FACULTY OF AGRICULTURE AND FORESTRY
DEPARTMENT OF FOOD AND NUTRITION
EKT-series 1950

IN SITU FORTIFICATION OF VITAMIN B12 IN GRAIN MATERIALS BY FERMENTATION WITH PROPIONIBACTERIUM FREUDENREICHII

CHONG XIE

Department of Food and Nutrition
University of Helsinki
Finland

EKT-series 1950

In situ fortification of vitamin B12 in grain materials by fermentation with *Propionibacterium freudenreichii*

Chong Xie

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki for public examination in Metsätalo room 4, Unioninkatu 40, on 7th August 2020, at 12 noon.

Helsinki 2020

Custos: Associate Professor Kati Katina
Department of Food and Nutrition
University of Helsinki, Finland

Supervisors: Associate Professor Kati Katina
Department of Food and Nutrition
University of Helsinki, Finland

Professor Vieno Piironen
Department of Food and Nutrition
University of Helsinki, Finland

Docent Pekka Varmanen
Department of Food and Nutrition
University of Helsinki, Finland

Pre-examiners: Professor Cornelia Witthöft
Department of Chemistry and Biomedical Sciences
Linnaeus University, Sweden

Dr Vesa Joutsjoki
Senior Scientist
Natural Resources Institute Finland, Finland

Opponent: Associate Professor Fabio Minervini
Department of Soil, Plant and Food Sciences
University of Bari Aldo Moro, Italy

ISBN 978-951-51-6355-4 (paperback)
ISBN 978-951-51-6356-1 (PDF)
ISSN 0355-1180

Unigrafia
Helsinki 2020

Abstract

Vitamin B12 is a micronutrient that is predominantly present in food of animal origin. Therefore, developing plant-origin food with a nutritionally relevant content of vitamin B12 can increase the dietary intake of this vitamin among people with limited consumption of animal products. Since its chemical synthesis is overly complicated and expensive, the commercial vitamin B12 used for food fortification is exclusively produced via a biotechnological process. As compared to fortification with this commercial form of vitamin B12, *in situ* fortification via fermentation can be a more cost-effective alternative. As a commonly consumed staple food, grains are excellent vehicles for enrichment with micronutrients. *Propionibacterium freudenreichii* is the only food-grade microorganism with the ability to produce vitamin B12. Because *P. freudenreichii* has a low growth rate and is sensitive to acidic conditions, sterilized grain materials have mostly been used so far to produce a high vitamin B12 content. The sterilization process, however, alters the technological properties of grain-based raw materials and decreases the feasibility of the process. The present thesis focuses on *in situ* fortification of vitamin B12 in native grain materials by fermentation with *P. freudenreichii*.

This study has demonstrated that fermentation of wheat flour, whole-wheat flour and wheat bran with *P. freudenreichii* resulted in a physiologically significant level of vitamin B12 (up to 155 ng/g dw) after 7 days. Whole-grain wheat flour and wheat bran had a higher content of vitamin B12 than refined wheat flour. However, the propagation of *Enterobacteriaceae* indicated that monoculture fermentation with *P. freudenreichii* cannot dominate the microflora, to guarantee microbial safety and control endogenous microbiota present in grain materials.

Thus, an effective co-culture of *Lactobacillus brevis* ATCC 14869 and *P. freudenreichii* was established through a pre-screening to ensure microbial safety. During co-fermentation in wheat bran, *P. freudenreichii* produced a high level of vitamin B12 (ca. 183 ng/g dw on day 3). Moreover, controlling pH during fermentation could greatly enhance the vitamin B12 production (up to 332 ng/g dw on day 3). Meanwhile, *L. brevis* showed a strong inhibition on the propagation of *Enterobacteriaceae* during fermentation, as expected.

The wider applicability of the established co-culture was demonstrated by fermenting 11 types of grain materials, including cereals, pseudocereals and legumes, with *P. freudenreichii* and *L. brevis*. *P. freudenreichii* produced a nutritionally significant level of vitamin B12 in most of the grain materials. The highest production was found in the rice bran (ca. 742 ng/g dw), followed by the buckwheat bran (ca. 631 ng/g dw), after fermentation. Meanwhile, the addition of *L. brevis* was able to dominate indigenous microbes during fermentation and thus greatly improve microbial safety during the fermentation of different grain materials.

Overall, this thesis demonstrates that the fermentation of grain materials with *P. freudenreichii* and an appropriate co-culture, such as *L. brevis*, is a promising way to provide vitamin B12 in non-sterilized grain-based materials, without compromising microbial safety. Meanwhile, selecting raw materials that provide optimal conditions for *P. freudenreichii* can significantly improve the efficacy of vitamin B12 synthesis.

Acknowledgements

This study was carried out at the Department of Food and Nutrition, Faculty of Agriculture and Forestry, University of Helsinki during 2016-2020. The work was funded by China Scholarship Council, Academy of Finland and Dissertation Completion Grant from University of Helsinki. These financial supports are greatly appreciated.

My sincere gratitude goes to my main supervisor, associate professor Kati Katina. Thank you for introducing me to the field of grain technology and giving me your insightful advices, critical comments and warm encouragement. I also would like to thank my co-supervisors, professor Vieno Piironen and Docent Pekka Varmanen, for providing constructive advices and valuable guidance throughout my study. I feel so lucky to have three of you as my supervisors and I am sure what I learned from you will be extremely helpful in my future career.

I kindly thank my follow-up group members Dr. Riikka Juvonen and Dr. Pekka Lehtinen for their valuable feedback and kind assistance on my work. I am also obligated to Professor Cornelia Witthöft and Dr Vesa Joutsjoki for their critical evaluation of this dissertation.

I owe my gratitude to Docent Rossana Coda, Dr. Minnamari Edelmann, Dr. Bhawani Chamlagain and Dr. Paulina Deptula for their guidance on experimental work and valuable contributions in planning of my research and preparation of my manuscripts.

Other colleagues from our group, adjunct Professor Frederick Stoddard, Docent Tuula Sontag-Strohm, Docent Ndegwa Henry Maina, Dr. Elisa Arte, Dr. Noora Mäkelä, Dr. Xin Huang, Dr. Yujie Wang, Yaqin Wang, Mikko Immonen, Hanna Nihtilä, Prabin Koirala and Hanna Ahola, are thanked for creating such a pleasant and positive working atmosphere.

In addition, I would also like to express my gratitude to the technical staffs: Outi Brinck, Jutta Varis, Taru Rautavesi, Miikka Olin and Mikko Kangas. I sincerely

thank my Chinese friends at the department: Dr. Yan Xu, Dr. Yulong Bao, Dr. Zhen Yang, Dr Chang Liu, Bei Korpela, Yuemei Zhang, Hongbo Zhao, Jian Lyu, Fengyuan Liu, Xiaocui Han for your kind support in my PhD study and daily life.

Last but not least, I owe my dearest gratitude to my dearest family. To my wife Ke Xu, I am so grateful for all of your support for these years. Without you, I would not be where I am today. To my lovely daughter Ruxu Xie, thank you for bringing me endless happiness and I am sorry for frustrating you by "not right now, daddy is writing dissertation" every now and then. To my parents, thank you for your unconditional love and support which meant the world to me.

Helsinki, June 2020

Chong Xie

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscript:

I. Xie, C., Coda, R., Chamlagain, B., Edelmann, M., Deptula, P., Varmanen, P., Piironen V. and Katina, K. (2018). *In situ* fortification of vitamin B12 in wheat flour and wheat bran by fermentation *with Propionibacterium freudenreichii*. *Journal of Cereal Science*, 81, 133-139.

II. Xie, C., Coda, R., Chamlagain, B., Varmanen, P., Piironen V. and Katina, K. (2019). Co-fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in wheat bran for *in situ* production of vitamin B12. *Frontiers in Microbiology*, 10, 1541.

III. Xie, C., Coda, R., Chamlagain, B., Edelmann, M., Varmanen, P., Piironen V. and Katina, K. Fermentation of grain materials with *Propionibacterium freudenreichii* and *Lactobacillus brevis* for Vitamin B12 fortification, *submitted*

The author's contributions

I, II and III Chong Xie planned the study together with the other authors. He performed most of the experiments and had the main responsibility for interpreting the results and preparation the manuscripts. He acted as the corresponding author of the papers.

