaore et al., 2006).

1.1.1. Microalgae Production

Photoautotrophic production is the most common method for microalgae growth since it is an economic and technically feasible method. It can be carried in open pond systems or in photobioreactors (PBRs).

The open pond systems can be classified into natural waters (lakes, lagoons and ponds) or wastewater or artificial ponds or containers. The most widely used artificial pond is called raceway pond and is constituted by a closed loop and oval shaped channel that allows recirculation, whose deep usually ranges from 0.2 to 0.5 m. They have a mixing and recirculation system to stabilize algae growth and productivity. In this system, algae broth and nutrients are added in the beginning of the raceway and circulate until the harvest spot, using a system of paddlewheels to prevent sedimentation. Although CO_2 can be provided by the surface air, a supplying system can be installed (Terry and Raymond, 1985). This method is the most cost-effective for large scale production, requiring low energy inputs and having an easy maintenance. On the other hand, since it is exposed to the environment, it has a high risk of contamination, disturbing monoculture cultivation and is susceptible to seasonal temperature differences, uncontrolled exposure to CO_2 and sunlight (Brennan and Owende, 2010, Safi et al., 2014).

Photobioreactor systems include tubular, flat plate or column reactors. They are constituted by a set of glass or plastic tubes that can be aligned in different ways, whose diameter is usually smaller than 0.1 m. The algae culture recirculates within the tubes using a mechanical pump or airlift system (Brennan and Owende, 2010). Closed photoreactors were designed as an alternative to the open pond system, to overcome some of its limitations. They are more effective since allow growth under controlled parameters, including pH, light intensity, temperature, CO_2 concentration, achieving higher biomass productivity, and are suitable for culturing sensitive species since they are designed and optimized considering the strain that will be cultivated. Moreover, these systems are less susceptible to contaminations and have more light availability. However, this system implies high design and operation costs (Safi et al., 2014, Narala et al., 2016).

Microalgae can also be grown in heterotrophic systems, where sunlight is not required and the biomass is fed by supplying organic carbon sources, such as glucose, acetate, glycerol and glutamate (Safi et al., 2014). In this case, the culture is grown in a

stirred tank reactor or fermenter which offers more scale up opportunities due to a smaller surface volume ratio. These systems are advantageous since they allow more growth control, and to achieve higher cells densities, reducing the harvesting costs (Brennan and Owende, 2010). Sugars availability can be a disadvantage and increase the production costs (Brennan and Owende, 2010, Barros et al., 2019).

Scale-up represents a costly and time-consuming process for the production of microalgae. Since some microalgae species, such as *C. vulgaris*, are capable of growing heterotrophically and the heterotrophic production improves growth performance, these systems can be used to seed PBRs or raceways, providing high quality inocula, which results in a reduction of time and occupancy area (Barros et al., 2019).

1.1.2. *Chlorella vulgaris* and its Role in Nutrition

The eukaryotic green microalgae, *C. vulgaris,* is one of the most relevant microalgae. It was discovered in 1890 by Martinus Willem Beijerinck and was described as the first microalgae with a well-defined nucleus (Safi et al., 2014).

Chlorella vulgaris has a spherical cell morphology, with 2-10 μm of diameter. Its cell wall rigidity and composition can differ between each growth phase, and even when the cell is finally mature, it can vary according to environmental conditions. These cells cytoplasm is composed by water, soluble proteins and minerals. Additionally, it harbours several organelles, including mitochondria and a single chloroplast. This microorganism presents asexual reproduction and multiplies by auto-sporulation (Safi et al., 2014).

Microalgae can play a significant role in human nutrition since they are a good source of carbohydrates, proteins, lipids, nucleic acids, vitamins and minerals and each molecule content is highly related to the algae strain. Microalgae protein content can compete with conventional protein sources and are reported to possess very high contents, that can vary from 42 to over 70% (Barkia et al., 2019). *Chlorella vulgaris* has a protein content that varies from 51 to 58% of dry matter and an amino acid profile that equals high-quality proteins, namely lactoglobulin, egg albumin, and soy (Becker, 2007).

Carbohydrates are represented by reducing sugars and polysaccharides that include starch and cellulose (Safi et al., 2014). The content of carbohydrates in *C. vulgaris* varies from 12 to 17% of dry matter (Becker, 2007) and starch is the most abundant carbohydrate (Safi et al., 2014).

The lipid fraction of microalgae receives more commercial attention. Microalgae are usually composed by C16 and C18 saturated and unsaturated fatty acids and some fatty acids with longer carbon chains, such as omega fatty acids (Barkia et al., 2019). Humans and higher plants are unable to synthetize polyunsaturated fatty acids (PUFA) of more than 18 carbons and they are obtained from food sources, such as fish and fish oil which can present a risk due to possible accumulation of toxins. Microalgae can represent an alternative for the obtention of these essential fatty acids (Spolaore et al., 2006). *Chlorella vulgaris* has a lipid content of 14 to 22% of dry matter (Becker, 2007) and the analysis of its lipid fraction content showed that 31% are saturated fatty acids (SFA), mainly palmitic acid, 21% are monounsaturated fatty acids (MUFA), mainly oleic acid 18:1ω9, and 35% PUFA, with predominance of ω3 acids (Batista et al., 2013).

Carotenoids such as β-carotene, astaxanthin, lutein, zeaxanthin, lycopene and bixin are used commercially. They are used as food colorants, as additives for animal feed and also have applications in cosmetics. They also have a therapeutic relevance since they can function as provitamin A to be converted in vitamin B and have anti-inflammatory properties (Spolaore et al., 2006). *Chlorella vulgaris* pigment analysis showed a total pigment content of 1.2%. Lutein and zeaxanthin were the main carotenoids found and it was observed the presence of both Chlorophyll a and b, typical of Chloropyceae (Batista et al., 2013).

1.2. Lactic Acid Bacteria

Lactic acid bacteria (LAB) were found in 1873, when Joseph Lister isolated a bacterial pure culture, *Bacterium lactis*, that was later renamed to *Lactococcus lactis*. This species was used mainly in milk fermentation process and the first works with LAB were essentially associated with dairy products. Over time, commercial starter cultures were

developed. Moreover, it was discovered that LAB were spread in more habitats (Narvhus and Axelsson, 2003).

The LAB group comprises 16 genera within the phylum Firmicutes that include *Streptococcus*, *Lactococcus*, *Vagococcus*, *Enterococus*, *Leuconostoc*, *Oenococcus*, *Weissela*, *Lactobacillus*, *Carnobacterium*, *Pediococcus*, *Tetragenococcus* and *Aerococcus* (Hatti-Kaul et al., 2018, Narvhus and Axelsson, 2003). Bacteria from this group are Gram-positive, non-spore formers and most of the species are non-motile. They are non-respiring, since they are incapable of producing porphyrin groups, and are catalase negative. The G+C content is under 50% and morphologically they can be cocci, coccobacilli and rods, with exception of *Aerococcus*, *Pediococcus* and *Tetragenococcus* that form tetrads (Narvhus and Axelsson, 2003).

The nutritional requirements are complex and the presence of a carbohydrate suitable for fermentation is imperative to active growth. The product of fermentation is lactic acid and it can be produced through a homofermentative pathway, producing more than 95% of lactic acid, or through a heterofermentative pathway, with the additional production of acetic acid, ethanol and carbon dioxide and, in limiting conditions, mixed fermentation can occur (Narvhus and Axelsson, 2003).

Although the anaerobic *Bifidobacterium* genus (phylum Actinobacteria) is phylogenetically and physiologically unrelated to the genus previously referred, it is also a member of the LAB group, being considered a probiotic (Narvhus and Axelsson, 2003, Hatti-Kaul et al., 2018).

1.2.1. Probiotic Lactic Acid Bacteria

Probiotics are living microorganisms that potentially have a beneficial effect in human and animal health if consumed in an adequate dose and these benefits are strain specific. They were likely introduced in human nutrition trough fermented foods and today, their consumption is associated with fermented milk, powder, capsule or tablet forms (Anadón et al., 2016).

Probiotic microorganisms mainly belong to LAB group, being *Lactobacillus* and *Bifidobacterium* the most common genera (Anadón et al., 2016). Some probiotic LAB species are mentioned in Table 1.1.

Table 1.1 List of common probiotic lactic acid bacteria (Anadón et al., 2016).

Phylum	Genus	Species
Firmicutes	*Lactobacillus*	*L. acidophilus, L. amylovorus, L. brevis, L. bulgaricus, L. casei, L. cellobiosus, L. crispatus, L. curvatus, L. delbrueckii spp. bulgaris, L. fermentum, L. gallinarum, L. helveticus, L. johnsonii, L. lactis, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus*
	Streptococcus	*Streptococcus salivaris spp. thermophiles*
	Lactococcus	*Lac. lactis cremoris*
	Leuconostoc	*L. mesenteroides*
	Pediococcus	*P. pentosaceus, P. acidilactici*
Actinobacteria	*Bifidobacterium*	*B. adolescentis, B. animalis, B. bifidum, B. breve, B. essensis, B. infantis, B. laterosporum, B. thermophilum, B. longum*
Firmicutes	*Enterococcus*	*E. fecalis, E. faecium*

The identification of probiotic species is a complex process and a set of guidelines have been proposed to guaranty their safety. These include strain identification, functional characterization and safety assessment with *in vitro* testing, animal and human studies, and finally, double blind, randomized, placebo-controlled (DBPC) testing to validate efficacy. Microorganisms should be able to survive to the passage through gastrointestinal tract, at low pH and in the presence of bile salts, adhere to the intestinal epithelial cells and stabilize the intestinal microbiota. They must be non-pathogenic, capable of surviving in foodstuffs and in pharmacopoeia lyophilized preparations, have a fast multiplication, whether with permanent or temporary colonization of the gastrointestinal tract. Finally, they need to have generic specificity (FAO/WHO, 2002).

The use of probiotics in clinical health is a promising area since they have a vast set of beneficial effects. The first benefit to be presented is anti-pathogenic activity that has high relevance since unlike antibiotics, probiotics have not a negative effect in the gut microbiota balance. Probiotic microorganisms produce short chain fatty acids (SCFA),

that are involved in the maintenance of colonic lumen optimal pH, bacteriocins and peptides, that interfere with membrane permeability, ethanol, organic acids, diacetyl, acetaldehydes and hydrogen peroxide (H_2O_2), that is involved in oxidation mechanisms. Moreover, their presence stimulates host defence pathways such as defensins and cationic peptides production pathways. Finally, they compete with pathogens for nutrients and growth (Kerry et al., 2018).

1.3. Vitamin B12

The term vitamin B12 refers to all cobalt corrinoid compounds, including those from cobalamin group. Vitamin B12 was discovered in early 1920, by two American physicians, Minot and Murphy, as a cure for a liver disorder named pernicious anaemia, and they referred it as an "extrinsic factor" (Minot and Murphy, 1926). After 20 years of research the "extrinsic factor" was finally isolated from the liver as a red crystalline compound by two pharmaceutical companies, one directed by Folkers at Merck in the USA, and the other by Smith at Glaxo in the UK, and it was designated vitamin B12 (Rickes et al., 1948). Vitamin B12 was also identified in milk powder, beef extract, and in several bacteria genera culture broths (Rickes et al., 1948).

After these discoveries, a biologically active coenzyme form of pseudo-vitamin B12 was crystallized (Barker et al., 1958). Then, the three-dimensional structure of vitamin B12 (cyanocobalamin) was determined and five years later, the structure of coenzyme B12 (adenosylcobalamin) (Hodgkin et al., 1955, Hodgkin et al., 1956, Hodgkin et al., 1957, Lenhert and Hodgkin, 1961). A second biologically active form of vitamin B12, methylcobalamin (MeCbl) was also determined and this form is a cofactor of methionine synthase (Guest et al., 1962). Following these findings, more cobalamin-dependent enzymes and their action mechanisms have been identified and studied (Martens et al., 2002).

1.3.1. Chemical Structure and Properties

The term vitamin B12 represents, as previously mentioned, a cobalt corrinoid family, particularly the cobalamin group, and is widely found in two naturally occurring forms that are referred to as coenzyme B12: adenosylcobalamin (AdoCbl) (Figure 1.1) and methylcobalamin (MeCbl). Coenzyme B12 has a molecular mass of 1,580 Da. Commonly, vitamin B12 also refers to cyanocobalamin (CNCbl), an industrially produced form, and the cyano group (CN) is obtained through an extraction procedure that removes this compound from bacterial cultures (Martens et al., 2002).

This molecule is constituted by a central element, a corrin ring containing four pyrrole ligands. The Cobalt (III) ion is chelated by nitrogen atoms donated by the four pyrrole rings. Two additional ligands are covalently linked to the cobalt ion, a lower (alpha) ligand, which is a N7-atom donated from 1,3-dimethyl-2-phenyl-2,3-dihydro-1H-benzoimidazole (DMBI), and an upper (beta) ligand, that can be a cyano, adenosyl, methyl or hydroxy group, forming different analogues. All the acetamide groups are projected in the upper ligand direction, while all the propionamide groups are projected in the benziminazole group direction (Hodgkin et al., 1956, Martens et al., 2002). Pseudovitamin B12, a biologically inactive form, occurs when instead of DMBI, adenine is installed as a lower ligand (Anderson et al., 2008, Stupperich and Nexø, 1991).

Vitamin B12 appears in the form of dark red crystals or as a red crystalline powder, being odourless and tasteless. Its melting point exceeds 300ºC and when exposed to air it can absorb up to 12% of water, being hygroscopic (Laird and Molloy, 2014). In aqueous solution it is stable in a range of pH values between 4.0 to 6.5 (Taub and Lieberman, 1953). Concerning stability, cyanocobalamin is the most stable form of the vitamin B12 analogues. It can be submitted to 120 ºC during autoclave sterilization, is relatively stable at 100 ºC for a few hours and is also stable in both air and dry forms. Methylcobalamin and adenosylcobalamin are stable in aqueous solution in dark conditions and can be submitted to 100 ºC for 20 min (Jägerstad and Arkbåge, 2003). In acidic or alkaline conditions, hydrolysis of the propionamide and acetamide side chains of vitamin B12 to carboxylic acid groups can occur, originating inactive products (Schnellbaecher et al., 2019). In highly acidic environment, hydrolysis of the phosphate bonds originates cobamide and DMBI, which can be further degraded into other products

(Bonnett, 1963). At alkaline pH values and elevated temperatures, cyclization of the acetamide side chain of the B ring generates a γ-lactam, the biologically inactive dehydrovitamin B12 (Bonnett et al., 1957).

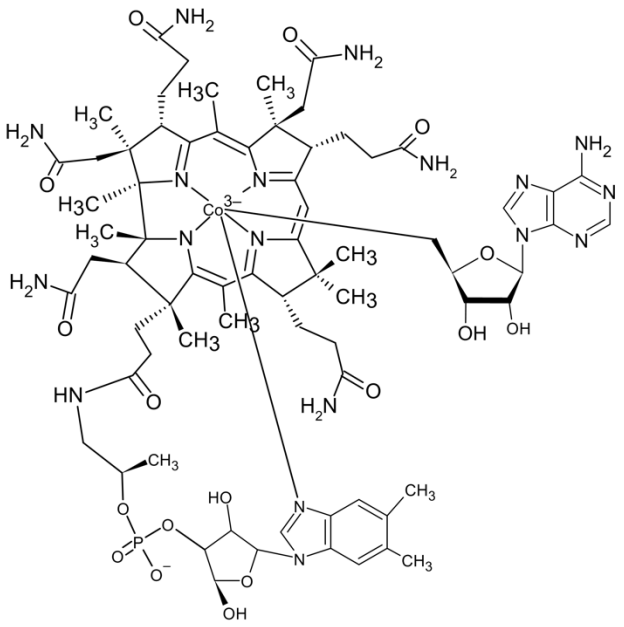


Figure 1.1. Chemical structure of coenzyme B12 (adenosylcobalamin); chemical structure (designed using Advanced Chemistry Development, Inc. (ACD)/ChemSketch; ACD/Labs 2019.2.1, Canada).

All forms of cobalamin are sensitive to ultraviolet and visible light (Demerre and Wilson, 1956). In aqueous solution, cyanocobalamin is photolyzed to the most photostable form, hydroxocobalamin, and in seconds of UV-A exposure, the biologically active forms of cobalamin, methylcobalamin and adenosylcobalamin, are converted to hydroxocobalamin as well (Juzeniene and Nizauskaite, 2013, Ahmad et al., 1992). Thiamine was described as a vitamin B12 destroying agent. At high temperature and pH values, thiamine degradation products induce cobalamin degradation (Feller and Macek, 1955). Moreover, the highest level of degradation occurs when thiamine and niacinamide are present simultaneously, since niacinamide induces the release of a substance from thiamine that interferes with cobalamin stability (Blitz et al., 1956). Finally, heavy metals, strong oxidizing and reducing agents can also affect the natural forms of cobalamins, including hydroxo-, aqua-, methyl-, or adenosylcobalamin (Jägerstad and Arkbåge, 2003).