Abbreviations

AdoCbl	Adenosylcobalamin
ALA	Aminolevulinic acid
ANOVA	One-way analysis of variance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection, USA
CFU	Colony forming units
CNCbl	Cyanocobalamin
DMBI	5,6-dimethylbenzimidazole
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dw	dry weight
GRAS	Generally recognized as safe
HOCbl	Hydroxocobalamin
HPAEC-PAD	High performance anion exchange chromatography with pulse amperometric detection
HPCE	High-performance capillary electrophoresis
HPLC	High performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
LAB	Lactic acid bacteria
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MeCbl	Methylcobalamin
MRS	de Man, Rogosa and Sharpe
MS	Mass spectrometry
PAB	Propionic acid bacteria
PLS	Partial least squares
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TTA	Total titratable acidity
UHPLC	Ultra-high performance liquid chromatography
VRBGA	Violet red bile glucose agar
YEL	Yeast extract-lactate
YM	Yeast malt

Table of Contents

1 Introduction

Vitamin B12 is synthesized only by certain microorganisms and it is accumulated in the tissues of higher predatory organisms (Martens et al., 2002). Therefore, the main source of dietary vitamin B12 are animal-origin products such as milk, meat, eggs and fish (Watanabe, 2007). Deficiency in vitamin B12 may result in various health problems, such as megaloblastic anemia, cognitive impairment, memory loss, stupor and psychosis (Hunt et al., 2014). Although clinical vitamin B12 deficiency is uncommon at present, globally, subclinical deficiency of vitamin B12 is commonly present, especially among people with a low intake of animal products (Green et al., 2017; Smith et al., 2018). Moreover, the current trend of replacing animal-based food products with plant-based ingredients may decrease global dietary vitamin B12 intake (Marsh et al., 2011). Therefore, developing vitamin B12-fortified plant-based food products is increasing vital for the future food system.

In situ fortification, via fermentation with selected starter cultures, is an economical and efficient way to introduce micronutrients into food. Up to now, *Propionibacterium freudenreichii* is the only food-grade microorganism known to synthesize active vitamin B12 (Piwowarek et al., 2018). *P. freudenreichii* is traditionally utilized as the secondary starter culture in Swiss-style cheeses, for its key role in the formation of both the flavor and the "eyes" that characterize such cheeses by converting lactic acid into propionic acid, acetic acid and CO_2 (Langsrud and Reinbold, 1973). In recent decades, *P. freudenreichii* has been studied for *in situ* fortification of vitamin B12 in some food products, such as sauerkraut (Babuchowski et al., 1999), lupine tempeh (Signorini et al., 2018) and kefir (Van Wyk et al., 2011).

Grains are ideal vehicles for food fortification because they are the most consumed staple food around the world. Recently, a nutritionally relevant amount of vitamin B12 was produced by fermentation with *P. freudenreichii* in autoclaved aqueous barley or wheat matrices (Chamlagain et al., 2017). However, heat treatment often leads to inferior technological properties in grains, due to altered functionality of proteins (especially enzymes) and starch (Mann et al., 2013).

Therefore, it is worthwhile to study the *in situ* production of vitamin B12 during fermentation with *P. freudenreichii* in native grain materials. *P. freudenreichii* is a slow-growing bacterium, compared to microorganisms commonly present in microflora of grains, such as lactic acid bacteria (LAB). Thus, its ability to produce a nutritionally relevant level of vitamin B12 in native grain materials is still unknown. Another question that needs to be studied is whether *P. freudenreichii* is able to compete with and dominate the potential pathogens in grains, to guarantee microbial safety during fermentation.

The overall aim of this thesis was to study the *in situ* fortification of vitamin B12 in grain-based materials, by fermentation with *P. freudenreichii*. The literature review provides an overview of the structural features, metabolic functions, dietary sources and analytical methods of vitamin B12. A brief introduction of the main types of grain (cereals, pseudocereals and legumes) and their fermentation is also presented. In addition, an overview of the microbial biosynthesis (mainly by *P. freudenreichii*) of vitamin B12 is given and the challenges of *in situ* fortification in grains are discussed. The experimental methods and results in three publications (studies I-III) are summarized. Finally, the significance of *in situ* fortification in native grain materials and the method to enhance vitamin B12 production during fermentation are discussed. The requirement for co-fermentation with lactic acid bacteria, during *in situ* fortification, is likewise explained.

2 Review of the literature

2.1 Vitamin B12

2.1.1 Chemical structure

Vitamin B12 (Figure 1), or cobalamin, refers to a group of corrinoids having a cobalt ion in the center of the corrin ring and two axial ligands, the lower and upper ligand, coordinated to the cobalt ion (Combs and McClung, 2017). The upper ligand can be a methyl group, a 5'-deoxyadenosyl group, a hydroxyl ion or a cyano group, thereby forming a methylcobalamin (MeCbl), a 5'-deoxyadenosylcobalamin (AdoCbl), a hydroxocobalamin (HOCbl) or a cyanocobalamin (CNCbl). Among these, MeCbl and AdoCbl are the two biologically active forms of vitamin B12. Yet CNCbl and HOCbl can be converted into these two active forms to exert a biological effect. The cobalt-carbon bonds of MeCbl and AdoCbl break under light exposure, leading to the formation of HOCbl (Obeid et al., 2015). CNCbl is the commercial form commonly used in food fortification and supplements, because it has the highest stability of all the forms (Martens et al., 2002).

The lower ligand of active vitamin B12 is 5, 6-dimethylbenzimidazole (DMBI), which is additionally linked to ribose-3-phosphate through a glycosidic link. There are some cobalt-containing corrinoids that otherwise share a similar structure with vitamin B12, yet differ in the lower ligand (Watanabe et al., 2002). Although these compounds are active in microorganisms, they are inactive in the human body (Stupperich and Nexo, 1991).

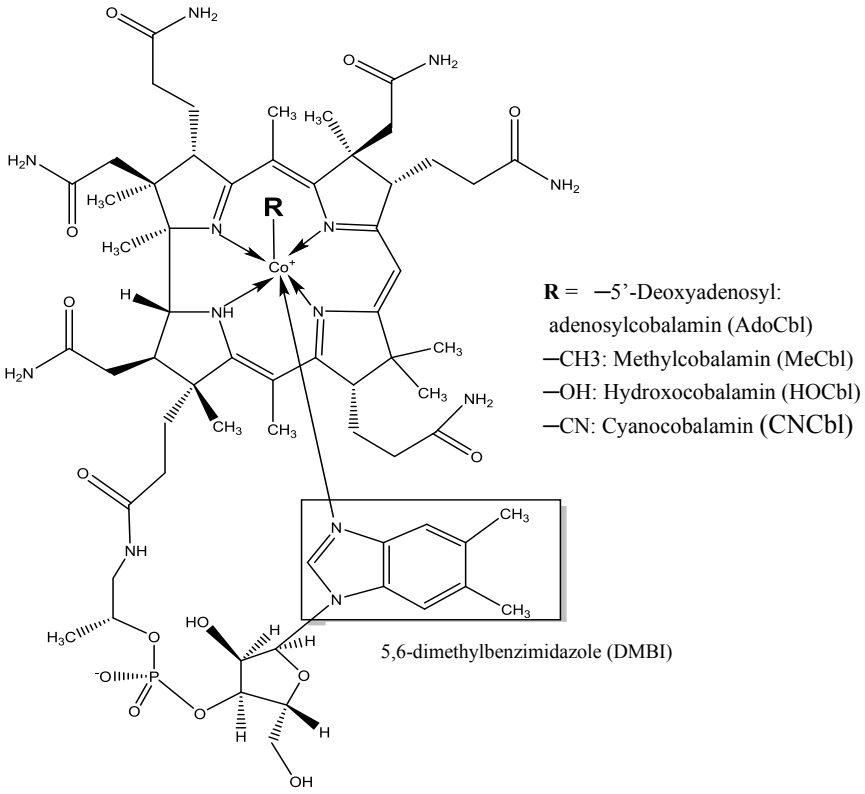

Figure 1. Structure of vitamin B12, with the lower ligand DMBI in the box and the list of 4 alternative upper ligands (R)