1.3.2. Biosynthetic Pathway

1.3.2.1. Vitamin B12 De Novo and Salvage Pathways

The biosynthesis of vitamin B12 can occur through the *de novo* or salvage pathways. Within *de novo* pathway, vitamin B12 can be synthetized via an oxygen-dependent or an oxygen-independent pathway. The aerobic mechanism may be found in microorganisms such as *Pseudomonas denitrificans* while the anaerobic mechanism may be found in *Bacillus megaterium*, *Propionibacterium shermanii* and *Salmonella* Typhimurium (Martens et al., 2002). At least 30 genes are involved in this vitamin *de novo* synthesis (Roth et al., 1993).

The vitamin synthesis begins in the tetrapyrrole synthesis pathway, with 5-aminolevulinic acid (ALA), synthesized either via C4 or C5 pathway, as a first committed precursor. Two molecules of ALA are then condensed to form porphobilinogen and this reaction is followed by the polymerization and cyclization of four porphobilinogen molecules, forming uroporphyrinogen III (Fang et al., 2017, Martens et al., 2002).

In both aerobic and anaerobic mechanisms, uroporphyrinogen III is converted into precorrin-2 through a C-2 and C-7 methylation (Martens et al., 2002). This reaction is catalysed by a methyltransferase encoded by gene *cobA* (Warren et al., 2002). From this point on the pathways diverge since the cobalt ion is added at different phases (Raux et al., 1999, Martens et al., 2002). In the oxygen- independent pathway, cobalt ion is inserted into precorrin-2 (Martens et al., 2002). Chelatase CysG or independent precorrin-2 cobalt chelatase CbiK can catalyse this reaction in *Salmonella enterica*, and cobaltochelatase CbiX in *B. megaterium* (Warren et al., 2002). This early insertion results in a high number of cobalt-complexes as intermediates (Martens et al., 2002). The chelation in the oxygen-dependent pathway occurs nine steps later, and hydrogenobyrinic acid a,c-diamide is the substrate of a two component system cobalt chelatase that includes a fist component, CobN protein, and a second, CobS and T complex (Martens et al., 2002, Warren et al., 2002).

The ring contraction and subsequent C-20 removal in both pathways also presents differences. In the oxygen-dependent pathway, C-20 atom of precorrin 3-A is oxidized by molecular oxygen with release of C-20 as acetate, in a reaction catalysed by CobG protein, whereas in the oxygen-independent pathway the complexed cobalt ion may

assume different valence states and allows oxidation and the C-20 atom is released as acetaldehyde. The two pathways converge when adenosylcobyric acid is converted into adenosylcobinamide (Martens et al., 2002). The last step involves the formation of an α-ribazole through the transference of a phosphoribosyl residue of nicotinic acid mononucleotide to DMBI, which is posteriorly linked to GDP-adenosylcobinamide. Guanosine 5'-monophosphate (GMP) is released in this reaction, giving origin to a coenzyme B12 molecule (Zayas and Escalante-Semerena, 2007, Martens et al., 2002).

The salvage pathway is a system that allows bacteria and archaea to salvage exogenous cobinamide, being a more cost-effective way to produce cobalamin. *Escherichia coli* is an example of a microorganism that has lost *de novo* cobalamin production capacity across evolution but can synthetize it through the salvage pathway (Fang et al., 2017). Gram-negative bacteria have a highly specific BtuBFCD system which translocates exogenous corrinoids from the environment, in an effective way. BtuB protein is a Ca-dependent transporter located in the outer membrane and its function is to deliver the corrinoid to BtuF protein, a periplasmic corrinoid-binding. BtuF delivers cobinamide to the ABC transporter, BtuCD, located in the inner membrane. Once inside the cell, cobinamide is adenosylated and converted into adenosylcobinamide. In bacteria, adenosylcobinamide serves as substrate for a bifunctional enzyme detaining kinase and guanylyltransferase activities (CobU, in S. Typhimurium or CobP, in *P. denitrificans*), and adenosylcobinamide-GDP is generated. The last two steps necessary for the production of adenosylcobalamin are identical to the *de novo* pathway (Escalante-Semerena, 2007).

The vitamin B12 *de novo* and salvage pathways are represented in Figure 1.2.

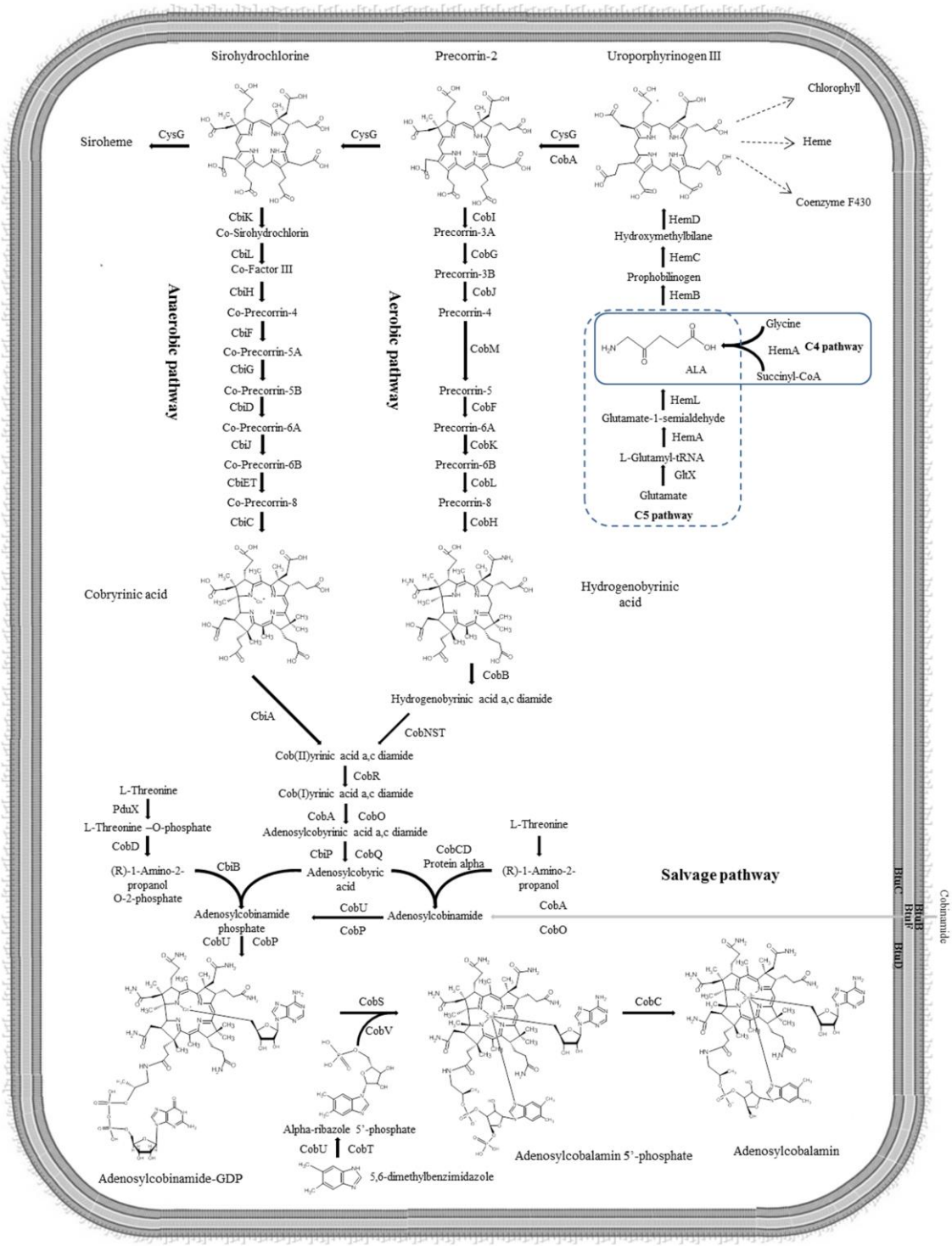


Figure 1.2. Vitamin B12 *de novo* and salvage pathways (designed using ACD/ChemSketch; ACD/Labs 2019.2.1; Adapted from Fang et al. (2017).

1.3.2.2. Biosynthesis Regulation

Cobalamin biosynthesis is mainly regulated by riboswitches. Riboswitches are RNA elements situated in 5′-UTR of specific prokaryotic mRNAs that modulate the expression of certain genes in response to changing concentrations of metabolites. These regulatory elements are constituted by two functional structural domains, in which one serves as a natural aptamer, binding the target metabolite with high selectivity, and the other domain is an expression platform that suffers allosteric changes in RNA structure to control expression of the adjacent gene or operon (Nahvi et al., 2004). The mechanism of inhibition is activated when coenzyme-B12 concentrations are high. In that case, the nascent mRNA binds to a coenzyme-B12 molecule which induces an allosteric change in structure. This change allows the intrinsic terminator stem to form, the coding region of the mRNA is not generated and transcription stops at that point (Mandal and Breaker, 2004).

Riboswitches may be interesting targets for enhancing vitamin B12 production. Propionibacterium strain UF1 abundantly produces vitamin B12, and the biosynthesis is regulated by VB12, being regulated within the *cobA* operon through the *cbiMCbl* riboswitch. It was demonstrated that the ablation of the *cbiMCbl* riboswitch increased the production of vitamin B12 (Li et al., 2020).

1.3.3. Vitamin B12 Production

It was necessary more than 10 years and 100 researchers to achieve vitamin B12 full chemical synthesis, by Woodward and Eschenmoser in 1973. Vitamin B12 is commercially produced through fermentation processes using selected genetically modified microorganisms. The chemical synthesis of this compound is not a viable process since it evolves 70 steps, being highly complex and expensive (Martens et al., 2002).

To obtain high vitamin B12 production yields a random mutagenesis strategy is used being obtained by submitting microorganisms to mutagenic agents that comprise UV light, ethyleneimine, nitrosomethyluretane or N-methyl-N′-nitro-N-nitrosoguanidine and

finally, selecting the most suitable strains. *Propionibacterium freudenreichii*, *Rhodopseudomonas protamicus*, *Prop. shermanii*, *Ps. denitrificans*, *Nocardia rugosa*, and *Rhizobium cobalaminogenum* are well characterized vitamin B12 producers (Kang et al., 2012, Bykhovskii et al., 1998).

Since *Prop. shermanii* and *Ps. denitrificans* strains produce vitamin B12 in high levels, these bacteria are frequently used in industrial production. *Propionibacterium* strains have the advantage of possessing a generally recognized as safe (GRAS) status. Nonetheless, *Ps. denitrificans* is more used in industrial settings (Martens et al., 2002).

The *Propionibacterium* strains employed in vitamin B12 production are microaerophilic and high yields are obtained when low oxygen concentrations are used. The fermentation is divided in two phases: in the first three days bacteria grow anaerobically to produce the vitamin B12 precursor cobamide (vitamin B12 without DMBI) and in the next one to three days aeration is initiated to allow the production of DMBI which will be linked to cobamide. During this process, it is necessary to neutralize propionic acid to stabilize the pH value at 7. In the process using *Ps. denitrificans*, which has an oxygen-dependent growth, the culture is aerated throughout all the fermentation process (Eggersdorfer, 1996).

1.3.4. Metabolic Engineering

Large scale industrial production of cobalamin occurs through fermentation. The most used strains have some setbacks, for instance, they often require long fermentation cycles, along with complex and expensive culture media. Most research has focused in optimizing the fermentation process and employed mainly traditional methods, such as random mutagenesis. Therefore, there is the need to find suitable genetics systems for strain engineering and to extend the knowledge in metabolic engineering (Fang et al., 2017).

Some techniques can be used to enhance cobalamin production, including gene over-expression, to enhance a desired chemical reaction, gene downregulation or inactivation, to improve precursor supply or remove by-products and competing reactions, and protein engineering, to improve enzymes specificity, solubility and stability, limiting inhibition by a determined compound or product (Fang et al., 2017). These techniques can be

applied to strains with commercial value, and some studies have already been performed in *Prop. freudenreichii* in order to enhance cobalamin production through gene over-expression and introduction of heterologous genes (Piao et al., 2004a, Piao et al., 2004b).

The construction of heterologous biosynthetic pathways using model organisms can also be a promising strategy to produce cobalamin. *Escherichia coli* has been widely employed as a microbial cell factory and it may be used as host for vitamin B12 heterologous production (Fang et al., 2017). The *de novo* cobalamin synthesis in *E. coli* has already been reported in a few studies (Fang et al., 2018, Raux et al., 1996, Ko et al., 2014).

1.3.5. Vitamin B12 Biosynthesis by Lactic Acid Bacteria

The first LAB found to produce vitamin B12 was *Lactobacillus reuteri* CRL 1098, since this strain was described as capable of metabolizing glycerol in a B12 free medium (Taranto et al., 2003). The compound produced by this strain was identified as pseudocobalamin, a vitamin B12 analogue (Santos et al., 2007). Furthermore, other strains including *L. reuteri* DCM 20016 (Santos et al., 2008), *L. reuteri* JCM 1112, *L. reuteri* CRL 1324 and 1327 (strains isolated from human vagina) were identified as corrinoids producers (LeBlanc et al., 2011).

The genomic sequences of two other *L. reuteri* strains were unveiled. *L. reuteri* F275 (type strain DSM 20016), isolated from human faeces, contained the cobalamin biosynthesis cluster associated with glycerol anaerobic catabolism, whereas *L. reuteri* 100-23, isolated from the mouse intestine, did not comprise the *cob* or the glycerol metabolism genes. Of the LAB genomes studied thus far, only *L. reuteri* DSM 20016/JCM 1112 and the Biogaia strain *L. reuteri* ATCC 55730 contained *pdu-cob* genes. Moreover, preliminary studies indicated that the *pdu-cob* cluster is present in *Lactobacillus coryniformis* and *Lactobacillus murinus* (LeBlanc et al., 2011). Some cobalamin biosynthesis genes were also shown to be present in *Lactobacillus buchneri* ATCC 11577, *Lactobacillus hilgardii* ATCC 8290, *L. coryniformis* KCTC 3167, *L. coryniformis* KCTC 3535 and *Lactobacillus brevis* ATCC 27305 strains (Capozzi et al., 2012).

It has already been shown that *L. coryniformis* strains CECT 571 and 394, isolated from goat milk, could produce reuterin through an enzymatic pathway that requires the presence of the cobalamin-dependent glycerol dehydratase enzyme. Moreover, *L. coryniformis* strain CECT 571 could also synthesize cobalamin (Martín et al., 2005). Considering these findings, a study was performed using *L. coryniformis* CRL 1001 strain, in which it was observed that the testing strain could produce corrinoids with cobalamin activity, mainly pseudocobalamin. Furthermore, it was possible to conclude, through genomic studies, that the complete anaerobic biosynthesis pathway of coenzyme B12 was present. This was the first facultative heterofermentative strain described with biosynthetic capacity (Torres et al., 2016).

In a previous study, in which LAB isolated from Japanese pickles were investigated, *L. coryniformis* and *L. plantarum* strains revealed a vitamin B12 production of 1.8 and 2.0 mg L^{-1}, correspondingly, in a vitamin B12-free medium. In addition, one strain of *P. pentosaceus* and another of *L. brevis* also showed production capacity, even though at lower levels (0.4–0.6 mg L^{-1}) (Masuda et al., 2012). *L. plantarum* strains had already demonstrated vitamin B12 production potential, since it was observed that strains isolated from the fermented food kanjika could synthetize this vitamin (Madhu et al., 2010). In a phenotypic and genotypic screening of human-originated lactobacilli concerning vitamin B12 production capacity, two *L. plantarum* strains (BHM10, isolated from human milk, and BCF20, isolated from child faeces) were identified as producers after the *cbiK* gene was detected (Bhushan et al., 2016).

The research for more cobalamin producers has contributed to the expansion of this group to new species. A strain isolated from healthy breast-fed infants fresh faecal samples, identified as *L. fermentum* CFR 2195, revealed vitamin B12 production capacity, achieving a yield of 29.45 $ng_{\text{vitamin B12}}$ g^{-1} of the dry biomass (Basavanna and Prapulla, 2013). Furthermore, a gene cluster involved in cobalamin biosynthesis was identified in *Lactobacillus rossiae* DSM 15814, with high degree of homology with the *L. reuteri* 20026 cluster. This suggests that this strain has vitamin B12 *de novo* synthesis capacity (De Angelis et al., 2014).

Enterococcus spp. with cobalamin production capacity were recently reported. Eight vitamin B12 producing strains were isolated from infant faeces, with five belonging to *Enterococcus* genus. Three isolates were identified as *E. faecium* and two as *Enterococcus faecalis*. Of the remaining isolates, two corresponded to *L. casei* and one to *L. plantarum*. The results revealed that *E. faecalis* LZ83 and *E. faecium* LZ86 strains

presented the highest cobalamin production and phylogenetic analysis revealed high similarity with *E. faecalis* ATCC 19433 and *E. faecium* ATCC 19434 commercial strains, correspondingly. Both strains from the study have shown to produce active forms of cobalamin (Li et al., 2017).