2.1.2 Absorption and physiological function in the human body

In the human body, vitamin B12 is mainly absorbed by receptor-mediated endocytosis, with the help of three homologous carrier proteins: haptocorrin, intrinsic factor and transcobalamin (Nielsen et al., 2012). First, dietary vitamin B12, released in the upper gastrointestinal tract, is bound to haptocorrin, which is mainly secreted from the salivary glands (Morkbak et al., 2007). The binding of vitamin B12 to haptocorrin is believed to protect the vitamin from hydrolysis by the acidic environment in the stomach (Allen et al., 1978). In the duodenum, haptocorrin is degraded by pancreatic enzymes. The released vitamin B12 is then captured by intrinsic factor, which is resistant to the enzymes from the pancreas (Nielsen et al., 2012). Finally, the intrinsic factor–vitamin B12 complex in the terminal ileum is absorbed through receptor-mediated endocytosis and vitamin B12 released from the complex is then transferred into the blood stream, with the

help of holo-transcobalamin II protein (Quadros et al., 1999). However, there is another pathway for vitamin B12 absorption, in which vitamin B12 is absorbed by diffusion across the epithelial ileum without requiring intrinsic factor or its receptors. This pathway is observed in the case of ingesting a large amount of vitamin B12, such as by taking vitamin B12 supplements and about 1% of the ingested vitamin B12 can be absorbed in this way (Herbert, 1988).

In mammal cells, vitamin B12 functions as a cofactor in metabolic reactions in the form of AdoCbl or MeCbl (Green and Miller, 2013). MeCbl is involved in the synthesis of myelin, by acting as a coenzyme in the methylation of homocysteine to form methionine (Pawlak et al., 2012). Moreover, by activating folate in the methylation, MeCbl also affects the synthesis of DNA (Selhub and Paul, 2011). AdoCbl is involved in the catabolism of cholesterol, odd-chain fatty acids and some branched amino acids, by acting as a cofactor for methylmalonyl-CoA mutase (Takahashi-Iniguez et al., 2012).

2.1.3 Dietary sources of vitamin B12

Vitamin B12 biosynthesis is restricted to microorganisms. It is accumulated mainly in the bodies of higher predatory organisms, in the natural food chain (Martens et al., 2002). Therefore, the main sources of dietary vitamin B12 are animal-origin food products, including milk, meat, eggs, shellfish and fish, as listed in Table 1 (Watanabe, 2007). Some plant-origin foods, such as edible algae or cyanobacteria (blue-green algae), have been reported to contain marked amounts of vitamin B12 (up to 3.3 μg/g), consisting, however, largely of forms unavailable to mammals (Watanabe et al., 2002; Edelmann et al., 2019). Another types of plant-origin foods that contain vitamin B12 are fermented or chemically fortified foods. For instance, soybean tempeh, a fermented product that originated in Indonesia, contains 0.7 to 8 μg/100 g fresh weight of vitamin B12, due to the contaminating bacteria during fermentation (Nout and Rombouts, 1990).

Table 1. Content of vitamin B12 (μg/100 g fresh weight) in some foods (Stabler and Allen, 2004; Watanabe et al., 2014)

Types of food	Foods	Vitamin B12
Red meat	Beef	2.6
	Pork	0.8
	Goat	1.2
	Lamb	3.0
Poultry products	Chicken	0.2
	Turkey	0.4
	Duck	0.3
	Egg	1.3
Milk and dairy products	Cow milk	0.4
	Yogurt	0.5
	Cheese	2.9
Sea foods	Fish	3.0-8.9
	Oyster	18.7
	Clam	97
	Shrimp	1.5
Others	Tempeh	0.7-8
	Natto	0.1-1.5
	Dry nori	32-78

2.1.4 Deficiency in vitamin B12

Deficiency in vitamin B12 may result in various symptoms, such as fatigue, anemia, distal sensory impairment, cognitive impairment, memory loss, stupor and psychosis, depending on the degree and duration of the deficiency (Hunt et al., 2014). Globally, the ratio of clinical vitamin B12 deficiency, with classic hematological or neurological manifestations, is relatively low (Carmel, 2011). However, a high frequency (30% to 60%) of vitamin B12 subclinical deficiency, seen in all subgroups of the population, is thought to be a potential public health issue (Carmel, 2011; Pawlak et al., 2013; Smith et al., 2018).

The two main causes of vitamin B12 deficiency are malabsorption and inadequate intake (Allen, 2009). In addition, smoking, alcoholism, long-term use

of certain drugs and various diseases such as malaria, HIV infection and tuberculosis may also lead to vitamin B12 deficiency (Green et al., 2017). Malabsorption is the main cause of vitamin B12 deficiency among the elderly, which may result from autoimmune disease pernicious anemia, gastrointestinal surgery or atrophy of the gastric mucosa (Campbell et al., 2003). Inadequate intake of vitamin B12 is mostly due to a lack of access to products of animal origin (Allen, 2009). For instance, more than half of vegans are reported to be deficient in vitamin B12 and even with supplementation, vegans may still be at risk of deficiency (Herrmann et al., 2003; Gilsing et al., 2010). Among vegetarians, vitamin B12 deficiency is also prevalent, especially among the elderly (up to 90%), pregnant women (up to 62%) and children (up to 86%; Pawlak et al. 2013).

2.1.5. Determination of B12 in foods

The determination of vitamin B12 in food products is challenging for various reasons. These including its low content in food and sensitivity to light, the presence of binding proteins and inactive forms and the contamination of bacterial vitamin B12 producers (Kumar et al., 2010). Therefore, highly sensitive and specific methods are needed for its quantification. Processes like extraction, isolation, purification and cyanation are usually employed for more precise measurement (Chamlagain et al., 2015; Watanabe and Bito, 2018). Traditionally, vitamin B12 is determined by a microbiological assay, which is a turbidimetric method based on the growth of certain vitamin B12-dependent bacteria (Kelleher and Broin, 1991). This method is time-consuming and sometimes unreliable because tested bacteria may also thrive on various inactive vitamin B12 analogs (Watanabe and Bito, 2018).

In the past few decades, numerous chromatographic methods have been developed for vitamin B12 measurement, including thin layer chromatography (TLC), high-performance capillary electrophoresis (HPCE) and gas or liquid chromatography (Lambert et al., 1992; Sundin and Allen, 1992; Kumar et al., 2010). Various studies have shown that high-performance liquid chromatography (HPLC) is a reliable method for the determination of vitamin B12 in different food types and that a better sensitivity and efficiency can be obtained by using the ultra high-performance liquid chromatography (UHPLC) method (Gimenez, 2014;

Zironi et al., 2014; Chamlagain et al., 2015). Therefore, an HPLC method with immunoaffinity extraction has been adopted as the official method of the Association of Official Analytical Chemists (AOAC), internationally (Campos-Gimenez et al., 2012). Meanwhile, mass spectrometry (MS) has also been used with HPLC to analyze vitamin B12, especially for its identification, and has led to various newly identified inactive corrinoid compounds from foods (Alsberg et al., 2001; Chamlagain et al., 2015; Bito et al., 2016; Watanabe and Bito, 2018; Edelmann et al., 2019).

2.2 Fermentation of grains

Grains are small, hard, dry seeds or fruits harvested from certain plants for food, feed or industrial utilization. Every year, more than 2.6 billion tons of grains are produced worldwide (Table 2), including cereals, pseudocereals and legumes. They supply the majority of food energy and about half of the proteins consumed on Earth (Graybosch, 2016).

2.2.1 Cereals and cereal side streams

Cereal grains comprise the entire fruit, or caryopsis, of cereal plants belonging to the *Poaceae* family (Haard, 1999). The main types of cereals are maize (*Zea mays*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*), oat (*Avena sativa*) and millet (Wrigley, 2016). Structurally, the seed portion of a cereal grain consists of three parts: the seed coat or bran (testa), the storage organ or nutritive reserve part (endosperm) and the germ (Haard, 1999). Basically, the endosperm comprises most of the whole cereal kernel and the percentages of the germ and bran components vary among different cereal types. Some cereals, like barley, millet and sorghum, are mainly used as wholegrain products. In other cereals, like wheat and rice, the starchy endosperm is generally separated from the outer layers and consumed as milled flour (wheat) or refined kernels (rice).