1.3.6. Vitamin B12 Auxotrophy in Microalgae

Over 40 years, vitamin B requirements of different species of microalgae have been investigated and more than half of the surveyed species are cobalamin-dependent. Moreover, this requirement is widespread in unrelated phyla, which suggested that auxotrophy appeared independently trough evolution (Croft et al., 2006).

Cobalamin has a major impact in methionine metabolism, that was found to be different between cobalamin-dependent and -independent algae. In the first group, vitamin B12-dependent methionine synthase genes (*metH*) are present, while in the other group expression of vitamin B12-independent methionine synthase genes (*metE*) occurs. However, some species, such as *Chlamydomonas reinhardtii*, can express both methionine synthase isoforms. In this scenario, *metH* is expressed both in the presence and in the absence of vitamin B12, while *metE* is only expressed in its absence. This indicates that the B12-dependent form of methionine synthase is preferred, since it has a higher catalysis rate (Croft et al., 2005, Helliwell et al., 2011).

Considering that microalgae are not capable of producing cobalamin, they must acquire it from the environment. At least 10 ng L^{-1} of vitamin B12 is necessary to allow growth. However, the level of cobalamin is 2–6 ng L^{-1} in fresh water and 3 ng L^{-1} in sea water. These cobalamin levels do not support algae growth, which indicates that they must obtain it from bacteria, trough symbiotic relations (Croft et al., 2005).

An evidence of these findings is the capability of *Halomonas* spp. to provide vitamin B12 to red alga *Porphyridium purpureum* and dinoflagellate *Amphidinium operculatum*, supporting their growth (Croft et al., 2005). *Dinoroseobacter shibae* hability to deliver, not only vitamin B12, but also vitamin B1 (thiamine), is another symbiosis example (Wagner-Dobler et al., 2010). Finally, a mutualism relation

established by the green algae *Lobomonas rostrata* and the bacterium *Mesorhizobium loti* was also described. It has been shown that the algae could not grow in the absence of the symbiotic bacterium or vitamin B12. Furthermore, the bacterium was dependent on the algae as a carbon source (Kazamia et al., 2012).

It is generally accepted that vitamin B12 is produced only by prokaryotic microorganisms. However, a study performed by Watanabe *et al*. (1991) demonstrated that the green algae *C. reinhardtii* cells, grown axenically in cobalamin-free Allen's medium, contained cobalamin. This is an evidence that *C. reinhardtii* is not only capable of accumulating cobalamin, but also to perform its *de novo* production (Watanabe et al., 1991).

1.3.6.1. *Chlorella vulgaris* as a Vitamin B12 Carrier

Chlorella spp. cells are used in dietary supplementation. *Chlorella* tablets contain substantial quantities of B12. The content can vary between <0.1 to approximately 415 μg/ 100 g dry weight (Bito et al., 2016, Watanabe and Bito, 2018b).

Many *Chlorella* species, including *C. vulgaris,* were already shown to not require vitamin B12 to grow (Croft et al., 2005). Nonetheless, in an experiment performed by Watanabe *et al.* (1997), it was demonstrated that *Chlorella* cells have the capability to uptake exogenous vitamin B12. Results obtained in the same experiment also showed that cyanocobalamin is not required for growth of *C. vulgaris* cells being, however, capable of stimulating it. Finally, it was observed that cells grown in a cyanocobalamin-free medium contained biologically active cobalamin analogues, which suggested that *C. vulgaris* cells have *de novo* cobalamin coenzymes synthesis capacity (Watanabe et al., 1997).

In order to determine which form of vitamin B12 was present in *C. vulgaris*, Kumudha et al. (2015) developed an experiment where *C. vulgaris* cells were grown in vitamin B12-free bold basal media (BBM). Different techniques, including tandem mass spectrometry (MSMS), selected ion recording (SIR), multiple reaction monitoring (MRM), gold nanoparticle (AuNPs) and RNA aptamer, high performance liquid

chromatography (HPLC), microbiological assay, and chemiluminescence were used and methylcobalamin was confirmed to be the existing form of the vitamin.

1.3.7. Biological Activity in Bacteria and Archaea

Vitamin B12 has a role in reactions that occur exclusively in bacteria and archaea which are mostly relevant to support the anaerobic growth of these microorganisms. Essentially, this molecule intervenes in the catalysis of molecular rearrangements that balance redox reactions (Roth et al., 1996).

With the exception of *E. coli*, in enteric bacteria vitamin B12 is necessary for 1,2-propanediol, ethanolamine and glycerol fermentation. These substrates suffer an intramolecular rearrangement to form an aldehyde and these reactions are performed by adenosylcobalamin-dependent enzymes. Propanediol dehydratase converts 1,2-propanediol to propionaldehyde. Ethanolamine ammonia lyase converts ethanolamine to acetaldehyde and ammonia and in some situations, acetaldehyde can be a carbon and energy source through acyl-CoA. Glycerol dehydratase converts glycerol to β-hydroxypropionaldehyde and this reaction balances the reducing character of the glycerol dehydrogenase catalysed reaction (Roth et al., 1996, Martens et al., 2002).

This vitamin is involved in other B12-dependent reactions such as acetyl-CoA synthesis via the Wood/Ljungdahl pathway, where acetyl-CoA is synthesized from two CO_2 molecules. It mediates the transference of methyl groups from methyltetrahydrofolate to CO dehydrogenase. It also participates in reactions essential for DNA synthesis, specifically the ones mediated by adenosylcobalamin-dependent reductases (class II) that provide free-radicals. In methane-producing archaea, corrinoids allow the transference of methyl groups from methanogenic substrates (methanol, methylamines and acetate) to coenzyme M thiol group (Martens et al., 2002, Roth et al., 1996).

1.3.8. Biological Activity in Humans and Animals

Vitamin B12 presents a biological role in humans and animals since it is required by two enzymes, namely, (R)-methylmalonyl-CoA mutase and methionine synthase (Martens et al., 2002).

The enzyme (R)-methylmalonyl-CoA mutase intervenes in propionyl-CoA metabolism that is obtained through degradation of thymine, valine, methionine and odd-chain fatty acids. In this process, propionyl-CoA suffers a carboxylation to (S)-methylmalonyl-CoA form and is epimerized to the (R)-methylmalonyl-CoA. A rearrangement performed by adenosylcobalamin-dependent (R)-methylmalonyl-CoA mutase occurs and succinyl-CoA is formed, being integrated in the tricarboxylic acid cycle. When vitamin B12 is absent, methylmalonylCoA intermediate accumulates and a hydrolase cleaves the D-isomer to coenzyme A and methylmalonic acid, originating acidosis. On the other hand, propionyl-CoA levels increase and it condenses with oxaloacetic acid to form 2-methylcitric acid, an aconitase inhibitor (Martens et al., 2002).

Methionine synthase, a MeCbl-dependent enzyme, catalyzes homocysteine methylation through the transference of a methyl group from 5-methyltetrahydrofolate, originating methionine and tetrahydrofolate (Banerjee and Matthews, 1990). In cases of vitamin B12 deficiency, methionine levels remain normal, which may be explained by an increased activity of an enzyme betaine-homocysteine methyltransferase, that neither requires cobalamin nor folate (Allen et al., 1993). Although the level of methionine is unchangeable, the deficiency of this vitamin induces the increase of methyltetrahydrofolate and the decrease of tetrahydrofolate levels. This results in low levels of folates that are used in DNA synthesis and it is called methylfolate trap. These events have been related with diseases like megaloblastic anaemia (Martens et al., 2002).

1.3.9. Dietary Requirements and Deficiency in Humans

The Recommended Dietary Allowance (RDA) for vitamin B12 is defined as the necessary amount of vitamin to satisfy the necessities of healthy individuals in a specific stage of life. The RDA for adults and other age groups was established by the Food and

Nutrition Board of the Institute of Medicine (IOM) (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, 1998). The RDA for different age groups and specific conditions is presented in Table 1.2.

Table 1.2. Recommended dietary allowance (RDA) for vitamin B12 (Institute of Medicine and Food and Nutrition Board 1998).

Group	RDA (μg day^{-1})
1-3 years	0.9
4-8 years	1.2
9-13 years	1.8
14-18 years	2.4
19-70 years	2.4
> 70 years	2.4
Pregnant women	2.6
Lactating women	2.8

The Estimated Average Requirement (EAR) consists in the value of daily intake that satisfies the vitamin B12 requirement in half of the individuals in a determined life-stage or group. The Recommended Nutrient Intake (RNI) for vitamin B12, is calculated as the EAR value plus 2 standard deviations (SD). The Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) adopted the same EAR values proposed by the IOM. Moreover, the RNI values presented are equivalent to RDA values presented by the IOM (FAO/WHO, 2002). The EAR and RNI values for each group are listed in Table 1.3.

Table 1.3. Estimated average requirement (EAR) and Recommended Nutrient Intake (RNI) for vitamin B12 (FAO and WHO 2004).

Group	EAR (µg day^{-1})	RNI (µg day^{-1})
0-6 months	0.3	0.4
7-12 months	0.6	0.7
1-3 years	0.7	0.9
4-6 years	1.0	1.2
7-9 years	1.5	1.8
10-18 years	2.0	2.4
19-65 years	2.0	2.4
> 65 years	2.0	2.4
Pregnant women	2.2	2.6
Lactating women	2.4	2.8

Table 1.4. Vitamin B12 adequate intake (AI) for vitamin B12 (EFSA panel on dietetic products, nutrition, and allergies 2015).

Group	AI (µg day^{-1})
7-11 months	1.5
1-3 years	1.5
4-6 years	1.5
7-10 years	2.5
11-14 years	3.5
15-17 years	4.0
> 18 years	4.0
Pregnant women	4.5
Lactating women	5.0

The European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) derived Dietary Reference Values (DRV) for cobalamin, considering the available data related to cobalamin intake and its status, functions and health consequences. It was concluded that the uncertainties with respect to the cut-off values of the indicators for cobalamin insufficiency did not allow to establish an Average Requirement (AR), being an Adequate Intake (AI) for cobalamin defined instead (EFSA Panel on Dietetic Products and Allergies, 2015). The AI values are summarized in Table 1.4.

1.3.10. Cellular Absorption

Vitamin B12 is a water-soluble vitamin, with a transport mechanism being necessary for passing through the cellular membrane, process in which three binding proteins, intrinsic factor, haptocorrin, and transcobalamin II, are involved (Banerjee, 2006).

The absorption of vitamin B12 begins when the vitamin is released from food and reaches the stomach, where it binds to haptocorrin, produced in the salivary glands. This protein protects vitamin B12 from acid degradation due to the low stomach pH. Once in the duodenum, a change in the pH value occurs, haptocorrin is degraded and the vitamin binds to an intrinsic factor, produced in parietal cells. This complex endocytosis into enterocytes is mediated by cubam receptor. Once inside lysosomes, the intrinsic factor is degraded and vitamin B12 is released. The free vitamin leaves the enterocyte by multidrug resistance protein 1 (MDR1), being transported to the portal circulation, where it circulates complexed to transcobalamin II, its transporter. The internalized vitamin is essentially stored in the liver and a part is excreted in bile (Green et al., 2017, Brito et al., 2018, Banerjee, 2006).

1.3.11. Food Sources

Vitamin B12 is produced by bacteria and archaea, while plants and animals do not possess such capacity. However, after being synthetized, vitamin B12 accumulates in animal tissues, thus being a good source. In some cases, vitamin B12 may occur in plants and mushrooms through microbial interactions. Humans acquire vitamin B12 produced by bacteria mainly through the consumption of meat, specially ruminants and fish (Watanabe and Bito, 2018b). The consumption of meat, milk and fish is associated with the increase of vitamin B12 content in serum (Brouwer-Brolsma et al., 2015).

The meat consumed by humans can be derived from ruminant animals, such as beef, veal and lamb, or from omnivorous animals, namely pork and poultry (Brouwer-Brolsma et al., 2015). Ruminants' diet is essentially constituted by plants, which have no vitamin B12 content. However, they possess vitamin B12 synthetizing bacteria inhabiting

their stomachs. The vitamin produced in the stomach is absorbed in the intestine, transferred to blood and stored in liver and muscles, or secreted into milk (Ortigues-Marty et al., 2005). Furthermore, omnivores include animals in their diet, which are vitamin B12 sources. The vitamin B12 content is higher in ruminant meats than in omnivorous meats. Although in milk the content is not high, particularly in comparison to meat, it is an important source since milk or dairy products are highly consumed by populations (Watanabe and Bito, 2018b). The vitamin B12 content of some animal derived products is presented in Table 1.5.

Table 1.5. Vitamin B12 content in animal-derived foods (Watanabe and Bito, 2018b).

Product	Vitamin B12 (μg $100g^{-1}$)
Cow Raw Liver	52.8
Cow Raw Meat	1.0-2.0
Pork Raw Liver	25.2
Pork Raw Meat	0.5
Chicken Raw Liver	44.4
Chicken Raw Meat	<0.5
Cow Milk	0.35
Sheep Milk	0.71
Goat Milk	0.06
Round Herring Viscera	37.5
Round Herring Meat	12.2
Edible Bivalves	60
Edible Snails	20

Fish and shellfish are also a good source of vitamin B12. In aquatic environment, this vitamin can be taken by phytoplankton, that is preyed by zooplankton. Zooplankton is consumed by small fish, that serve as feeding to the bigger predatory fish, where the content of vitamin B12 is high (Watanabe and Bito, 2018b). Shellfish, namely edible bivalves and snails, also have a relevant content of vitamin B12 (Watanabe and Tanioka, 2014).

1.3.12. Vitamin Deficiency

Vitamin B12 deficiency is manifested at several levels. The consequences may occur at haematological level, including macrocytic or megaloblastic anaemia, leukopenia, pancytopenia, thrombocytopenia and thrombocytosis, or at neurological level, such as peripheral neuropathy and areflexia. In cases of a more severe deficiency, dementia diseases and psychosis may occur (Shipton and Thachil, 2015, Langan and Goodbred, 2017).

The deficiency in vitamin B12 may have many causes, being pernicious anaemia one of them, since in such condition there is a destruction of the parietal cells, in which the intrinsic factor is produced. The low intake of vitamin B12 due to a poor diet can also lead to its deficiency. Currently, food-bound cobalamin malabsorption (FBCM) is the most frequent cause, in which there is a difficulty in releasing the vitamin from transport proteins (Shipton and Thachil, 2015).

Deficiency can be diagnosed by quantification of some biomarkers, namely, total serum vitamin B12, homocysteine, methylmalonic acid (MMA) and total serum holo-transcobalamin. Methylmalonic acid is the most reliable marker for determination of the B12 status in all age groups with normal renal function. In a vitamin B12 deficiency scenario, (R)-methylmalonyl-CoA mutase is inactive, leading to an increase of MMA, which enters blood circulation in a free form (Hannibal et al., 2016).

Vitamin B12 deficiency is very common in elderly people, affecting about 5% of the population aged 65 to 74 years and over 10% of those aged above 75 years (Clarke et al., 2004). As age increases, there is a higher risk of developing atrophic gastritis with a decrease in acid pepsin production, which is important for the cleavage of protein-bound vitamin B12 from the natural chemical form of vitamin B12. Thereby, it is recommended, for people aged over 50, the consumption of foods fortified with crystalline B12 or B12-containing supplements (Park and Johnson, 2006, Baik and Russell, 1999).

In vegetarians, vitamin B12 deficiency is a common occurrence, since the consumption of animal foods is very low or absent, and the vitamin presence in plant foods is very scarce. Within this group, vegans have the highest risk, as they do not ingest any food of animal origin (Rizzo et al., 2016, Pawlak et al., 2014).

Data from a systematic review of the literature reporting the cobalamin serum concentration among people following vegetarian diets, showed that 0% to 86.5% of adults and elderly, approximately 45% of infants, 0% to 33.3% of children and

adolescents, and 17% to 39% of pregnant women presented vitamin B12 deficiency (Pawlak et al., 2014). In order to prevent this, dietary/pharmacological solutions are recommended, such as the consumption of supplements and fortified products (Mądry et al., 2012).

1.3.13. Detection Methods

The methods used to detect and quantify vitamin B12 are mostly based on microbiological and chemical principles. Over the years, an interest in developing and improving this screening techniques has been growing in order to obtain fast and precise results with less costs, that be suitable for a wide range of samples (Tsiminis et al., 2017).

The first technique used as vitamin B12 assay was based on assessing microbial growth in a determined sample and comparing it with a calibrated growth curve. The microorganism used in the assay must require the presence of externally supplied corrinoids to grow (Tsiminis et al., 2017). Some strains were tested, including *Lactobacillus lactis* Dorner ATCC 8000, *Lactobacillus leichmannii* ATCC 7830, *L. leichmannii* ATCC 4797, and *Euglena gracilis*. Since *L. lactis Dorner* ATCC 8000 in certain conditions does not require vitamin B12 to grow, being difficult to obtain reproductible results and the tests with *E. gracilis* only showed consistent results when using pure preparations, it was concluded that this microorganism is not the most suitable for this assay (Hoffmann et al., 1949, Ford, 1952, Harrington, 2019). *L. leichmannii* ATCC 7830 presented consistent results being the most frequently used strain (Harrington, 2019).

Skeggs et al. (1950) developed a method that was used for decades for vitamin B12 determination. *L. leichmannii* culture was incubated in a skim-milk-based medium for 24 h with regulated pH. The culture was then added to tubes containing solution test or standard (basal medium) that were posteriorly autoclaved for 15 minutes at 120 ºC. Finally, the results were obtained turbidimetrically, after 24 h in incubation at 37 ºC, and the samples growth can be compared with a calibrated growth rate of the microorganisms

when exposed to known concentrations of cobalamin The method was improved overtime and the detection limit has been lowered to 20 pg mL^{-1} (Kelleher and Broin, 1991).

Vitamin B12 can also be detected using immunoassays, methods that appeared in 1982, which principle relies on using specific antibodies to bind to a target molecule onto a substrate (Frater-Schroder et al., 1982). A method for transcobalamin detection in serum was developed. Rabbit antibodies that attach to cobalamin are immobilized on a cover slip and a biotinylated detection antibody, that reacts with horseradish peroxidase-avidin, is introduced. Vitamin B12 and labelled enzyme compete for antibody binding sites and this results in a colour reaction that decreases with increasing concentration of vitamin B12. This method has a limit of detection (LOD) of <1.6 $pmol.L^{-1}$ (~2.2 pg mL^{-1}) (Nexo et al., 2000). Nonetheless, the LOD for ELISA Vitamin B12 quantification for food samples is higher (0.3 ng mL^{-1}) than for serum samples, according to the manufacturer's indication (Vitamin B12 ELISA, Immunolab GmbH, Kassel, Germany).

Other methods like HPLC, capillary electrophoresis, radioisotope and MSMS are used for vitamin B12 detection. Although these are sensitive techniques, they do not allow fast and low-cost cobalamin quantification. Therefore, optical detection techniques as chemiluminescence, absorption and fluorescence, surface plasmon resonance and Raman spectroscopy, can present themselves as an alternative. This subject was extensively reviewed by Tsiminis et al. (2017).

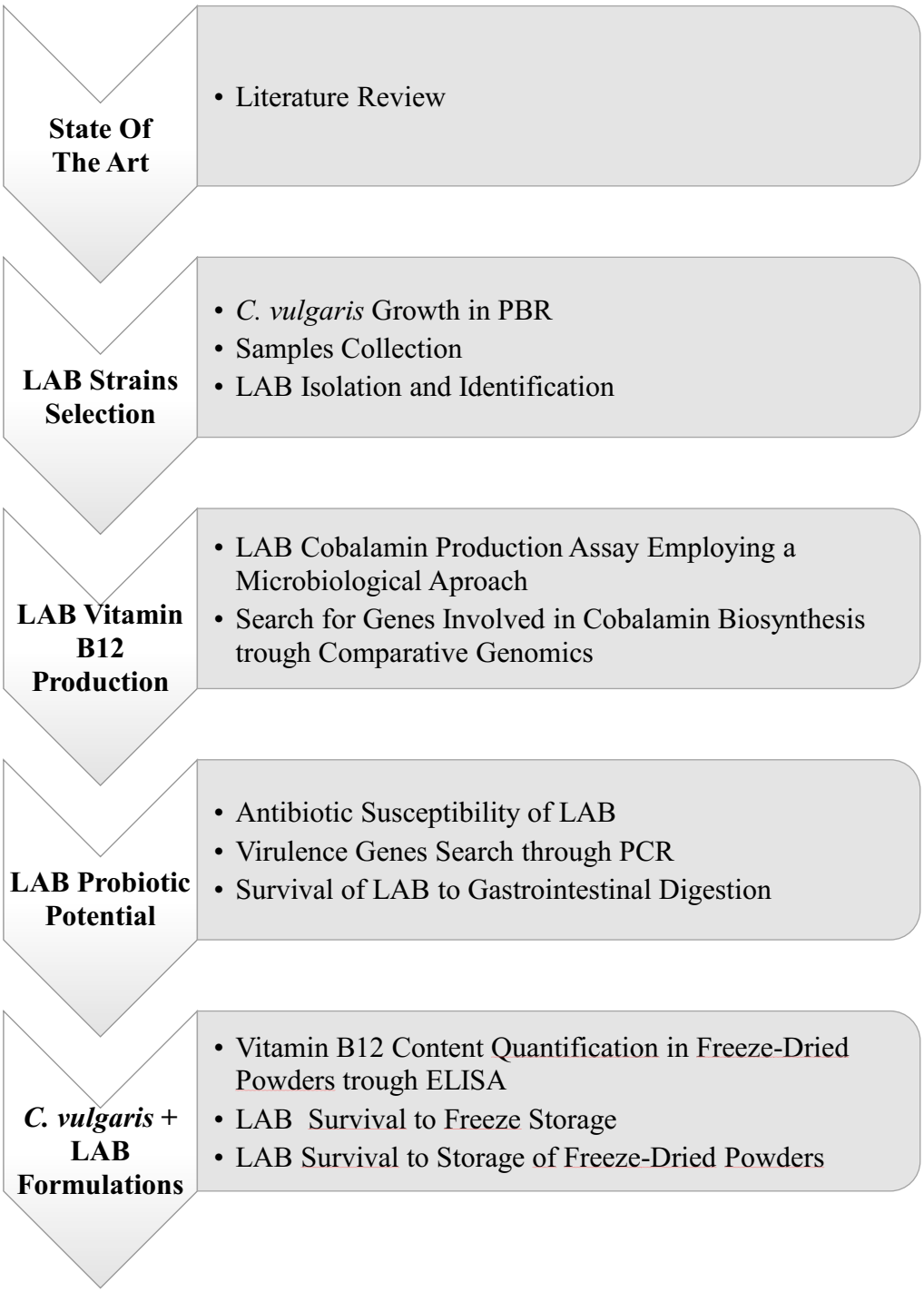
2. OBJECTIVES

It is known that vitamin B12 deficiency is very common, especially among the elderly people. It may also occur due to previous conditions, such as pernicious anaemia or FBCM, and even from poor diet intake, as occurs in people following strict vegetarian diets. To prevent this, is recommended the consumption of supplements or fortified products.

Vitamin B12 was previously detected in *C. vulgaris* culture produced in photobioreactors by Allmicroalgae Company – Natural Products S.A., a company whose purpose is developing microalgae solutions for consumption and dietetic supplements. Upon these findings, an interest in increasing vitamin B12 content in *C. vulgaris* culture emerged.

Thus, the main objective of this project is to identify potential probiotic LAB from *C. vulgaris* culture and assess their vitamin B12 biosynthesis capacity. Once potential producers are identified, another aim of this work is to develop a LAB and microalgae mixed culture with an enhanced cobalamin content.

3. THESIS ROADMAP



4. MATERIALS AND METHODS

4.1. *Chlorella vulgaris* Production in Allmicroalgae Facilities

Chlorella vulgaris was produced in Allmicroalgae Company – Natural Products S.A. (Pataias, Portugal) facilities, under both heterotrophic and autotrophic conditions. The culture was transferred from a master cell bank to a 50 mL Erlenmeyer and grown heterotrophically for two days. Then, the 50 mL Erlenmeyer's culture was used to inoculate a 250 mL Erlenmeyer's and it was grown for 1 day. After this period, the 250 mL Erlenmeyer's culture was used to inoculate a 5 L fermenter. The fermenter was operated in fed-batch mode and glucose was used as carbon source. Temperature was maintained at 28 ºC and pH was adjusted to 6.5 by the addition of an ammonium solution. After three days, the culture of two 5 L fermenters was transferred to a 5000 L fermenter and grown for four days. At this stage, the heterotrophic route was completed, and the culture of the 5000 L fermenter was used to feed a 100 m^3 PBR, only containing culture media, that functions as an autotrophic system (Barros et al., 2019, Martins, 2019).

Re-inoculation can occur in outdoor reactors, and in this scenario the culture from a 5000 L fermenter enters the reactor already containing microalgal culture. In this stage, ammonia is used as nitrogen source and pH is controlled through continuous aeration and CO_2 injection to a value between 5.5 and 6.5. During autotrophic growth, cultures are exposed to natural circadian light and therefore are submitted to a dark cycle (16 h of light per day). The culture is harvested when cells protein and chlorophyll content is sufficiently high (Barros et al., 2019, Martins, 2019).

4.2. Isolation and of Lactic Acid Bacteria from Bioreactor's Microbiota

In order to identify LAB among the *C. vulgaris* bioreactor's microbiota, samples from four bioreactors (L2, L5, L7 and L8) were recovered over time and microbiological counts were performed. Samples were serially diluted in Ringer´s solution (1:10) and subsequentially 100 µL of each dilution was spread on de Man Rogosa and Sharpe (MRS) Agar. Colonies were enumerated after 72 h of incubation at 30 ºC.

To recover LAB from MRS Agar plates, isolated colonies were transferred to new MRS Agar plates by streaking. After three days at 30 ºC, Gram staining, catalase and oxidase tests were performed to characterize cells of the isolated colonies.

To identify the isolated LAB, colonies were transferred to MRS broth and after growth for 18 h at 30 ºC the genomic DNA was extracted from the broth culture. The extraction was performed according to GRS Genomic DNA Kit Bacteria (GRiSP Research Solutions, Porto, Portugal) following the manufacturer's instruction.

A polymerase chain reaction (PCR) assay for the amplification of 16S rRNA gene was carried out according to (Lane, 1991). Amplification reactions were performed in a T-100^{TM} Thermal Cycler (Bio-Rad, Richmond, CA). PCR products obtained were then purified using PCR DNA and Band Purification kit (GRiSP Research Solutions, Porto, Portugal) according to the manufacturer's instructions. Finally, samples were outsourced to Eurofins Genomics company (Ebersberg, Germany) for sequencing. Sequences analysis was performed using Geneious Prime version 2020.0.5 software (Biomatters, West Auckland, **N**ew Zealand).

4.3. Determination of Vitamin B12 Production by Lactic Acid Bacteria

For the determination of cobalamin production, test cultures and both positive and negative control cultures (*L. rossiae* DSM 15814 and *L. plantarum* DSM 20205, resctivelly) were inoculated into vitamin B12-free assay medium (Merck, Buenos Aires, Argentina). After incubation for 18 h at 30 ºC, 50 µL of the culture was transferred to 5 mL of fresh vitamin B12-free assay medium. This procedure was repeated each 18 h, for 8 days (eight reinoculations). The last culture media was submitted to 115 ºC for 10 min to disrupt cell membrane and release intracellular content. The media was filtered using a 0.2 µm Minisart Syringe Filter to remove cell debris.

A vitamin B12 stock solution was used to prepare vitamin B12-free assay medium supplemented with different cobalamin concentrations, in order to obtain a calibration curve. All tubes were submitted to the same temperature treatment as the test and control tubes.

Lactobacillus leichmannii DSM 20355*,* formerly *Lactobacillus delbrueckii subsp. lactis,* a strain that requires vitamin B12 to grow, was grown in MRS broth for 24 h at 37 ºC in microaerophilic conditions and transferred two times in the same media. The culture was washed three times in Ringer's solution and the cell level was adjusted to 10^4 CFU ml^{-1} through abs determination at 600 nm, and 1% of the culture was inoculated in all the tubes containing different cobalamin concentrations. The tubes were subsequentially incubated for 72 h at 37 ºC in microaerophilic conditions. The optical density was determined at 600 nm.

4.4. Next Generation Sequencing

Lactococcus lactis E31, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51 complete genomes were sequenced at Eurofins genomics (Ebensberg, Germany), by a Genome Sequencer Illumina HiSeq technology, using NovaSeq 6000 S2 PE150 XP sequencing mode.

Paired-end fastq files were used to assemble the bacterial genomes at INIAV (Instituto Nacional de Investigação Agrária e Veterinária, I. P.) bioinformatics server, using SPAdes (St. Petersburg genome assembler) version SPAdes-3.12.0-Linux (Nurk et al., 2013). The fasta files with the generated contigs were used to search for orthologous proteins involved in cobalamin synthesis. Protein sequence alignments were carried with BLASTX, using ncbi-blast-2.10.1 + -1.x86_64.rpm version in a Linus environment (Altschul et al., 1997) against a protein fasta file of a partial sequence of *Lactobacillus reuteri* CRL 1098 cobalamin gene cluster, deposited with assession no. AY780645.1 at NCBI (https://www.ncbi.nlm.nih.gov/nuccore/AY780645.1/), using a cut-off E-value of 10^{-4}.

4.5. Assessment of Lactic Acid Bacteria Probiotic Potential

4.5.1. Susceptibility of Lactic Acid Bacteria to Antibiotics

Minimal inhibitory concentration (MIC) of different antibiotics was determined according to the liquid dilution method described by Wiegand et al. (2008).

Lactococcus lactis, *L. brevis*, *L. plantarum*, *P. pentosaceus* and the control strain *Enterococcus faecalis* DSM 2570 were grown on MRS agar. The inoculum was prepared to contain 10^8 CFU mL^{-1}, according to a growth method using overnight cultures, where a correlation between OD at 600 nm and an overnight culture microbial number for each isolate was established. For the antibiotic testing it was used a broth microdilution approach. Antibiotic dilutions were prepared for ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol. Bacterial suspensions were added to the wells to obtain a final inoculum of 10^5 CFU mL^{-1}, the microplate was incubated at 30 ºC and the MIC were determined. The antibiotics used and the cut-off values considered were those established by EFSA FEEDAP Panel (2018).

4.5.2. Identification of Virulence Genes

The selected isolates (*Lac lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51) were tested for harbouring of virulence genes trough PRC reactions. The virulence genes searched included *agg* (agregation substances), *esp* (enterococcal surface protein), *gelE* (gelatinase), *efaAfs* and *efaAfm* (cell wall adhesin), *cylA* (cytolysin activator serine protease), *cylB* (cytolysin secretion/tranporter), *cylM* (post-translational modification cytolysin), with primers and PCR conditions designed accordingly to Eaton and Gasson (2001). Primers and PCR conditions were also established for searching genes $cylL_L$ and $cylL_S$ (cytolysin structural subunits) as described in Semedo et al. (2003). *E. faecalis P1* strain was used as positive control for *agg* and *gelE* genes, *E. faecalis* F2 for *cylA*, *cylB*, *cylM*and *efaAfs* genes, *E. faecalis* DS 16 for $cylL_L$ and $cylL_S$ *genes and E. faecium* F10 and *E. faecalis* P36 to *efaAfm* and *esp* genes, respectively.

4.5.3. Determination of Haemolytic Capacity

Cells of the selected isolates (*Lac. lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51) were grown in MRS broth overnight and subsequently streaked on Blood Agar and incubated at 30 ºC for 72 h. The formation of a transparent area around colonies was an indicator of β-hemolysis, the presence of a greenish area around colonies was an indicator of α-hemolysis and the absence of any area around the colonies was an indicator of γ-hemolysis (Semedo et al., 2003).

4.5.4. Screening of Biogenic Amine Production by Lactic Acid Bacteria

Biogenic amine production by the isolates of LAB in study was performed accordingly to (Bover-Cid and Holzapfel, 1999). Lactic acid bacteria strains were sub-cultured 5 to 10 times in MRS broth containing 0.1% of each precursor amino-acid (tyrosine free base, histidine monohydrochloride, ornithine monohydrochloride and lysine monohydrochloride) and 0.005% of pyridoxal-5-phosphate.

All bacteria were streaked on the screening medium with and without amino acids (control) and incubated for 4 days at 30 ºC, under aerobic conditions. A positive reaction was identified when colonies gained purple colour.

4.6. Survival of Lactic Acid Bacteria Through Gastrointestinal Digestion

The isolated LAB were submitted to a stimulation of the gastrointestinal digestion to assess their survival. Samples were digested using a static *in vitro* gastrointestinal digestion model, accordingly to (Brodkorb et al., 2019).

All the isolates were grown in MRS broth for 18 h at 30 ºC. After the incubation period, cells were washed two times in Ringer's solution and concentrated five times. To assess the protective effect of *C. vulgaris* along the gastrointestinal digestion, spray dried

powder of organic *C. vulgaris* (Allmicroalgae, Portugal) was added with a 1.5 (w/v) ratio to the Simulated Salivary Fluid (SSF), considering the total volume achieved at the end of the procedure.