Table 2. Worldwide production of some grains in 2017, in millions of metric tons (adopted from FAOSTAT).

Types of grain	Production	Types of grain	Production
Cereals		Legumes	
Maize	1135	Soybean	353
Wheat	772	Fava bean	4.8
Paddy rice	770	Lupine	1.6
Barley	147		
Sorghum	58	Pseudocereals	
Millet	28	Buckwheat	3.8
Oat	26	Quinoa	0.1
Rye	14		

Brans are major by-products of the cereal milling process. Although brans are rich in some valuable nutrients, including vitamins, minerals and dietary fiber, only a small proportion of bran is utilized in the food chain, for several reasons. First, adding bran in baking application weakens the dough structure and decreases bread volume and crumb elasticity (Sanz Penella et al., 2008; Noort et al., 2010). Moreover, the addition of cereal brans may introduce a bitter and pungent taste as well as a dark color, which make the products less appealing to consumers (Heiniö et al., 2016). As the outer layer of the kernel, brans may also contain a higher level of potential pathogens, mycotoxins and toxic metals than refined flours (Thielecke and Nugent, 2018). However, the utilization of bran or wholegrain materials in the food chain is currently recommended for a healthier diet and a more sustainable food system.

2.2.2 Pseudocereals and legumes

Pseudocereals are the starchy seeds from various non-cereal plants. Globally, quinoa (*Chenopodium quinoa Willd*.), buckwheat (*Fagopyrum esculentum*) and amaranth (*Amaranthus hypochondriacus*) are the most popular pseudocereals (Fletcher, 2016). Generally, pseudocereals have a higher protein and ash content and a better amino acid profile than wheat (Alvarez-Jubete et al., 2009). Moreover,

some dietary benefits such as lowering plasma low-density lipoprotein cholesterol and hypoglycemic activity, together with their gluten-free status have led to a global popularity for developing healthy products with pseudocereals (Alvarez-Jubete et al., 2010). Yet the utilization of pseudocereals in foods, especially in bakery products, is challenging due to their detrimental effects on technological properties and the presence of bitter compounds like saponins and tannins (Gobbetti et al., 2019).

Legumes are the dried seeds from the plants of the family Fabaceae (Graybosch, 2016). As the second-largest food crop worldwide, after cereals, there is increasing interest in legumes because they can provide high protein content with high biological value and relevant levels of vitamins, minerals and phenolic compounds (Roy et al., 2010). Moreover, it has been found that frequent consumption of legumes may be linked to lower risks of type 2 diabetes, cardiovascular disease and obesity (Jenkins et al., 2012; Hosseinpour-Niazi et al., 2015).

2.2.3 Fermentation of grain materials

Fermentation is the oldest form of food biotechnology and fermented food products are still very much present, providing about one-third of the global human diet (Campbell-Platt, 1994). A variety of fermentations have been developed, to process grain materials. Notably, bread, the end product of dough fermentation, provides more nutrients to the world population than any other single food source (Peña, 2002). The fermentation of grain materials is typically started by mixing flour with a certain amount of water. The addition of water increases the water activity, activating endogenous enzymes and making constituents available for microorganisms, either deliberately added or present as contaminants (Hammes and Gänzle, 1998). Grain fermentation is affected by various factors, such as water content, fermentation condition and the type of grain (Hammes et al., 2005).

In general, cereals are good substrates for fermentation because they contain high levels of carbohydrates, minerals, vitamins and other growth factors to support the growth of microbes (Salovaara and Simonson, 2004). However, the content of free sugars in cereals is generally at a relatively low level (2%-5%) and sugars are only able to support the early phase of the fermentation process (Salovaara and Simonson, 2004). Therefore, starch-hydrolyzing enzymes such as

α-amylase and some amylolytic microbes are required to degrade starch into simple sugars for the metabolism of microorganisms during fermentation (Nkhata et al., 2018). Meanwhile, minerals in grains may also not be readily available for microorganisms, because they are complexed with phytate and its hydrolysis by phytase begins only when pH value is lower than 5.5 (Hammes et al., 2005). Some antimicrobial substances such as phenolic acids and flavonoids may also be present in cereal materials, especially in whole-grain flours, because these substances are mainly found in the bran layer (Sekwati-Monang et al., 2012; Calinoiu and Vodnar, 2018). Therefore, the fermentation of cereal grains containing a high level of these substances, such as some sorghum species, can be challenging (Svensson et al., 2010).

Cereal brans, pseudocereals and legumes contain abundant nutrients and can provide many health benefits. However, their application in food is somehow limited by their technological, sensory and/or anti-nutritional weaknesses. Fermentation is believed to be one of the most sustainable and promising options, to attenuate the negative effect of these materials on the technological and sensory features of food products (Gobbetti et al., 2019). The fermentation of these materials can also lead to many nutritional benefits, including increasing folate, free phenol compounds and free amino acids (Katina et al., 2007; Kariluoto et al., 2014; Arte et al., 2015).

2.3 *In situ* fortification of vitamin B12 in grain materials by fermentation

Food fortification is a cost-effective way to supply micronutrients to the public. As the staple food for most people, grain materials are excellent vehicles for nutritional fortification. The fortification of grain products with micronutrients, including vitamin B12, has been conducted for many years and resulted in outstanding improvements to human health (Allen L, 2006; Allen et al., 2010).

2.3.1 Microbial producers of vitamin B12

Since chemical synthesis is both technically too challenging and too expensive, the commercial vitamin B12 used for the food fortification is exclusively produced via a biotechnological process, which includes microbial fermentation, extraction, purification and conversion to cyanocobalamin (Martens

et al., 2002). Instead of adding purified vitamin B12 to food, *in situ* fortification via fermentation with selected microorganisms during food processing can be a more cost-effective approach. Additional benefits, such as longer shelf life and enhanced flavor, can be also introduced during fermentation.

Although microorganisms from more than 20 genera have shown the ability to produce vitamin B12, *Propionibacterium freudenreichii* and *Pseudomonas denitrificans* are most used in industrial production due to their high vitamin B12 productivity (Hugenholtz and Smid, 2002). It is reported that certain lactic acid bacteria (LAB) strains, such as *Lactobacillus reuteri* and *Lactobacillus plantarum*, are able to produce vitamin B12 (Taranto et al., 2003; De Angelis et al., 2014). However, other studies have proven that *lactobacilli* can produce only pseudovitamin B12, which is not active in the human body because its lower ligand is adenine rather than DMBI (Crofts et al., 2013; Santos et al., 2007). Up to now, *P. freudenreichii* remains the only "generally recognized as safe" (GRAS) microorganism that is suitable for the *in situ* fortification of active vitamin B12 (Mogensen et al., 2002; Piwowarek et al., 2018).

2.3.2 *Propionibacterium freudenreichii*

Propionic acid bacteria (PAB) are Gram positive, mesophilic, aerotolerant Actinobacteria with a peculiar metabolism that produces propionic acid as the main metabolic end-product (Thierry et al., 2011). *P. freudenreichii* has a long history of utilization as the starter culture in Swiss-style cheeses. This is due to its key role in the cheeses' flavor and in their distinctive "eyes" formation, which is achieved by converting lactic acid into propionic acid, acetic acid and CO_2 (Langsrud and Reinbold, 1973). Strains from *P. freudenreichii* can be divided into two subspecies, based on their abilities to reduce nitrates and to metabolize lactose. Among these, the *P. freudenreichii subsp. freudenreichii* strains can reduce nitrates but cannot use lactose. Meanwhile, strains from *P. freudenreichii subsp. shermanii* are able to metabolize lactose but cannot reduce nitrates (Thierry et al., 2011).