In the oral phase, bacterial solutions were diluted with a 1:1 ratio with SSF containing $CaCl_2(H_2O)_2$ (1.5 mM) and incubated for 5 min at 37 ºC. Enumeration on MRS agar plates was performed at 0 and 5 min of the oral phase. Simulated gastric fluid (SGF) containing $CaCl_2(H_2O)_2$ (0.15 mM) and pepsin (2000 U mL^{-1}) was added to achieve a 1:1 ratio and the pH adjusted to 3.0 to initiate gastric phase. The solutions were incubated at 37 ºC for 2 h. Bacteria counts were determined on MRS agar plates at 45, 90 and 120 min of the gastric phase. Finally, for the intestinal phase, simulated intestinal fluid (SIF) containing $CaCl_2(H_2O)_2$ (0.15 mM), bile solution (10 mM) and pancreatin (100 U mL^{-1}) was added to obtain a final ratio of 1:1 and incubated for 2 h at 37 ºC. Bacteria were enumerated on MRS agar plates at 45, 90 and 120 min of the intestinal phase. All incubation periods were performed in a shaking (300 rpm) water bath at 37 ºC.

4.7. Optimization of Organic Medium for Growth of Lactic Acid Bacteria

For the optimization of the industrial Organic medium provided by Allmicroalgae (used for *C. vulgaris* cultivation) for the growth of LAB, *Lac. lactis* was grown in MRS broth for 18 h at 30 ºC. The cells were harvested by centrifugation, resuspended in Ringer's solution and inoculated in different formulations of Organic medium (pH adjusted to 6.4) to obtain a final concentration of 10^4 cell mL^{-1}. The formulations developed are presented in table 3.1. The best formulation determined for growth of *Lac. lactis* E32 was tested for growth of *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51.

Table 3.1. *Organic medium formulations with varying concentrations of glucose, yeast extract, peptone and iron (II) sulphate heptahydrate tested for LAB growth optimization.*

Formulation	Glucose (g L^{-1})	Yeast Extract (g L^{-1})	Peptone (g L^{-1})	Iron (II) Sulphate Heptahydrate (g L^{-1})
Fa	10	2.5	5	0.02
Fb	-	2.5	5	0.02
Fc	10	2.5	-	0.02
Fd	10	-	5	0.02
Fe	10	2.5	5	-
Ff	10	-	-	-
Fg	-	-	5	-
Fh	-	2.5	-	-
Fi	-	-	-	0.02
Ha	15	5	-	-
Hb	15	3.75	-	-
Hc	15	2.5	-	-
Ja	10	5	-	-
Jb	10	3.75	-	-
Jc	10	2.5	-	-
Ka	5	5	-	-
Kb	5	3.75	-	-
Kc	5	2.5	-	-
L	5	10	-	-
M	-	10	-	-
N	-	15	-	-

4.8. Lactic Acid Bacteria and *Chlorella vulgaris* Lyophilization and Vitamin B12 Quantification

Lactococcus lactis E32, L. brevis G31, and *P. pentosaceus* L51 cultures were inoculated in organic medium formulations N (supplemented with glucose 15 g L^{-1}) and Fh (supplemented with 2.5 g L^{-1}) (Table 3.1 pH adjusted to 6.4), and incubated for 18 h at 30 ºC.

Chlorella vulgaris culture with 55 g L^{-1} of dry weight provided by Allmicroalgae Company – Natural Products S.A (Pataias, Portugal) was submitted to a pasteurization processes at 70 ºC for 15 min to decrease microbial population. After pasteurization, a mixture in a proportion 1:1 of bacteria and algal culture was prepared. The single algal culture was diluted with organic media with the same proportion, prior to pasteurization process.

Chlorella vulgaris axenic culture grown in Vitamin B12 free Bold Basal medium for 2 months at room temperature with light and aeration conditions was used directly for lyophilization.

Before lyophilization, *Lac. lactis E32, L. brevis G31*, and *P. pentosaceus* L51 and the correspondent LAB and microalgae culture were frozen at -80 ºC overnight. The frozen samples were dried-frozen at a temperature -42 ºC and vacuum pressure 0.4 torr, for 48h using a lyophilizer (SB4 Armfield, UK). For the samples containing *Lac. lactis*, *L. brevis* or *P. pentosaceus*, viable cell counts were determined weekly for 1 month by plate count method. The dried powder was resuspended in Ringer solution and the suspensions were serially diluted, plated on MRS agar medium and incubated at 30 ºC for 2 days.

The vitamin B12 content in dried-frozen powder was determined using ELISA method for Vitamin B12. To perform cellular lysis, powder was resuspended in 1 mL of water, glass beads were added, and the samples were submitted to vortex for more than 12 min. After centrifugation at 8000 rpm for 10 min, Carrez I (potassiumhexacyanoferrate(II)-3-hydrate, 150 g L^{-1}) and Carrez II (zincsulfate-7-hydrate, 300 g L^{-1}) solutions were added to the supernatants, and centrifugated for 8000 rpm for 5 min. The aqueous phase was collected and used for vitamin B12 quantification. Quantification was carried using the ELISA Vitamin B12 Kit (Immunolab GmbH, Kassel, Germany), according to the manufacturer's instructions.

4.9. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics 27.0 (New York, United States).

5. RESULTS AND DISCUSSION

5.1. Identification of Lactic Acid Bacteria

A total of 130 LAB were isolated from cultures of *C. vulgaris* grown in three photobioreactors (table 5.1). Ninety-five of these isolates were identified as *Lac. lactis*, 16 as *E. casseliflavus*, 14 as *L. plantarum*, three as *P. pentosaceus* and two as *L. brevis*.

Table 5.1. *Identification of lactic acid bacteria isolated from C. vulgaris cultivation bioreactors.*

Species	Origin	Number of Isolates
Lactococcus lactis	L2 and L5 bioreactors	95
Enterococcus casseliflavus	L2 bioreactor	16
Lactobacillus plantarum	L2, L5 and L7 bioreactors	14
Pediococcus pentosaceus	L5 bioreactor	3
Lactobacillus brevis	L5 bioreactor	2

Four isolates were selected for further characterization, namely *Lac. lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51.

5.2. Vitamin B12 Production by Lactic Acid Bacteria

The selected LAB isolates, *Lac. lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51 were secreened for their vitamin B12 production capacity following a microbological aproach for quantification. The results (Figure 5.1) indicated that *Lac. lactis* E31, *L. brevis* G31 and *P. pentosaceus* L51 are capable of producing vitamin B12: 6.18 ± 1.08 pg mL^{-1}, 5.47 ± 0.37 pg mL^{-1} and 28.19 ± 2.27 pg mL^{-1}, respectivelly. Moreover, *P. pentosaceus* L51 achieved a production level equivalent ($P>0.05$) to *L. rossiae* DSM 15814 (29.30 ± 1.21 pg mL^{-1}), the strain used as positive control. *Latococcus lactis* E32 and *L. brevis* G31 have shown to produce cobalamin at the same level. In constrast to these results, *L. plantarum* G12 was considered as a non-producer strain since detected levels of cobalamine (0.21 ± 0.17 pg mL^{-1}) were similar ($P>0.05$) to

those detected for the negative control (*L. plantarum* DSM 20205). Even though *L. plantarum* DSM 20205 has shown a residual cobalamin production (0.32 ± 0.05 pg mL^{-1}), this could be due to the fact that the test microorganism *L. leichmannii* DSM 20355 used in the microbiological assay for vitamin B12 detection can replace cobalamin by deoxyribosides and deoxynucleotides (the alkali-resistant factor) or pseudocobalamin to grow, leading to an overestimation of values (Watanabe and Bito, 2018a).

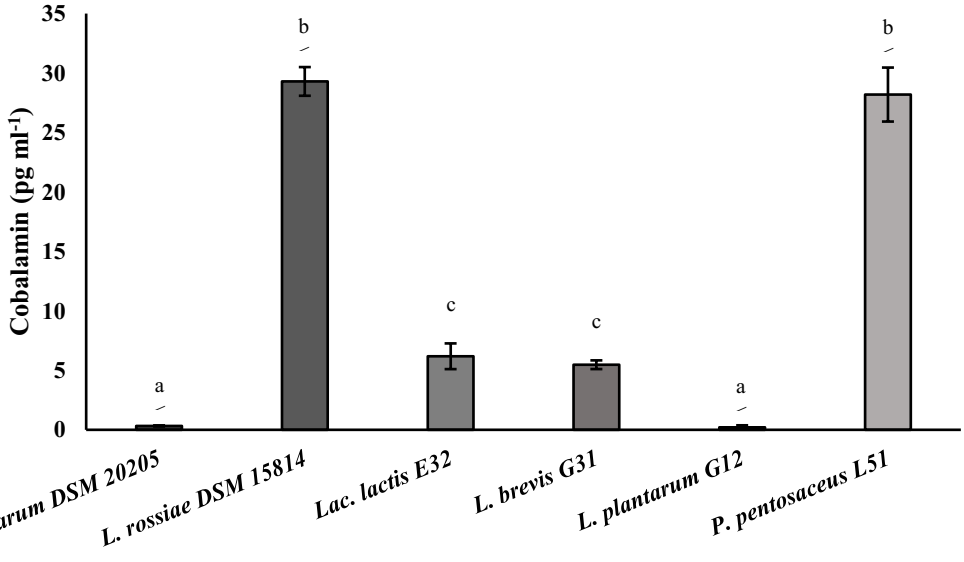


Figure 5.1. Cobalamin production by *Lac. lactis E*32*, L. brevis* G31*, L. plantarum* G12 and *P. pentosaceus* L51. *L. plantarum* DSM 20205 and *L. rossiae* DSM 15814 were used as negative and positive control, respectively.

Lactobacillus brevis and *P. pentosaceus* have been reported to produce between 0.4 to 0.6 pg mL^{-1} by Masuda et al. (2012). These values are at the level of the production determined in this study for the negative control strain *L. plantarum* DSM 20205. However, we must take into consideration that the assay medium used in the study mentioned (Nissui basal medium) was different from the medium used in the present study, which can have an impact on the results. Although *L. plantarum* G12 had no production capacity, other *L. plantarum* strains have been identified as potential cobalamin producers. In the same study, *L. plantarum* CN-225 produced 2.0 pg mL^{-1} of vitamin B12 (Masuda et al., 2012). A *L. plantarum* strain isolated from kanjika showed a production of 13 ng of vitamin B12/ g of dry biomass when determined by an ELISA method (Madhu et al., 2010). *Lactobacillus plantarum* strains BHM10, isolated from human milk, and BCF20, isolated from child faeces, showed a production between 0.5

and 0.8 pg/mL, after growth in VBAM (Bhushan et al., 2016). Once again, it is verified that different growth medium can have an impact on the quantification results. So far, there is no other study reporting a *Lac. lactis* strain capable of producing B12.

The production of pseudocobalamin by a LAB strain *L. reuteri* CRL1098 was already reported by Santos et al. (2007), and this analogue is not bioavailable for humans and other mammals (Stupperich and Nexø, 1991). Hence, another technique like HPLC, MSMS or nuclear magnetic resonance spectroscopy should be employed to discriminate the analogue produced by the selected strains.

5.3. Genotypic Confirmation of Vitamin B12 Biosynthesis

The phenotypic results obtained were confirmed by comparative genomic analysis against *L. reuteri* CRL 1098 cobalamin synthesis cluster, that was previously described by Santos et al. (2008).

Four genes involved in different steps of the biosynthetic pathway were identified in *Lac. lactis* E32, *L. brevis* G31 and *L. plantarum* G12 genome (Table 5.2): *hemL, cbiT, cobD* and *cobC. hemL* is involved in uroporphyrinogen III synthesis; *cbiT* and *cobD* are involved in adenosylcobinamide synthesis; and *cobC* is involved in lower ligand synthesis. Although *hemL* was not found in *P. pentosaceus* L51, *cobA*, a gene also involved in adenosylcobinamide synthesis, was also located.

Although the E-values considered were low, which provides confidence in claiming that the genes being searched are present, the percentage for the sequence identity of the codified proteins was always bellow 30%, with exception of corrinoid adenosyltranferase, encoded by *cobO*, that showed an identity of 38%. Furthermore, only a few genes involved in cobalamin synthesis were found. Nonetheless, other genes related to cobalamin synthesis, that are present in the same cluster, were detected. For instance, all strains harboured *cbiO*, that is related to the synthetic pathway since it codifies a cobalt import ATP-binding protein.

Propanediol dehydratase requires cobalamin as cofactor and the association of both *pdu* and *cbi-cob* clusters in the genome can emerge from this requirement (Morita et

al., 2008). *Pediococcus pentosaceus* L51, that has shown to be the most promising cobalamin producer, was the only strain harbouring *pduU* (2.00 E-32; 86%) and *pduV* genes (4.00 E-48; 55%), which codify propanediol utilization proteins. This can be considered an evidence that the cobalamin operon is present in the genome of this strain.

Table 5.2. Presence of the cobalamin biosynthesis genes in *Lac. lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51 genomes.

		***Lac. lactis* E32**		***L. brevis* G31**		***L. plantarum* G12**		***P. pentosaceus* L51**	
		E-value	**Identity**	**E-value**	**Identity**	**E-value**	**Identity**	**E-value**	**Identity**
Uroporphyrinogen III Synthesis	***gltX***								
	hemA								
	hemL	4.00 E-24	27%	4.00 E-24	27%	1.00 E-28	31%		
	hemB								
	hemC								
	hemD								
Adenosylcobinamide Synthesis	***cobA***							4.00 E-28	38%
	cysG								
	cbiK								
	cbiL								
	cbiH								
	cbiF								
	cbiG								
	cbiD								
	cbiJ								
	cbiE								
	cbiT	7.00 E-05	26%	2.00 E-05	26%	2.00 E-04	26%	7.00 E-04	27%
	cbiC								
	cbiA								
	cobR								
	cobO								
	cobQ								
	cobD	4.00 E-09	23%	3.00 E-09	23%	8.00 E-17	26%	1.00 E-08	32%
	cbiB								
Lower Ligand Synthesis	***cobU***								
	cobT								
	cobS								
	cobC	9.00 E-07	30%	9.00 E-07	30%	6.00 E-09	30%	3.00 E-06	24%

Taken all together, these results do not allow to conclude about the presence of the biosynthetic pathway on the subject strains. However, it must be taken into

consideration that the genome sequencing was carried with a short-read approach that faces some challenges in *de novo* assembly. Although Illumina generates accurate reads, sequencing errors may still occur, mostly in regions with high GC and AT content, and lead to misassemble. Additionally, the need of resorting to PCR results in uneven read depth and ultimately in the introduction of gaps during assembly. Finally, the presence of repetitive sequences in genomes can also present a problem since, in general, sequences generated are too short to cover an entire repetitive region. Therefore, all repeated sequences can be assembled together and consequently a singular sequence at the end of a repeat can end up being assembled to a sequence located in a distant part of the genome, forming a chimeric contig. These limitations could be overcome by using a complementary long read method, which produces larger contigs and is not PCR biased. In this scenario, a hybrid approach is used and short reads are assembled with long contigs (Sohn and Nam, 2018, Schatz et al., 2010).

The presence of genes involved in cobalamin synthesis was detected in *L. plantarum* G12, for which production of vitamin B12 was not revealed in the phenotypic assays. However, the detection of a few genes does not sustain that the complete biosynthetic pathway is present. Additionally, their occurrence in the genome does not mean that they are being expressed. Finally, it's important to notice that with the exception of *L. rossiae* DSM 15914, that was grown in microaerophilia, *L. plantaram* G12 and the other tested isolates were grown in aerobioses. Assuming that the presence of *cbiT* in the genome is an evidence that cobalamin production follows an anaerobic pathway, bacteria growth conditions could explain the negative results verified in the microbiological assay for *L. plantarum* G12 and the low production verified for *Lac. lactis* E32 and *L. brevis* G31.

5.4. Lactic Acid Bacteria Probiotic Potential

To assess the probiotic potential of *Lac. lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51, susceptibility to antibiotics and the presence of virulence genes was investigated. The antibiotic susceptibility profile was determined for ampicillin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin,

tetracycline, vancomycin and chloramphenicol. With the exception of vancomycin, all strains were sensitive to all the other tested antibiotics. While *Lac. lactis* E32 and *L. brevis* G31 have shown to be susceptible to vancomycin, the same was not observed for *L. plantarum* G12 and for *P. pentosaceus* L51, that presented resistance. However, susceptibility to vancomycin is not required according to EFSA recommendations (EFSA FEEDAP Panel, 2018) since intrinsic resistance to this antibiotic is widespread among these species (Campedelli et al., 2018, Singla et al., 2018). For the four tested strains, the virulence genes *agg*, *esp*, *gelE*, *efaAfs*, *efaAfm*, *cylA*, *cylB*, *cylM*, $cylL_L$ and $cylL_S$ were not detected.

According to EFSA, it is recommended to assess haemolytic capacity to evaluate a strain probiotic potential (FAO/WHO, 2002). Therefore, haemolytic activity of the four isolates was tested. *Lactococcus lactis* E32, *L. brevis* G31 and *P. pentosaceus* L51 revealed to have no haemolytic capacity, being considered γ-haemolytic. *Lactobacillus plantarum* G12 showed to be α-haemolytic, presenting partial haemolytic capacity.