In the metabolism of PAB, substrates are first oxidized to pyruvate. They are then reduced to propionate or oxidized to acetate and CO_2. In the pathway from pyruvate to propionate, the original enzyme of PAB, methylmalonyl-CoA mutase

(EC 5.4.99.2), requires vitamin B12 as the coenzyme to isomerise succinyl-CoA to methylmalonyl-CoA (Thierry et al., 2011). This may explain the intracellular accumulation of vitamin B12 in *P. freudenreichii* during fermentation. Besides vitamin B12, strains from *P. freudenreichii* are also widely used for the industrial production of propionic acid and other valuable compounds, such as bacteriocins and trehalose (Piwowarek et al., 2018). Some strains of *P. freudenreichii* have also been studied for developing functional food products because of their probiotic attributes (Cousin et al., 2011; Campaniello et al., 2015).

2.3.3 Vitamin B12 synthesis of *P. freudenreichii*

De novo biosynthesis of vitamin B12 in bacteria and archaea is a complex reaction, which requires more than 30 genes (Burgess et al., 2009). The biosynthesis of vitamin B12 in all microorganisms starts with the formation of δ-aminolevulinic acid (ALA), either from the Shemin pathway (C4 pathway) or the C5 pathway (Martens et al., 2002). In the Shemin pathway, ALA is formed by the condensation of glycine and succinyl-coenzyme A (CoA) catalyzed by ALA synthase (Shemin, 1969). In the C5 pathway, tRNA-bound glutamate is first reduced to glutamate-1-semialdehyde. The aldehyde is then converted, via an intramolecular shift of the amino group from the C-2 to the C-1, to form ALA (Piao et al., 2004). Notably, *P. freudenreichii* is found to be capable of obtaining ALA from both pathways (Iida and Kajiwara, 2000).

In the following steps, two molecules of 5-aminolevulinic acid are condensed to generate a pyrrole derivative, porphobilinogen, and 4 molecules of these pyrrole compounds polymerize to form uroporphyrinogen III (Piwowarek et al., 2018). The methylation of uroporphyrinogen III at C-2 and C-7 then leads to the formation of precorrin-2. Vitamin B12 producers diverge at this intermediate into two distinct routes, aerobic and anaerobic pathways, according to the timing of cobalt insertion and the method for ring contraction (Warren et al., 2002). In the aerobic pathway, which is applied by *P. denitrificans*, chelating of cobalt happens at the late phase and the C-20 atom of precorrin-3A is oxidized by molecular oxygen, with the subsequent release of C-20 as acetate (Martens et al., 2002). In the anaerobic pathway, which is applied by *P. freudenreichii*, cobalt is inserted at

the early stage and the process of ring contraction is mediated via the complexed cobalt ion, resulting in the release of C-20 as acetaldehyde (Moore et al., 2013).

The two pathways join again at the formation of adenosyl-cobyric acid and, thereafter, convert to adenosylcobinamide. The aminopropanol arm of formed adenosylcobinamide is then activated by guanosine triphosphate so that it is ready for the attachment of DMBI, the lower ligand of active vitamin B12 (Martens et al., 2002). The biosynthesis of DMBI in microorganisms diverges again into two completely different approaches: the aerobic route and the anaerobic route. In the former route, DMBI is derived from flavin mononucleotide, originating from riboflavin, by an oxygen-dependent transformation triggered by a single enzyme, BluB (Taga et al., 2007). In the anaerobic route, DMBI is put together from glycine, formate, S-adenosyl-L-methionine, glutamine and erythrose-4-phosphate (Warren et al., 2002). Synthesized DMBI is then activated into α-ribazole-phosphate by the CobT enzymes, so that it is ready for the attachment of the lower ligand to form the complete vitamin B12 (Trzebiatowski et al., 1994). In *P. freudenreichii*, a fused enzyme (BluB/$CobT_2$) is responsible for the production and activation of DMBI. The fusing of these two activities ensures the production of the active vitamin over the pseudovitamin (Deptula et al., 2015).

2.3.4 Challenges of *in situ* fortification of vitamin B12 with *P. freudenreichii*

Although *P. freudenreichii* has low nutritional requirements and is able to survive in various environments, its fermentation in grains for *in situ* fortification of vitamin B12 is still challenging. First, the optimum pH value for the cell growth and metabolism of *P. freudenreichii* is around 7.0 and an acidic environment (< pH 4.5) can totally inhibit its activity (Ye et al., 1996; Piwowarek et al., 2018). Unlike the fermentation of Swiss-type cheese, which typically has a pH higher than 5.0 (Fröhlich-Wyder et al., 2002), fermented native grain materials usually have a much lower pH (< 4.0) due to the accumulation of acids produced by LAB (De Vuyst and Neysens, 2005). The low pH may totally inhibit the metabolism of *P. freudenreichii*. Therefore, current research on vitamin B12 production in grain materials, via fermentation with *P. freudenreichii*, has focused mainly on sterilized grain materials that had a neutral to slightly acidic pH during fermentation. For instance, from 9 ng/g to 37 ng/g fresh weight of vitamin B12 were produced in

autoclaved aqueous barley and wheat aleurone matrices during fermentation, with pH ranging from 4.5 to 5.6 (Chamlagain et al., 2017). In lupine tempeh, up to 1230 ng/g dw of vitamin B12 were produced by co-fermentation of *Rhizopus oryzae* and *P. freudenreichii* at a pH ranging from 6.6 to 7.2 (Wolkers-Rooijackers et al., 2018). Heat treatment has a negative impact on the technological properties of grains, however, due to the changes in proteins (i.e. gluten and enzymes) and starch (Mann et al., 2013). Whether *P. freudenreichii* can produce a nutritionally relevant level of vitamin B12 in native grain materials needs to be studied. On the other hand, it is also unknown whether *P. freudenreichii* is able to control the propagation of potential pathogens in grains, to guarantee the microbial safety during fermentation.

Another bottleneck for *in situ* fortification of vitamin B12 is the need for cobalt and DMBI, which have been reported as limiting factors for the production during fermentation (Berry and Bullerman, 1966; Hugenschmidt et al., 2011; Chamlagain et al., 2016). Yet, their addition in food production is not permitted. Cobalt levels are generally low in grains, especially in cereals. They range from <10 to 60 ng/g dry weight (dw) (Ekholm et al., 2007) and the availability of cobalt in grain materials during fermentation is unknown. *P. freudenreichii* can *de novo* synthesize DMBI from riboflavin in the presence of oxygen. However, it is currently unknown whether the precursor (riboflavin) and oxygen in the fermentation of native grain materials are sufficient to support production.

3 Aims of the study

The main aim of this study was to investigate the *in situ* fortification of vitamin B12 in native grain materials by fermentation with *P. freudenreichii* and to improve the microbial safety of the fermentation by establishing a co-culture with *P. freudenreichii.*

The specific aims were:

1 To compare the *in situ* production of vitamin B12 in wheat flours and wheat bran by fermentation with *P. freudenreichii* (Study I)

2 To improve the microbial safety of the fermentation by establishing a co-culture with *P. freudenreichii* (Study II)

3 To study the vitamin B12 production by co-fermentation in different pH conditions (Study II)

4 To study the vitamin B12 production in different grains by co-fermentation (Study III)

4 Materials and methods

4.1 Strains and grain materials

In total, 9 bacterial strains were used in this study. These included 1 strain of *P. freudenreichii* (DSM 20271), 1 strain of yeast (*Saccharomyces cerevisiae*) and 7 strains of LAB as listed in Table 3. *P. freudenreichii* DSM 20271 is the type strain of species *Propionibacterium freudenreichii* and its complete genome sequence has been reported (Koskinen et al., 2015).

The cultures were cryopreserved (-80 °C) in 15% glycerol. The *P. freudenreichii* strain was propagated in yeast extract-lactate (YEL) broth (Malik et al., 1968) at 30 °C for 3 days. *S. cerevisiae* was propagated in yeast malt (YM) broth (Lab M, Lancashire, United Kingdom) and LAB were propagated in de Man, Rogosa and Sharpe (MRS) broth (Lab M) at 30 °C -37 °C for 1 day. After incubation, the cultures were centrifuged (3,200 × g, 10 min) and then suspended in sterile MillQ water before inoculation.