Lactic acid bacteria can produce biogenic amines, which are organic nitrogenous compounds formed during amino acids decarboxylation, that when consumed in high concentrations can have a toxic effect on health (Spano et al., 2010). Histamine, tyramine, putrescine, cadaverine and β-phenylethylamine result from the histidine, tyrosine, ornithine, lysine and β-phenylalanine decarboxylation, respectively. These are the most frequently biogenic amines found in foods and their synthesis is favoured by acidic environments (Linares et al., 2011). The biogenic amines production capacity of *Lac. lactis* E32, *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51 was assessed. The four strains were considered not to be biogenic amine producers.

5.5. Survival of Lactic Acid Bacteria Through Gastrointestinal Digestion

It was observed that pure cultures of *Lac. lactis E32* and *L. brevis* G31 have similar resistance to the gastrointestinal digestion; no significative differences ($P<0.05$) were verified at any stage of the digestion process (Figures 5.2A and 5.2B). The acid environment (pH 3) of the gastric phase did not affect cell viability; only a slight decrease was observed (0.14 ± 0.14 log CFU mL^{-1} for *Lac. lactis* E32 and 0.18 ± 0.16 log CFU

mL^{-1} for *L. brevis* G31). However, in the intestinal phase, an accentuated decline of 3.50 ± 0.57 log CFU mL^{-1} and 3.19 ± 0.07 log CFU mL^{-1}, respectively, was registered indicating that bile has a significant impact on cells survival ($P<0.05$). The addition of *C. vulgaris* conferred a high protective effect ($P<0.05$) against bile during the intestinal phase. At the end of the intestinal phase, reductions of 0.29 ± 0.55 log CFU mL^{-1} and 0.49 ± 0.51 log CFU mL^{-1}, were observed for *Lac. lactis* and *L. brevis*, respectively. At the end of the gastrointestinal digestion the presence of *C. vulgaris* resulted in an increase in the survival rate of *Lac. lactis* and *L. brevis* from 0.05 ± 0.05 % and 0.06 ± 0.01 %, respectively, to 67.47 ± 45.95 and 48.67 ± 48.67 %.

Lactobacillus plantarum G12 and *P. pentosaceus* L51 also demonstrated similar behaviours when submitted to the adverse conditions of the gastrointestinal digestion (Figures 5.2C and 5.2D). However, they have shown to be more resilient than *Lac. lactis* E32 and *L. brevis* G31. *Lactobacillus plantarum* G12 viable cell counts suffered a reduction of 0.21 ± 0.15 log CFU mL^{-1} during the complete digestion process. *Pediococcus pentosaceus* L51 suffered a reduction of 0.58 ± 0.04 log CFU mL^{-1} during the entire process. The survival rate of *L. plantarum* G12 and *P. pentosaceus* L51 was 63.81 ± 23.22 % and 30.88 ± 9.06 %, respectively. The protective effect of *C. vulgaris* upon *P. pentosaceus* L51 was verified, suffering a low reduction of 0.26 ± 0.07 log CFU mL^{-1} in the presence of *C. vulgaris*, with a survival rate of 56.02 ± 9.29%. Reduction of *L. plantarum* G12 in the presence or in the absence of *C. vulgaris* were not significantly ($P>0.05$) different; the survival rate of *L. plantarum* in the presence of *C. vulgaris* at the end of digestion was 96.22 ± 13.75%.

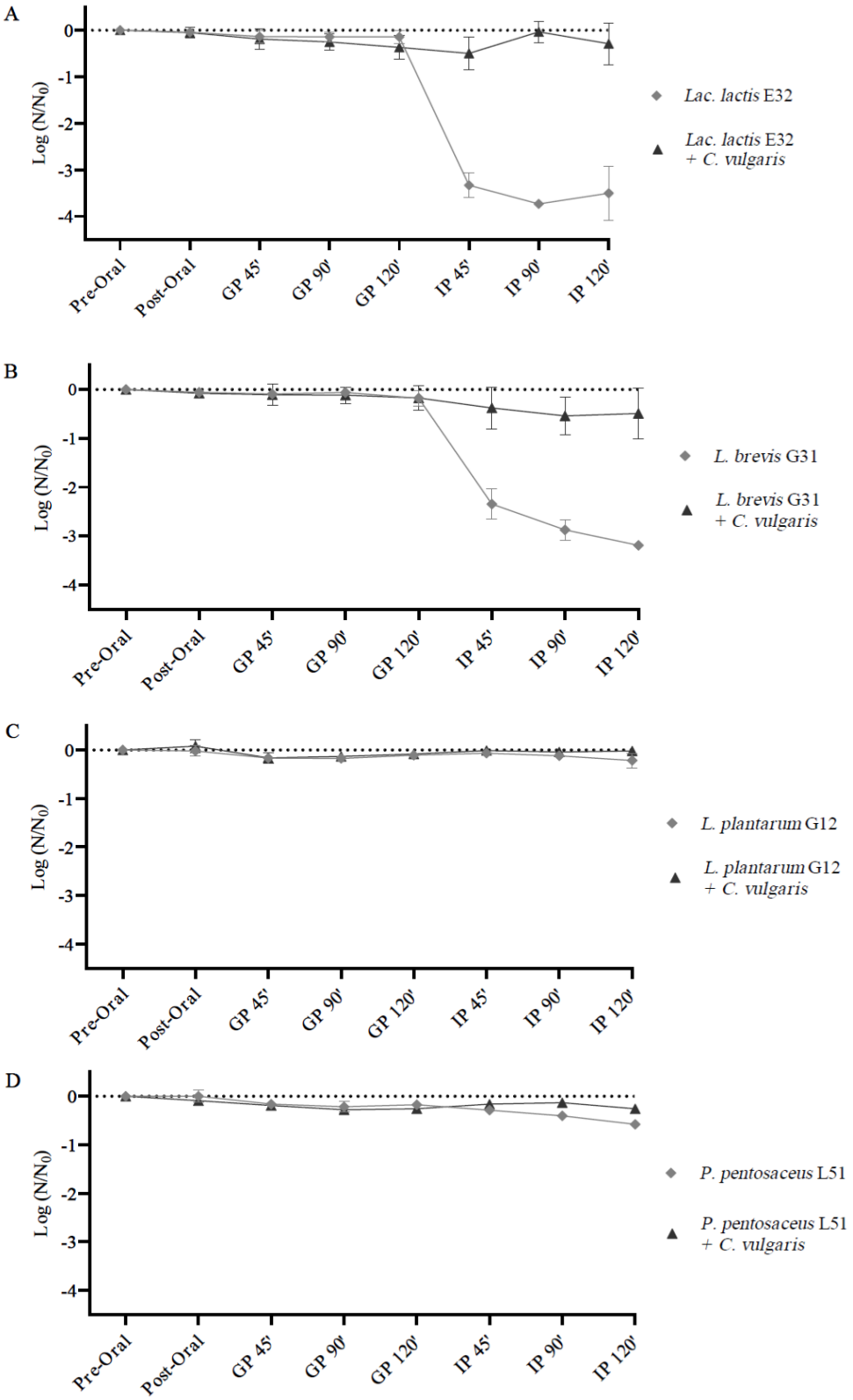


Figure 5.2. Effect of simulated gastrointestinal digestion on the viability of *Lac. lactis* E32 (A), *L. brevis* G31 (B), *L. plantarum* G12 (C) and *P. pentosaceus* L51 (D), each strain in the absence and in the presence of *C. vulgaris*.

Adouard et al. (2016) studied the resistance of *Lac. lactis* strain S3 (cheese origin) through the gastrointestinal digestion using the *in vitro* simulator DIDGI® system, mimicking stomach, duodenum and intestine compartments. *Lactococcus lactis* S3 (initial inoculum of 8.4 log CFU mL^{-1}) showed a decrease of 4 log CFU mL^{-1} at the end of the gastric phase. This decrease was only observed after 70 min, when a pH 2 was reached. After 110 min, in the duodenal phase, it was observed a decrease from 7.2 to 4.8 log CFU mL^{-1} and by the end of the small intestine phase, counts below the detection limit of the enumeration technique were reached; survivors detected only until 150 min. Fernandez et al. (2014) assessed the resistance of *Lac. lactis* ATCC 11454, to digestion, using a dynamic gastrointestinal TIM-1 model simulating four compartments, stomach (pH variated from 5.5 to 1.7), duodenum, jejunum and ileum. In the gastric phase, *Lac. lactis* maintained stability until a pH between 2 and 1.7 was reached, which resulted in a 4.5 log CFU mL^{-1} reduction, and finalized the gastric phase at levels of $5.6 \pm 3.6 \times 10^5$ CFU mL^{-1}. This strain demonstrated to be highly sensitive to bile stress, reaching the jejunal compartment at $2.5 \pm 3.2 \times 10^3$ CFU mL^{-1} and ileum compartment at $2.0 \pm 0.5 \times 10^4$ CFU mL^{-1}. At the end of the experiment a total reduction of 5.21 ± 0.32 log CFU mL^{-1} was observed. Prior to these studies, an *in vitro* model using human gastric (pH 2.5) and duodenal fluids collected form volunteers was applied to assess survival of bacterial cultures that included three *Lac. lactis* ssp. *cremoris* (Ar-1, milk origin and Ar-2, Bf-3, plant origins) and one *Lac*. *lactis* ssp. *lactis* (ML-8, milk origin) strains. The results showed that after exposure to gastric phase viability of both strains was reduced to below 10^2 CFU mL^{-1}. However, in some cases, it was verified a 1 to 2 log increase in the duodenal phase (Faye et al., 2012). In these studies, pH 2 or lower had a strong impact on *Lac. lactis* E32 cells viability. However, when cells were exposed to pH 3, they presented no significative reduction, which is in accordance with the results observed in the present study, whereas the gastric effect was tested at pH 3. Moreover, previous works had shown evidences that intestinal phase was a stress factor to *Lac. lactis* survival, which was corroborated by the results obtained, showing a reduction of more than 3 log CFU mL^{-1}.

The survival of *L. brevis* to gastrointestinal digestion was already assessed in a previous study. In Iseppi et al. (2019), *L. brevis* LP5, isolated from rectal samples, presented a survival rate of 80.4 ± 0.9 % after 1 h and 78.3 ± 2.0 % after 3 h of exposure to simulated gastric fluid at pH 2 and 83 ± 0.9 % after 1h, 78.3 ± 1.1 % after 3h and 79,86 $\pm$ 1.2 % after 4 h of exposure to simulated intestinal fluid. The survival of *L. brevis* G31

strain tested in this work, at the end of digestion, was 0.06 ± 0.01 %, which indicates a poor response to the gastrointestinal stress conditions.

In the present work, the results indicated that *L. plantarum* G12 was not sensitive to gastric or intestinal stress factors, which is not in accordance with previous works. Bove et al. (2012) used a simulated gastrointestinal model constituted by a progressive acidification gastric environment, to determine the resistance to orogastrointestinal transit of *L. plantarum* WCFS1 and some mutant strains grown in skim milk powder. The results showed that during the gastric phase, the viable cell counts were constant until pH 3.0 was reached. However, when pH dropped to 2.0 and 1.5, it was verified a loss of viability of 4 and 8 log CFU mL^{-1}, respectively. In the intestinal phase less than 1 log reduction was observed and, in the sample submitted to 1.5 pH, it was observed a slight recovery. Later, in a work using the same *L. plantarum* strain and gastrointestinal digestion model, bacteria survival was assessed in saline solution. In these conditions, the survival was more affected by the pH and when a pH 3 was achieved, a reduction of 6 log CFU mL^{-1} was verified. At the final part of the gastric phase (pH 2.0 and 1.5) a 8 log CFU mL^{-1} decrease was verified. Additionally, the intestinal stress affected the samples that responded well to the less acidic environment of the gastric phase (pH 6-4), with a loss of viability of 4 log CFU mL^{-1}. On the contrary, intestinal stress had a slight positive effect on the samples that were more affected by the pH reduction (pH 3-1.5), being verified a slight increase in cell counts (Bove et al., 2013). More recently, in a study performed using the modified simulation model described by Bove et al. (2013) to investigate the survival to gastrointestinal digestion of *L. plantarum* WCFS1 and six *L. plantarum* isolates from human faeces, it was demonstrated that upon bacteria exposure to gastric phase (pH 2) for 30 min, cell viability in saline solution decrease by approximately 3 log CFU mL^{-1}. Upon intestinal stress exposure for 1 h, cell viability decreased by 2 log CFU mL^{-1}. No significative differences were observed between strains. As it was previously observed, survival of bacteria in skim milk was more pronounced (Gheziel et al., 2019). However, when comparing the results obtained in this work with those previously obtained, we must take into consideration that earlier studies were carried with bacteria exposed to lower pH values and that the most accentuated reductions of cell viability essentially occurred at pH values lower than 3, environment that was not tested in the protocol followed in this study.

Some studies have been carried in order to characterize the ability of specific strains of *P. pentosaceus* to survive through the gastrointestinal digestion. In the study by Damodharan et al. (2015) gastrointestinal digestion resistance of *P. pentosaceus* strain KID7 was assessed using an assay with a variation of pH 3 to pH 2 during the gastric phase. At the end of the complete digestion process, it was only verified a reduction of 1 log CFU ml^{-1} comparing with initial counts. The survival of *P. pentosaceus* L1, isolated from pickled radish in simulated gastrointestinal juices was assessed by Cao et al. (2016). It was verified that after 3 h of gastric juice (pH 2) exposure a reduction of <0.3 log CFU mL^{-1} was observed. The same reduction was observed after 4 h of exposure to the intestinal juice. These results are in accordance with the evidences of this work, where it was observed a total reduction of 0.6 ± 0.4 log CFU mL^{-1} in the viability of *P. pentosaceus* L51 along the digestion assay. Additionally, previous data suggested that lowering pH to 2 in the gastric phase has not an impact on the survival of *P. pentosaceus* species.

For all the strains studied in this work it was possible to observe an evident increase in their survival through the simulated digestion in the presence of *C. vulgaris*. The positive effect of *C. vulgaris* on LAB survival was previously demonstrated by Ścieszka and Klewicka (2020). In this study four strains of *L. brevis* (ŁOCK 0944, 0980, 0992, and MG451814) were exposed to adverse environmental conditions, namely low pH and the presence of bile salts. It was demonstrated that the addition of *C. vulgaris* to the 24 h culture had a protective effect in the presence of bile salts. Nonetheless, at low pH the protection was strain dependent, being beneficial only for two out of the four strains investigated.

Ashwar et al. (2018) reported that the encapsulation of *L. casei* MTCC 297, *L. brevis* MTCC 01 and *L. plantarum* MTCC 021 with resistant starch type 4 (RS4), a portion of starch which escapes digestion in the small intestine being further fermented in the colon, had a protective effect on the bacterial survival through the simulated gastric and intestinal juices, comparatively to free bacteria. Since starch is the most abundant polysaccharide in *C. vulgaris* (Safi et al., 2014), it can be hypothesized that the protective effect of *C. vulqaris* is related to its starch composition .

5.6. Organic Medium Optimization for Lactic Acid Bacteria Growth

There was an interest by Allmicroalgae in the optimization of the industrial OM, used for *C. vulgaris* growth, to allow LAB cultivation. To achieve that goal, several formulations with varying composition in glucose, yeast extract, peptone and iron (II) sulphate heptahydrate were developed and the growth of *Lac. lactis* E32 was assessed and compared with growth obtained when cultivated in commercial MRS medium and OM without supplementation. Results are shown in Tables 5.3A and 5.3B**.**

It was verified that the organic medium formulation N, with yeast extract 15g L^{-1}, was the only one that did not present significative differences ($P>0.05$) in *Lac. lactis* E32 growth (4.94 ± 0.16 log CFU mL^{-1}), when compared with growth in MRS (4.98 ± 0.16 log CFU mL^{-1}). This result indicates that the addition of a nitrogen source has a major role in growth enhancement. Significative differences ($P<0.05$) were found in growth between Ff formulation, supplemented with glucose 10 g L^{-1} (2.99 ± 0.20 log CFU mL^{-1}) and M formulation, supplemented with yeast extract 10g L^{-1} (4.56 ± 0.11 log CFU mL^{-1}), suggesting that the addition of a nitrogen source has a major impact when compared to the addition of a carbon source. The addition of iron (II) sulphate heptahydrate (0.02 g L^{-1}) was not relevant ($P<0.05$) since growth was not enhanced, when compared to OM (2.17 ± 0.02 and 2.36 ± 0.07 log CFU mL^{-1}, respectively).

Table 5.3. (A) *Lac. lactis* E32 growth in organic medium with no supplementation (OM) and MRS commercial medium; **(B)** *Lac. lactis* E32 growth OM formulations, with varying composition in glucose, yeast extract, peptone, and iron (II) sulphate heptahydrate. Statistical analysis was carried using t-student test, comparing results obtained for OM or MRS with results obtained for each OM formulation. All results presented significative differences, except MRS and formulation N comparison (P>0.05).