Table 3. Origin of strains in this work

Strain	Origin*	STUDY
Propionibacterium freudenreichii DSM 20271	a	I, II, III
Lactobacillus brevis ATCC 14869	b	II, III
Lactobacillus reuteri DSM 20016	a	II
Leuconostoc pseudomesenteroides DSM 20193	a	II
Lactobacillus delcrueckii ATCC 8000	b	II
Weissella confusa F74	c	II
Leuconostoc mesenteroides I21	c	II
Weissella confusa DSM 20194	a	II
Saccharomyces cerevisiae H10	d	II

* a, strains from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany (DSMZ); b, strains from American Type Culture Collection, USA (ATCC); c, strains from our laboratory collection; d, strains from the culture collection of the University of Helsinki, Faculty of Agriculture and Forestry, Division of Microbiology and Biotechnology.

The grain materials were obtained from markets or provided by manufactures (detail information can be found in Studies I-III). Their nutrient composition and cobalt content are shown in Table 4.

Table 4. Origin, nutrient composition (% of fresh weight) and cobalt content (ng/g dw) of grain materials.

Materials	Origin	Protein	Fat	Fiber	Fermentable carbohy-drates	Cobalt	Study
Durum wheat flour*	Finland	14	2	4	65	3	I
Whole-wheat flour *	Finland	12	3	12	60	10	I
Wheat bran I*	Finland	14	6	54	11	10	I,II
Wheat bran II*	Finland	16	5	43	20	27	II
Rye bran *	Finland	15	4	39	26	20	III
Oat bran*	Finland	18	8	14	49	17	III
Rice bran *	USA	13	20	20	33	166	III
Sorghum flour**	Burkina Faso	10	5	10	65	183	III
Millet flour**	Burkina Faso	9	5	7	68	157	III
Buckwheat bran*	Europe	40	9	9	38	183	III
Quinoa flour*	Peru	14	6	6	57	85	III
Amaranth flour*	Germany	14	6	9	66	182	III
Fava bean flour**	Finland	31	2	9	42	696	III
Soybean flour*	China	38	20	13	18	68	III
Lupine flour*	France	43	12	28	10	29	III

* Nutrient compositions of the materials were provided by the manufacturers

** Nutrient compositions of the materials were analyzed by Eurofins (methods can be found in Study III)

Cobalt contents of all materials were determined by the Finnish Environment Institute as introduced in the section 4.6

4.2 Preparation and fermentation of batters

The preparation and fermentation of batters, as well as the sampling times in Studies I-III are presented in Figure 2. All the batters in this work were prepared by mixing flours with water. In Study I, batters were fermented with approximately 9.0 log colony forming units (CFU)/g of *P. freudenreichii*. A durum batter, enriched with 0.6 mg/g dw batter of cobalt chloride (Sigma-Aldrich, Steinheim, Germany), was also prepared. In Study II, wheat bran I was fermented with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 6.0 log CFU/g strain candidates, listed in Table 3, for the screening of co-cultures. During fermentation, the pH of each batter was adjusted to 6.0 every 12 h with 3M NaOH. Next, wheat bran II was used for four different fermentations: spontaneous fermentation with pH control; fermentation with ca. 9.0 log CFU/g *P. freudenreichii* and pH control; fermentation with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 6.0 log CFU/g *L. brevis* with pH control; fermentation with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 6.0 log CFU/g *L. brevis* without pH control. During fermentation, pH was controlled at 5.0 by the addition of 5 M NaOH solution. In Study III, 11 materials were fermented with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 6.0 log CFU/g *L. brevis*. In addition, buckwheat bran, oat bran, rice bran and sorghum flour were also studied for fermentation without starter cultures; fermentation with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 7.0 log CFU/g *L. brevis*; fermentation with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 8.0 log CFU/g *L. brevis*.

Three biological replicates were applied in all fermentations, except in the screening process of Study II, which was conducted in duplicate.

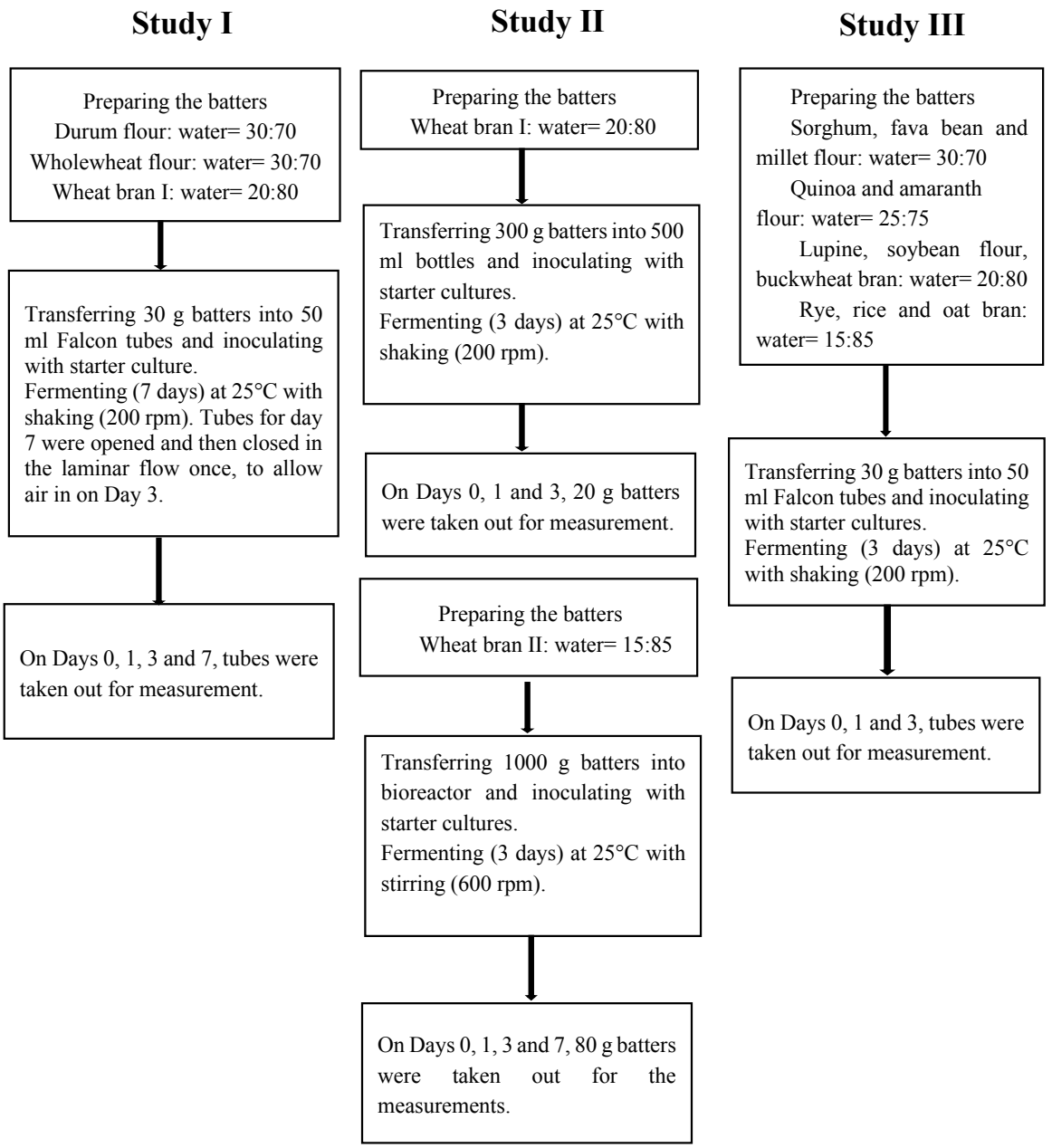

Figure 2. Batter preparation, fermentation condition and sampling time

4.3 Cell counts (Studies I-III)

To estimate the number of viable cells, the batters (10 g) were serial diluted by sterile saline solution (8.5 g/L of NaCl) and appropriate dilutions were plated on the agar plates. YEL plates, used for the cell count of *P. freudenreichii,* were anaerobically (anaerobic jars with Anaerogen, Oxoid, Basingstoke, UK) incubated for 4 days and aerobically incubated for 1 day at 30 °C. This led the colonies of *P. freudenreichii* to turn brownish and distinguishable from colonies of other microorganisms. MRS agar (Lab M) plates, supplemented with 0.01% of cycloheximide (Sigma Chemical Co., USA), were used for the cell count of LAB. Violet red bile glucose agar (VRBGA) plates (Lab M) were used for the cell count of total *Enterobacteriaceae*. MRS plates were cultivated at 30 °C for 48 h, but VRBGA plates were incubated at 37 °C for 48 h.

4.4 Determination of pH and TTA (Studies I-III)

The pH values were determined by a pH meter (Portamess 752 Calimatic, Knick, Berlin, Germany). For the measurement of total titratable acidity (TTA), batter samples of 10 g were mixed with 90 ml of distilled water and titrated against 0.1 M NaOH, to a final pH of 8.5, via a Mettler Toledo EasyPlus Titrator (Schott, Germany). TTA was expressed as the usage of 0.1 M NaOH (ml).

4.5 Determination of vitamin B12 (Studies I-III)

The vitamin B12 in batters was determined as cyanocobalamin using a UHPLC method after extraction and purification according to Chamlagain et al. (2015), with some minor modification as presented in subsequent sections. The identity of pseudovitamin B12 was confirmed by a mass spectrometry method, as described by Chamlagain et al. (2015).

4.5.1 Extraction

Batter samples (3 g) were weighed and mixed with 15 ml of extraction buffer (20.7 mmol/L of acetic acid and 8.3 mmol/L of sodium hydroxide, pH 4.5) and 100 µl of sodium cyanide (1% w/v in water). After extraction in boiling water for 30 min, the mixtures were cooled on ice and then incubated in a water bath (30 min, 37 °C) to break down starch by adding 300 µl of α-amylase (50 mg/ml; St Louis, MO, USA). After incubation, mixtures were centrifuged at 6,900 × g for 10 min. Supernatants were collected and the residues were re-suspended in 5 ml of

extraction buffer and centrifuged again. After combining the supernatants, the final volume was adjusted to 25 ml with the extraction buffer.

4.5.2 Purification

Ten milliliters of extracts were filtered (0.45 µm; Pall) and loaded into an immunoaffinity column (Easi-Extract; R-Biopharma; Glasgow, Scotland). After washing with MilliQ water (10 ml), the retentate in the column was eluted with 3.5 ml of methanol. The eluent was evaporated under the nitrogen flow at 50 °C. The residue was recovered in 300 µl of MilliQ water and filtered (0.2 µm; Pall) into a LC vial (Waters).

4.5.3 UHPLC measurement

A Waters UHPLC system (Milford, MA, USA) equipped with a photodiode array detector (210 - 600 nm) and a C18 column (Acquity HSS T3, 2.1 × 100 mm, 1.8 µm) was used for analysis of the cyanocobalamin content. The flow rate was set to 0.32 mL/min, with a column temperature at 30 ˚C. The mobile phase consisted of 0.025% trifluoroacetic acid (TFA) in water (solvent A) and of 0.025% TFA in acetonitrile (solvent B). The injection volume was 15 µL. Cyanocobalamin was detected at 361 nm and quantified, via an external standard method using a calibration curve with six points (0.4 to 8 ng). The presence of other corrinoids, such as pseudovitamin B12, was recognized in the chromatograms, based on their retention times and absorption spectra.

4.6 Determination of other components

The lactic acid, acetic acid and propionic acid content (Studies I-III) was determined by an HPLC method, according to Hugenschmidt et al. (2011), with minor modification. Riboflavin content (Studies I and II) was determined by UHPLC according to Chamlagain et al. (2016) with minor modification. The contents of monosaccharides (arabinose, galactose, xylose, glucose and fructose) were analyzed by high performance anion exchange chromatography, equipped with a pulse amperometric detection system (HPAEC-PAD) according to Xu et al. (2017) with minor modification. The cobalt content was determined by the Finnish Environment Institute, using the method based on microwave-assisted digestion and inductively coupled plasma mass spectrometry (ICP-MS) quantitation, as

described by Nóbrega et al. (2012). Detailed information on the methods can be found in Studies I-III.

4.7 Statistical analysis

Statistical analyses were performed with SPSS 21.0-23.0 for Windows (IBM Corporation, NY, USA). One-way analysis of variance (ANOVA) and Tukey's post hoc test were employed, to determine significant differences among the samples. The results were calculated based on three replicates and the level of statistical significance was defined at a p-value < 0.05. A multivariate data analysis was performed in Study III by Partial least squares regression (PLS), using Simca 15.0 (Umetrics AB, Malmö, Sweden). The nutrient and cobalt content, as well as the acidification parameters (pH, TTA and contents of acids), were chosen as the x variables and vitamin B12 content was chosen as the y variable.

5 Results

5.1 Growth of bacteria CFUs during fermentation (Studies I-III)

5.1.1 Growth of PAB CFUs during fermentation (Studies I-III)

Table 5 shows the cell density of PAB, during the first 3 days of fermentation in the batters with inoculation of *P. freudenreichii*. In general, the cell density of PAB ranged from 8.6 log CFU/g to 9.0 log CFU/g on Day 0 and increased to different levels during fermentation, depending on the materials and fermentation conditions. In Study I, the highest increase of the cell density of PAB from Day 0 to Day 3 was found in the durum flour batter (ca. 1 log cycle). In the wheat bran II, the cell density of *P. freudenreichii* increased by about 0.4 log cycle during fermentation with pH control, but remained constant throughout the fermentation without pH control (Study II). In Study III, the cell densities of PAB on Day 3 slightly decreased when the level of inoculated *L. brevis* increased from 6 log CFU/g to 8 log CFU/g.

Table 5. Cell density of PAB (log CFU/g) during fermentation.

Batters	Day 0	Day 1	Day 3	Study
Durum flour_P9	8.7±0.0	9.6±0.1	9.6±0.1	I
Whole-wheat flour_P9	8.7±0.1	8.7±0.1	8.7±0.0	I
Wheat bran I_P9	9.0±0.0	9.4±0.1	9.2±0.1	I
Wheat bran II_P9*	8.8±0.1	9.2±0.1	9.0±0.1	II
Wheat bran II_P9L6*	8.7±0.1	9.1±0.1	8.9±0.2	II
Wheat bran II _P9L6	8.6±0.1	8.6±0.2	8.5±0.1	II
Oat bran_P9L6	8.7±0.1	9.3±0.1	9.4±0.3	III
Oat bran_P9L7	8.8±0.1	9.3±0.1	9.3±0.1	III
Oat bran_P9L8	8.7±0.1	9.2±0.1	9.3±0.2	III
Rice bran_P9L6	8.8±0.0	9.4±0.0	9.5±0.1	III
Rice bran_P9L7	8.7±0.2	9.2±0.2	9.3±0.0	III
Rice bran_P9L8	8.8±0.1	9.3±0.1	9.3±0.2	III
Sorghum flour_P9L6	8.7±0.2	8.8±0.1	9.1±0.2	III
Sorghum flour_P9L7	8.8±0.1	9.0±0.1	8.9±0.1	III
Sorghum flour_P9L8	8.7±0.1	8.9±0.1	8.9±0.2	III
Buckwheat bran _P9L6	8.8±0.1	9.4±0.1	9.4±0.1	III
Buckwheat bran _P9L7	8.7±0.2	9.3±0.1	9.3±0.1	III
Buckwheat bran _P9L8	8.7±0.1	9.1±0.2	9.0±0.2	III

Abbreviations: Batters fermented with ca. 10^9 CFU/g *P. freudenreichii* (P9), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^6 CFU/g *L. brevis* (P9L6), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^7 CFU/g *L. brevis* (P9L7), and ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^8 CFU/g *L. brevis* (P9L8).

* means the batters with pH control

5.1.2 Growth of LAB CFUs during fermentation (Studies I-III)

Table 6 shows the cell density of LAB, during the first 3 days of fermentation in different materials. The cell densities of indigenous LAB in different materials varied from below the detection limit to ca. 3.0 log CFU/g prior to fermentation. In Study I, the cell densities of LAB were found to have increased by ca. 9.0 log cycles, in all batters, from Day 0 to Day 3. In Study II, the cell densities of LAB were ca. 10.0 log CFU/g in the batters with pH control and ca. 9.0 log CFU/g in the batter without pH control on Day 3. In Study III, the cell density of LAB at the end of fermentation was higher than 9.0 log CFU/g in all batters, except in the sorghum control batter and P9L6 batter, which both contained ca. 8.0 log CFU/g of LAB.