A

Medium	Log N/N_0 (CFU mL^{-1})
OM	2.36 ± 0.07
MRS	4.98 ± 0.16

B

OM Formulation	Glucose (g L^{-1})	Yeast Extract (g L^{-1})	Peptone (g L^{-1})	Iron (II) Sulphate Heptahydrate (g L^{-1})	Log N/N_0 (CFU mL^{-1})
Fa	10	2.5	5	0.02	4.17 ± 0.04
Fb	-	2.5	5	0.02	4.06 ± 0.05
Fc	10	2.5	-	0.02	4.01 ± 0.05
Fd	10	-	5	0.02	4.08 ± 0.09
Fe	10	2.5	5	-	3.46 ± 0.074
Ff	10	-	-	-	2.99 ± 0.20
Fg	-	-	5	-	3.19 ± 0.11
Fh	-	2.5	-	-	3.53 ± 0.53
Fi	-	-	-	0.02	2.17 ± 0.02
Ha	15	5	-	-	4.12 ± 0.08
Hb	15	3.75	-	-	4.18 ± 0.12
Hc	15	2.5	-	-	4.08 ± 0.12
Ja	10	5	-	-	4.09 ± 0.06
Jb	10	3.75	-	-	4.12 ± 0.09
Jc	10	2.5	-	-	4.12 ± 0.20
Ka	5	5	-	-	3.97 ± 0.01
Kb	5	3.75	-	-	4.14 ±0.07
Kc	5	2.5	-	-	4.05 ± 0.11
L	5	10	-	-	4.32 ± 0.04
M	-	10	-	-	4.56 ± 0.11
N	-	15	-	-	4.94 ± 0.16

After the N formulation had been selected, the growth of *L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51 was assessed and compared with growth in MRS medium and in organic medium with no supplementation. Growth of each strain was also compared with growth of *Lac. lactis* E32 in N formulation. The results are presented in Table 5.4.

Table 5.4. *Lac. lactis* E32*, L. brevis* G31, *L. plantarum* G12 and *P. pentosaceus* L51 growth in organic medium with no supplementation (OM), N formulation (supplemented with yeast extract 15 g L^{-1}) and MRS commercial medium. Statistical analysis was carried using t-student test.

Strain	Log N/N_0 (CFU mL^{-1})		
	OM	N	MRS
Lac. lactis E32	2.36 ± 0.07	4.94 ± 0.07	4.98 ± 0.16
L. brevis G31	2.43 ± 0.20	4.76 ± 0.13	4.94 ± 0.04
L. plantarum G12	1.41 ± 0.19	4.36 ± 0.10	5.74 ± 0.07
P. pentosaceus L51	1.30 ± 0.01	4.19 ± 0.15	5.13 ± 0.15

Lactobacillus brevis G31 (4.76 ± 0.13 log CFU mL^{-1}) and *Lac. lactis* E32 (4.94 ± 0.07 log CFU mL^{-1}) growth in N formulation (4.76 ± 0.13 log CFU mL^{-1}) was equivalent ($P>0.05$). This behaviour was verified for growth in OM and MRS as well. As it would be expected, no significative differences ($P>0.05$) were verified between *L. brevis* G31 cultivation in N formulation or in MRS. This was not verified for *L. plantarum* G12 and *P. pentosaceus* L51 growth. Therefore, they presented lower growth in N formulation (4.36 ± 0.10 and 4.19 ± 0.15 log CFU mL^{-1}, respectively) when compared with growth in MRS ($P<0.05$). Further work must be done to develop an organic medium formulation that allows optimal growth of *L. plantarum G12* and *P. pentosaceus* L51 strains.

5.7. Vitamin B12 Quantification in Lyophilised Lactic Acid Bacteria and *Chlorella vulgaris*

Chlorella vulgaris is used in animal feed and for human consumption and there is a great interest in increasing its vitamin B12 content in order to enhance the product nutritional value and counter cobalamin deficiency. To achieve this objective *C. vulgaris* paste provided by Allmicroalgae was mixed with LAB, comprising potential vitamin B12 producers *Lac. lactis* E32, *L. brevis* G31 and *P. pentosaceus* L51. This mixture was submitted to a lyophilization process. The vitamin B12 content was determined in microalgae and bacteria mixtures and in freeze-dried powders of pure bacterial cultures. The vitamin B12 content obtained when the bacteria were grown in two different organic medium formulations, N and Fh, was compared.

A significant (P<0.05) increase in cobalamin content from 326.86 ± 30.22 ng g^{-1} to 522.97 ± 23.09 ng $g^{-1}$was only observed upon addition of *P. pentosaceus* L51, grown in organic medium formulation N, to the microalgae culture (figure 5.3A). When formulation Fh was used, it was not verified any significative difference between cobalamin content in *C. vulgaris* and in mixtures comprising *C. vulgaris* and each bacterial strain added. The decrease observed in the vitamin B12 content of the mixture comprising *C. vulgaris* and *Lac. lactis* E32 was not expected. It can be hypothesised that the cellular lysis step in the assay method was not efficient. Therefore, to obtain more reliable results, the lysis process should be further optimized.

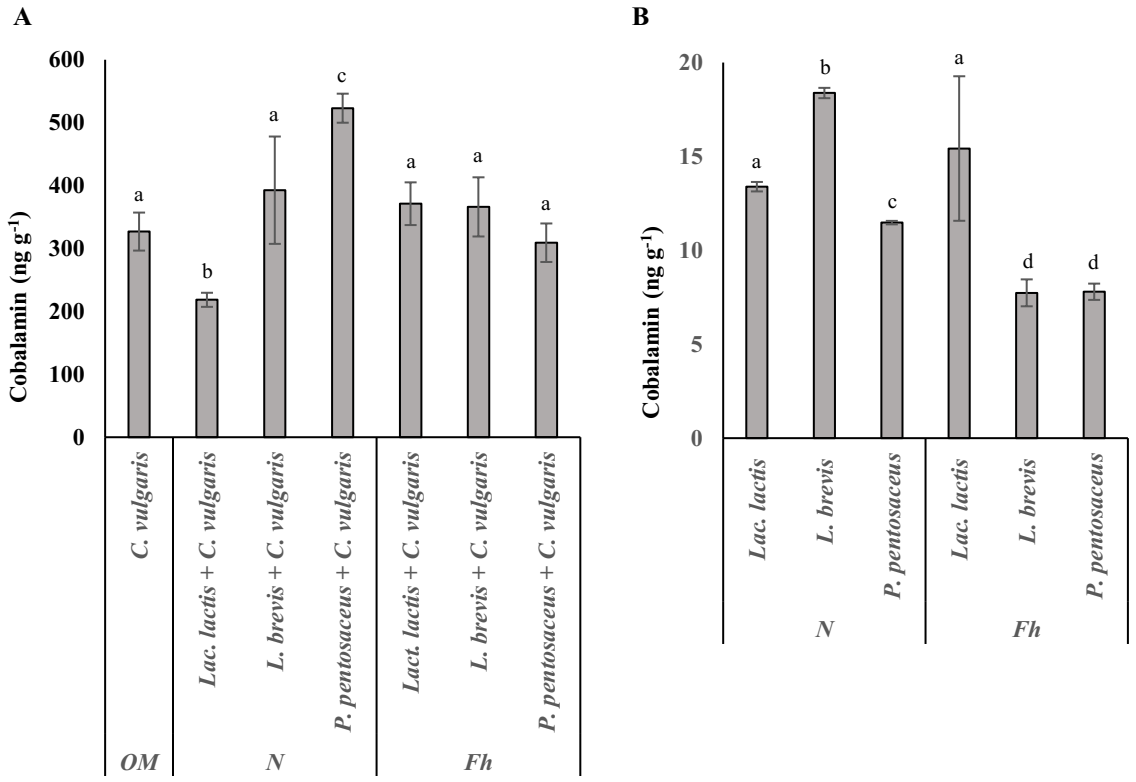


Figure 5.3. **(A)** Cobalamin content in freeze-dried *C. vulgaris* grown in organic medium and freeze-dried coculture comprising *C. vulgaris* and lactic acid bacteria (*Lac lactis* E32, *L. brevis* G32 or *P. pentosaceus* L51) grown in N or Fh organic medium formulation; **(B)** Cobalamin content in freeze-dried *Lac lactis* E32*, L. brevis* G32 or *P. pentosaceus* L51, grown in N or Fh organic medium formulation.

For bacteria single cultures, grown in formulation N, different (P<0.05) cobalamin values were determined for each strain (Figure 5.3B). *Lactobacillus brevis G31* reached the highest vitamin B12 content (18.37 ± 0.27 ng g^{-1}), followed by *Lac. lactis* E32 (13.38

± 0.25 ng g^{-1}) and *P. pentosaceus* L51 (11.55 ± 0.09 ng g^{-1}). *Lactococcus lactis* E32 grown in Fh formulation showed a vitamin B12 production at the same level as verified when grown in N formulation, with a cobalamin content of 15.42 ± 3.9 ng g^{-1}. The vitamin B12 content determined when *L. brevis* G31 (7.73 ± 0.72 ng g^{-1}) and *P. pentosaceus* L51 (7.79 ± 0.43 ng g^{-1}) were grown in Fh formulation was not significantly different ($P<0.05$) and was lower than the content determined when the strains were cultivated in N formulation. Nonetheless, as it was previously referred, it must be taken in consideration that the lysis step still requires optimization.

It was possible to determine a high amount of vitamin B12 in *C. vulgaris* culture, which can be due to three events: 1) During growth, exogenous vitamin B12 is added to the fermenter, in the heterotrophic growth phase, and it was already documented that *C. vulgaris* has uptake capability; 2) Once *C. vulgaris* culture is transferred to the PBR, the associated microbiome includes vitamin B12 producers, and production capacity can be disseminated among bacteria and 3) *C. vulgaris* can also produce cobalamin. This last possibility will be explored in the next section.

5.8. Production of Vitamin B12 by *Chlorella vulgaris* Grown Axenically

In the present work, after growing *C. vulgaris* in axenic culture in Bold Basal, a cobalamin free medium, vitamin B12 was quantified in lyophilized culture resorting to ELISA method. It was possible to determine a production of 10.22 ± 0.53 ng g^{-1}.

Evidence that *C. vulgaris* not only has a vitamin B12 uptake system, but also *de novo* biosynthesis capacity, has already been reported. Watanabe et al. (1997) reported a total cobalamin production of 27.42 ± 0.73 ng g^{-1} (wet weight) by *C. vulgaris* grown in a vitamin B12-free medium. The quantification of methylcobalamin in lyophilized *C. vulgaris*, grown in cobalamin-free bold basal medium, by microbiological, chemiluminescence and a gold nanoparticle (AuNPs) based aptamer analysis revealed a production of 29.87 ± 2 µg/100 g, 26.84 ± 2 µg/100 g and 28.02 ± 2 µg/100 g (dry weight), respectively (Kumudha et al., 2015). Although the determined cobalamin content in this work was lower in comparison with previous studies, these findings are in agreement with

earlier observations reporting *C. vulgaris* production capacity, since vitamin B12 was detected in absence of any exogenous source.

5.9. Survival of Lactic Acid Bacteria during Frozen and Freeze-Dried Storage

Freeze-drying, also known as lyophilisation, is a technique widely adopted to concentrate and store LAB cultures and to incorporating probiotics in food (Velly et al., 2014, Savini et al., 2010). The lyophilization processes involve two major stages. The first one consists in freezing the liquid sample which results in the formation of ice crystals. The second consists in a drying stage, where crystalline ice is removed by sublimation. Additionally to sublimation, a secondary drying step is necessary to remove unfrozen water by desorption (Kasper and Friess, 2011). Freezing can lead to osmotic stress and cells dehydration, while drying induces changes in the physical state of the membrane lipids and changes in proteins structure (Velly et al., 2014). Transition of membrane lipids from a liquid crystal to a gel phase occurs and during rehydration this event is reversed. However, during reversion membrane packing defects may occur which result in a leaker membrane (Leslie et al., 1995). Considering the stress induced in cells during this storage method, strains may experience a loss of viability. Therefore, in this work, survival of *Lac. lactis E32*, *L. brevis* G31 and *P. pentosaceus* L51 was assessed during frozen storage at -80 ºC and during storage in the freeze-dried state. Preserving of cells at -80 º C has proven to be more effective to prevent cell death than at higher temperatures, when no cryoprotection strategy is used (Foschino et al., 1996). Additionally, the survival of LAB in the above mentioned conditions in the presence of *C. vulgaris* was also investigated.

Survival during frozen storage at -80 ºC was assessed for a six weeks period for *Lac. lactis* E32, *L. brevis* G31 in pure cultures and in the presence of *C. vulgaris*. Survival of *P. pentosaceus* L51 and *P. pentosaceus* L51 in the presence of *C. vulgaris* was only studied for four weeks. These determinations for LAB previously grown in both organic medium N and Fh formulations.

The highest viability loss occurred for *Lac. lactis* E32 single culture, grown in N formulation, with a reduction of 2.72 ± 0.00 Log CFU mL^{-1} during 6 weeks of storage. For both *Lac. lactis* E32 and *L. brevis* G31 pure cultures grown in N formulation, it was possible to observe that a major reduction occurred after 1 weak (2.26 ± 0.02 Log CFU mL^{-1} and 1.51 ± 0.06 Log CFU mL^{-1}, respectively) and loss of viability stabilized in the following weeks (Figure 5.4A).

As it was observed for the other growth media, pure cultures of *Lac. lactis* E32 and *L. brevis* G31 suffered a considerable viability reduction ($P<0.05$) after 1 weak (1.21 ± 0.01 Log CFU mL^{-1} and 1.38 ± 0.01 Log CFU mL^{-1}, respectively). In samples comprising LAB grown in Fh formulation, *L. brevis* G31 presented the highest loss in cell viability, with a reduction of 1.43 ± 0.05 Log CFU mL^{-1} at the end of 6 weeks, followed by *Lac. lactis* E32 with a reduction of 1.29 ± 0.06 Log CFU mL^{-1} (Figure 5.4B).

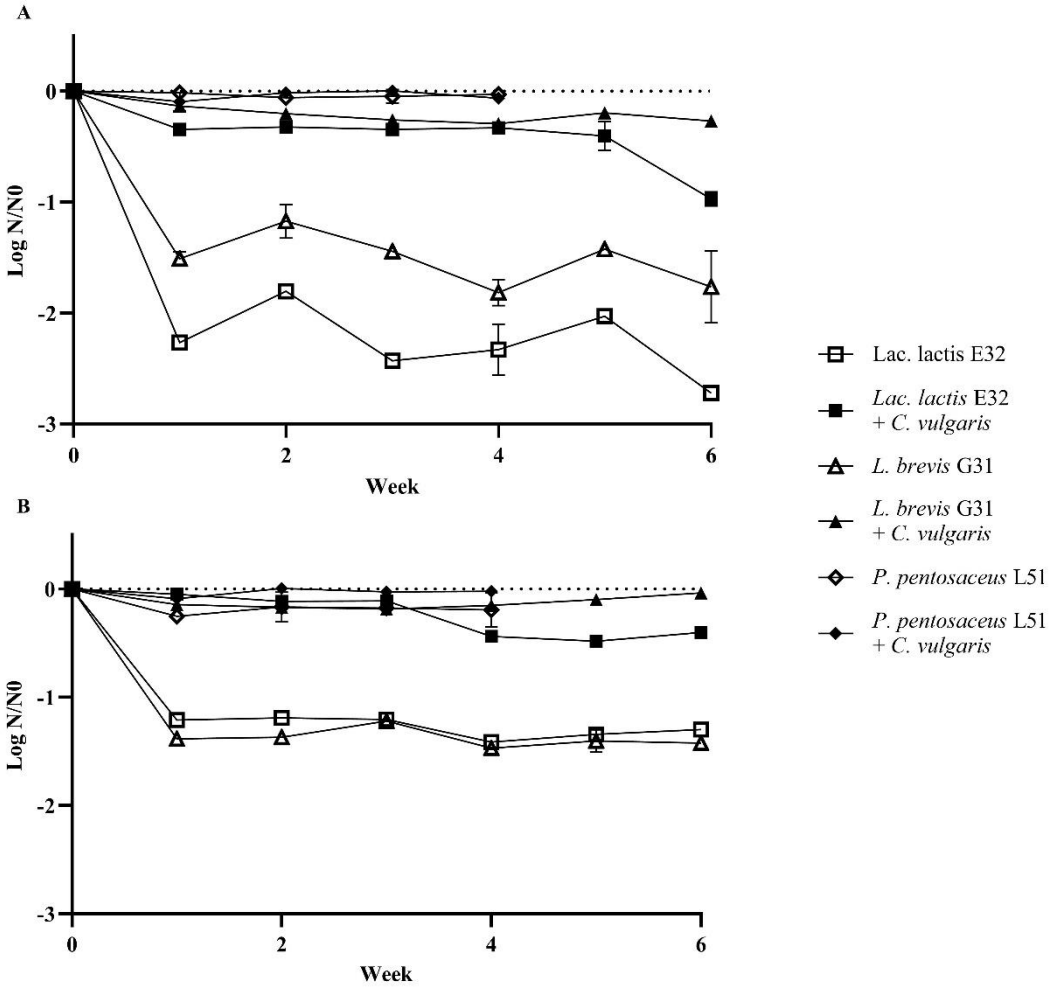


Figure 5.4. Effect of frozen storage at -80 ºC on viability (Log CFU mL^{-1}) of (A) *Lac. lactis* E32, *L. brevis* G31, *P. pentosaceus* L51 grown in organic medium N formulation, and their respective LAB and *C. vulgaris* mixture, and (B) *Lac. lactis* E32, *L. brevis* G31, *P. pentosaceus* L51 grown in organic medium Fh formulation, and their respective LAB and *C. vulgaris* mixture. *Lac. lactis* E32 and *L. brevis* G31 survival was assessed for a 6 weeks period and *P. pentosaceus* L51 survival was assessed for a 4 weeks period.

Pediococcus pentosaceus L51 has shown to be the most stable strain, and no significant differences in viable cell reduction were observed between the two growth media ($P>0.05$). The highest viability loss occurred in pure culture, grown in Fh formulation, where a reduction of 0.25 ± 0.02 Log CFU mL^{-1} was observed at the end of the first week, stabilizing in the next following two weeks.

The presence of *C. vulgaris* demonstrated a protective effect on the survival *Lac. Lactis* E32 and *L. brevis* G31 in comparison with pure cultures ($P<0.05$) but had no significative ($P>0.05$) impact on *P. pentosaceus* L51. The total viability loss of *Lac. Lactis* E32, *L. brevis* G31 (6 weeks) and *P. pentosaceus* L51 (4 weeks) in the presence of

C. vulgaris were 0.97 ± 0.07 Log CFU mL^{-1}, 0.27 ± 0.01 Log CFU mL^{-1} and 0.06 ± 0.02 Log CFU mL^{-1}, when LAB were grown in N formulation, and 0.40 ± 0.05 Log CFU mL^{-1}, 0.04 ± 0.02 Log CFU mL^{-1} and 0.02 ± 0.02 Log CFU mL^{-1}, when LAB were grown in Fh formulation.

The behaviour of LAB towards low temperature stress is species and strain dependent. It was proposed that cell size is related to resistance to freezing and that a lower surface area/ volume ratio is associated with a higher cell injury due to intracellular ice formation (Mazur, 1970). This occurs since in smaller and spherical cells, a higher cell surface facilitates cell water loss during cooling (Mazur, 1977). These events were observed by Fonseca et al. (2000), where smaller *Streptococcus thermophilus* showed greater resistance to freezing (-20 ºC) when compared to *L. lactis* strains, that in turn were more resistant to freezing than bigger *L. bulgaricus* cells. In the present work, *P. pentosaceus* L51 showed to be the most resistant strain to the freezing process, and in contrast with the other two strains that revealed an abrupt loss of viability at the end of the first week, no significant losses occurred. *Pediococcus* cells are spherical and with a diameter of 0.5-0.8 μm and *L. brevis* cells are rod-shaped with 0.7-1.0 × 2.0-4.0 μm (Holzapfel et al., 2006, Teixeira, 1999). This is in agreement with previous observation relating cell size with survival to freezing. However, since *Lac. lactis* cells possesses a diameter of 0.75-0.95 μm, it would be expected that *Lac. lactis* E32 behaved similarly to *P. pentosaceus* L51, which was not verified (Kokkinosa et al., 1998). Nonetheless, in previous experiments, freezing was performed at controlled cooling rates and the cooling rate was not controlled in this experiment.

Finally, the survival of each LAB strain during storage, when freeze-dried in pure culture or in the presence of *C. vulgaris* was studied. The results for LAB grown in N formulation (Figure 5.5A) show that one week after lyophilization, *Lac. lactis* E32 suffered a reduction of 1.23 ± 0.32 Log CFU g^{-1}, reaching a reduction of 2.83 ± 0.25 Log CFU g^{-1} at the end of the 6 weeks storage. For *L. brevis* G31 significant viability losses were only observed at the third week ($P<0.05$), and after 6 weeks of storage suffered a reduction of 1.77 ± 0.27 Log CFU g^{-1}. Survival of *P. pentosaceus* L51, was assessed for four weeks and during this period counts remained stable until the third week, being observed a significative reduction of 0.18 ± 0.05 Log CFU g^{-1} on cells viability at the end of the fourth week ($P<0.05$).

Although it was verified a protective effect on LAB when *C. vulgaris* was present during frozen storage, the opposite effect was observed for freeze-dried cultures of *Lac. lactis* E32 and *L. brevis* G31 (Figure 5.5A). At the third and fifth week, in the presence of *C. vulgaris,* counts of *Lac. lactis* E32 and *L. brevis* G31, respectively, reached a value below the enumeration method's LOD. Although *C. vulgaris* had a less harmless effect on *P. pentosaceus* L51, in the mixture comprising *P. pentosaceus* L51 and *C. vulgaris* this bacterial strain suffered a statistically relevant viability decrease ($P<0.05$) in the second week, achieving a total reduction of 0.76 ± 0.01 Log CFU g^{-1} by the end of the fourth week.

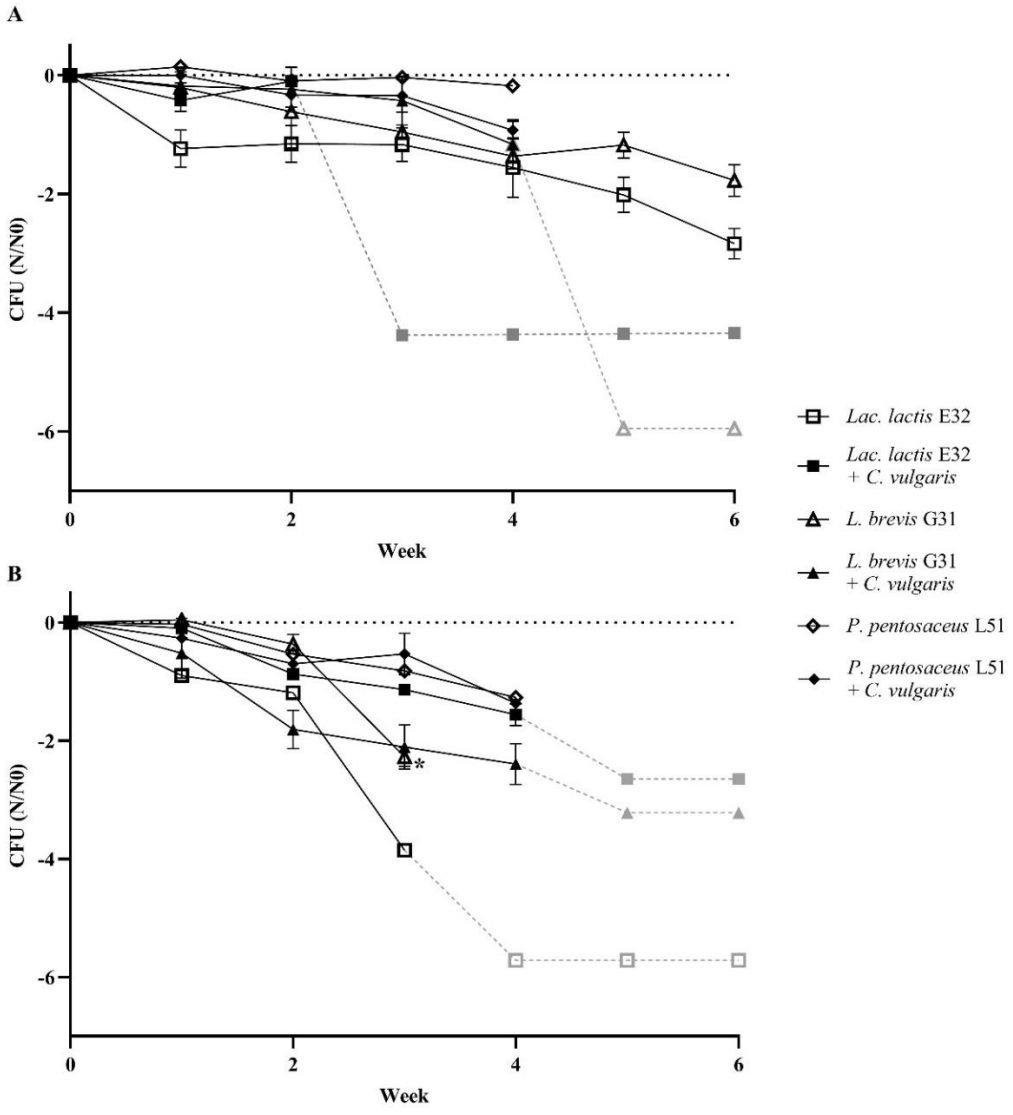


Figure 5.5. (A) Effect of lyophilization on viability (Log CFU g^{-1}) of *Lac. lactis* E32, *L. brevis* G31, *P. pentosaceus* L51 grown in organic medium N formulation, and their respective LAB and *C. vulgaris*

mixture, during freeze-dried storage; *Lac. lactis* E32 and *L. brevis* G31 survival was assessed for a 6 weeks period and *P. pentosaceus* L51 survival was assessed for a 4 weeks period; (B) Effect of lyophilization viability (Log CFU g^{-1}) of *Lac. lactis* E32, *L. brevis* G31, *P. pentosaceus* L51 grown in organic medium Fh formulation, and their respective LAB and *C. vulgaris* mixture. *Lac. lactis* E32 and *L. brevis* G31 survival was assessed for 4 weeks and *P. pentosaceus* L51 survival was assessed for 4 weeks; Streaked lines and grey symbols represent quantification below LOD; (*) last quantification due to sample lost.

When LAB were grown in Fh formulation (Figure 5.5B), it was observed that after three weeks, *Lac. lactis* E32 and *L. brevis* G31 suffered a viability reduction of 3.85 ± 0.05 Log CFU g^{-1} and 2.27 ± 0.16 Log CFU g^{-1}, correspondingly. It was not possible to determine cell counts in the *Lac. lactis* E32 freeze-dried powder from forth week forward since it was below method's LOD. *Lactobacillus brevis* G31 survival was also only assessed until the third week, since a low quantity of sample was obtained and additionally the sample was prone to Maillard reactions during storage. *Pediocoocus pentosaceus* L51 showed a total reduction of 1.26 ± 0.03 Log CFU g^{-1} at the end of four weeks.

Once more, it was verified that the presence of *C. vulgaris* has a negative impact on the survival of *Lac. lactis* E32 and *L. brevis* G31 samples (Figure 5.5B). In the presence of *C. vulgaris* viable cell counts of *Lac. lactis* E32 and *L. brevis* G31 significantly ($P<0.05$) decreased after four weeks, with an overall reduction of 1.56 ± 0.18 Log CFU g^{-1} and 2.39 ± 0.35 Log CFU g^{-1}, respectively. From the fifth week onwards, counts reached the LOD. The presence of *C. vulgaris* only negatively influenced the survival of *P. pentosaceus* L51 on the fourth week ($P<0.05$), being verified a reduction of 1.37 ± 0.01 Log CFU g^{-1}. Until the third week cell counts remained stable.

Overall, organic medium N formulation is more adequate as transport medium for lyophilization due to its higher solids content. Additionally, the obtained powders are not prone to Maillard reactions, and cell survival is higher after the third week when compared with Fh formulation.

The addition of protective agents has been studied to enhance bacterial survival after freeze-drying and some studies were already reported for *Lac. lactis*, *L. brevis* and *P. pentosaceus* strains. The addition of 10% of trehalose showed to have a protective effect on a *Lac. lactis* strain upon freeze-drying and long-term storage (Archacka et al., 2019). In Borges et al. (2013) a *P. pentosaceus* SB83 strain was lyophilized in skim milk and remained stable during storage for 9 months at room temperature; a higher survival

was obtained during storage at 4 ºC. Although skim milk has been shown to have a protective effect, Damodharan et al. (2015) observed that the addition of galactomannan to skim milk potentiated the survival of *P. pentosaceus* KID7 strain. In Zhao and Zhang (2005), yeast extract and sodium glutamate demonstrated to be the best protectants for a *L. brevis* strain. Additionally, in the same work, it was verified that the highest survival was achieved by adding 10% sucrose upon rehydration. Therefore, the addition of a protective agent could be a possibility to enhance cell survival, especially in *Lac. lactis* E32 and *L. brevis* G31 strains which have been shown to be more sensitive to lyophilization.

So far, the survival of dried LAB in the presence of *C. vulgaris* had not been investigated. More studies are necessary to understand the harmful effect of microalgae upon lyophilized LAB.

6. CONCLUSION

Lactic acid bacteria are widely used as probiotics due to their beneficial health effects. Vitamin B12 deficiency is a common condition that has several negative impacts on health since it is essential for many biological reactions. Several strains have been identified as cobalamin producers and there is an interest in developing new formulations to supress vitamin B12 deficiencies.

In the present work, LAB were isolated from *C. vulgaris* cultivated in photobioreactors. Of the four strains selected, *Lac. lactis* E32, *L. brevis* G31 and *P. pentosaceus L51* were identified as potential cobalamin producers, while *L. plantarum* G12 did not show production capacity. Through comparative genomics, genes *hemL, cbiT, cobD* and *cobC*, involved in different steps of the biosynthetic pathway, were identified in *Lac. lactis* E32, *L. brevis* G31 and *L. plantarum* G12 genome. In the *P. pentosaceus* L51 genome, although *hemL* was not found, *cobA* was identified. However, although the E-values considered were low, the percentage of sequence identity of most of the codified proteins was bellow 30%. Additionally, only a few genes involved in cobalamin synthesis were found, thus not being possible to conclude if biosynthetic pathway is present. Nonetheless, other genes related to cobalamin synthesis, found in the same cluster, were detected. For instance, all strains harboured *cbiO*, that codifies a cobalt import ATP-binding protein. *Pediococcus pentosaceus* L51, the most promising cobalamin producer, harboured *pduU* and *pduV* genes, which are involved in propanediol cobalamin-dependent utilization. Taken together, this can be considered evidence of the cobalamin operon presence.

Concerning probiotic potential, susceptibility of the isolates to antibiotics was within the stablished standards and the presence of virulence genes was not detected. Haemolytic capacity and biogenic amines production results were also within the requirements. All strains survived to gastrointestinal digestion, and *L. plantarum* G12 and *P. pentosaceus* L51 presented the highest survival rates. Additionally, the presence of *C. vulgaris* had a protective effect through digestion.

Upon selection of *Lac. lactis* E32, *L. brevis* G31 and *P. pentosaceus* L51 as potential vitamin B12 producers, it was assessed if adding each LAB culture to *C. vulgaris* could increase its vitamin B12 content. Adding *P. pentosaceus* L51 grown in

organic medium N formulation to *C. vulgaris* culture successfully increased vitamin B12 content in the mixed culture freeze-dried powder. Higher cobalamin content was detected in *L. brevis* G31 and *P. pentosaceus* L51 grown in organic medium N formulation than in *L. brevis* G31 and *P. pentosaceus* L51 grown in organic medium Fh freeze-dried powders. Nonetheless, the quantification method requires further optimization. Additionally, *C. vulgaris* grown axenically in cobalamin-free medium revealed capacity of synthetizing vitamin B12.

Finally, the effect of frozen storage and lyophilization processes on cell survival was studied. If *C. vulgaris* has a beneficial effect on the viability of LAB strains during frozen storage, the opposite effect was verified during storage of freeze-dried cultures. *Pediococcus pentosaceus* L51 demonstrated to be the more resistant to adverse conditions than *Lac. lactis* E32 and *L. brevis* G31.

In conclusion, given the fact that *P. pentosaceus* L51 showed the strongest evidence of possessing cobalamin biosynthesis capacity and showed a robust survival response to stress conditions, it can be considered a potential strain for developing high vitamin B12 formulations.

7. FUTURE WORK

Future research will focus on employing a vitamin B12 detection method that can distinguish between pseudocobalamin and biologically active cobalamin, such as HPLC or MSMS. The impact of changing bacteria growth condition to anaerobiosis on cobalamin production will be assessed. Additionally, further genomic studies addressing the presence of genes involved in the cobalamin synthesis will be carried, using a third-generation sequencing technique as PacBio, to complement sequencing results obtained with Illumina.

The lyophilization processes will be optimized trough the addition of protective agents that enhance cell survival. Moreover, the negative impact of *C. vulgaris* culture in freeze-dried LAB powders will be revaluated after the addition of protective agents. The vitamin B12 quantification methodology will be also optimized, in other to obtain more reliable results on cobalamin content in freeze-dried powders.

Considering the aim of using *C. vulgaris* and LAB cocultures as fortified foods, exploiting LAB probiotic potential, in the future, the capability of the isolated strains to adhere and colonize enterocytes will be assessed. Additionally, characterization of LAB probiotic potential will be further explored. Finally, it will be determined vitamin B12 stability during gastrointestinal digestion.

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