Table 6. Cell density of LAB (log CFU/g) during fermentation of different batters

Batters	Day 0	Day 1	Day 3	Study
Durum flour_P9	nd**	9.2±0.0	8.8±0.0	I
Durum flour_CT	nd	9.2±0.0	8.7±0.1	I
Whole-wheat flour_P9	nd	8.6±0.1	9.0±0.1	I
Whole-wheat flour_CT	nd	8.6±0.2	8.5±0.1	I
Wheat bran I_P9	nd	9.2±0.1	9.2±0.1	I
Wheat bran I_CT	nd	9.3±0.1	9.1±0.1	I
Wheat bran II_CT*	2.7±0.3	9.8±0.2	9.8±0.2	II
Wheat bran II_P9*	3.0±0.2	9.7±0.2	9.6±0.1	II
Wheat bran II_P9L6*	6.3±0.2	10.2±0.0	10.3±0.2	II
Wheat bran II _P9L6	6.4±0.2	9.6±0.2	9.1±0.1	II
Oat bran_CT	nd	7.1±0.1	9.1±0.1	III
Oat bran _P9L6	5.7±0.1	8.4±0.1	9.2±0.1	III
Oat bran _P9L7	6.9±0.1	8.3±0.1	9.3±0.1	III
Oat bran _P9L8	8.1±0.1	9.4±0.1	9.3±0.1	III
Rice bran_CT	nd	7.4±0.1	8.1±0.2	III
Rice bran _P9L6	5.8±0.1	8.1±0.1	8.9±0.2	III
Rice bran _P9L7	7.0±0.0	8.0±0.2	9.3±0.2	III
Rice bran_P9L8	8.0±0.1	9.1±0.1	9.5±0.2	III
Sorghum flour_CT	2.3±0.3	6.6±0.1	7.9±0.1	III
Sorghum flour_P9L6	5.9±0.0	8.1±0.1	8.1±0.1	III
Sorghum flour_P9L7	6.8±0.2	8.9±0.1	9.1±0.1	III
Sorghum flour_P9L8	7.9±0.1	8.8±0.1	9.0±0.1	III
Buckwheat bran_CT	2.8±0.2	8.4±0.0	9.4±0.1	III
Buckwheat bran _P9L6	5.7±0.2	9.1±0.1	9.3±0.0	III
Buckwheat bran _P9L7	7.2±0.1	9.2±0.1	9.3±0.1	III
Buckwheat bran _P9L8	8.1±0.2	9.1±0.1	9.4±0.2	III

Abbreviations: Batters fermented with ca. 10^9 CFU/g *P. freudenreichii* (P9), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^6 CFU/g *L. brevis* (P9L6), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^7 CFU/g *L. brevis* (P9L7), and ca. 10^9 CFU/g *P. freudenreichii*

and ca. 10^8 CFU/g *L. brevis* (P9L8). CT means the control batter, fermented without starter culture.
* means the batters with pH control
**nd = not detected

5.1.3 Growth of *Enterobacteriaceae* CFUs during fermentation (Studies I-III)

The cell density of *Enterobacteriaceae* was below the detection limit in the oat and rice bran, and varied from 4.5 log CFU/g to 5.5 log CFU/g in the other materials before fermentation (Table 7).

In Study I, the cell density of total *Enterobacteriaceae* increased by about 2 log cycles from Day 0 to Day 1 in all batters and decreased thereafter. The cell density of *Enterobacteriaceae* became undetectable in the durum batter on Day 3 (Study I). However, in the whole-wheat flour and wheat bran I batters, the cell densities of *Enterobacteriaceae* still ranged from 4.0 to 5.0 log CFU/g on Day 3 and inoculated *P. freudenreichii* did not show any inhibition on *Enterobacteriaceae* (Study I).

To establish a co-fermentation culture to control the propagation of *Enterobacteriaceae, P. freudenreichii* was co-fermented with different microorganisms (one strain of yeast and seven strains of LAB) in the wheat bran. As shown in Supplemental Table 3 of Study II, batters co-fermented with LAB generally had a lower cell density of *Enterobacteriaceae* than did batters co-fermented with yeast or fermented with *P. freudenreichii* monoculture. The lowest cell density of *Enterobacteriaceae* was observed in the batter co-fermented with *L. brevis* and *P. freudenreichii* (Supplemental Table 3, Study II).

In the wheat bran II fermentation, the cell density of *Enterobacteriaceae* decreased to ca. 3.3 log CFU/g on Day 1 in the batters with inoculation of *L. brevis* and remained stable thereafter when pH was controlled (Study II). Without pH control, the cell density of *Enterobacteriaceae* decreased to ca. 2.8 log CFU/g on Day 3 (Study II). In Study III, a lower cell density of *Enterobacteriaceae* than that of the control batter was detected during fermentation of each material, when *L. brevis* was inoculated. In the fermentation of all materials, the cell density of *Enterobacteriaceae* decreased when *L. brevis* inoculum was increased.

Table 7. Cell density of *Enterobacteriaceae* (log CFU/g) during fermentation

Batters	Day 0	Day 1	Day 3	Study
Durum flour_P9	4.9±0.0	6.8±0.1	nd	I
Durum flour_CT	4.9±0.0	6.6±0.1	nd	I
Whole-wheat flour_P9	4.5±0.1	7.3±0.1	5.3±0.1	I
Whole-wheat flour_CT	4.5±0.1	7.4±0.1	5.2±0.2	I
Wheat bran I_P9	4.5±0.1	6.9±0.0	4.1±0.1	I
Wheat bran I_CT	4.5±0.1	6.8±0.1	4.1±0.0	I
Wheat bran II_CT*	4.8±0.0	6.1±0.1	3.7±0.3	I
Wheat bran II_P9*	4.7±0.1	6.0±0.1	3.7±0.1	II
Wheat bran II_P9L6*	4.7±0.1	3.3±0.3	3.4±0.4	II
Wheat bran II _P9L6	4.8±0.1	3.4±0.3	2.8±0.1	II
Oat bran_CT	nd**	7.6±0.1	4.7±0.2	III
Oat bran _P9L6	nd	6.4±0.2	4.4±0.2	III
Oat bran _P9L7	nd	4.8±0.2	3.9±0.1	III
Oat bran _P9L8	nd	4.3±0.1	nd	III
Rice bran_CT	nd	7.8±0.1	8.7±0.2	III
Rice bran _P9L6	nd	5.4±0.2	3.5±0.4	III
Rice bran _P9L7	nd	4.2±0.2	nd	III
Rice bran_P9L8	nd	4.3±0.1	nd	III
Sorghum flour_CT	5.4±0.1	9.0±0.1	8.9±0.1	III
Sorghum flour_P9L6	5.5±0.2	8.8±0.1	9.2±0.2	III
Sorghum flour_P9L7	5.4±0.0	7.9±0.1	nd	III
Sorghum flour_P9L8	5.5±0.1	5.4±0.1	nd	III
Buckwheat bran_CT	4.4±0.2	8.6±0.1	5.4±0.2	III
Buckwheat bran _P9L6	4.3±0.2	6.5±0.2	5.1±0.0	III
Buckwheat bran _P9L7	4.4±0.1	5.5±0.1	nd	III
Buckwheat bran _P9L8	4.3±0.1	5.1±0.2	nd	III

Abbreviations: Batters fermented with ca. 10^9 CFU/g *P. freudenreichii* (P9), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^6 CFU/g *L. brevis (*P9L6), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^7 CFU/g *L. brevis (*P9L7), and ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^8 CFU/g *L. brevis (*P9L8). CT means the control batter, fermented without starter culture.

* means the batters with pH control

**nd = not detected

5.2 Chemical changes during fermentation (Studies I-III)

5.2.1 Changes of monosaccharide contents during fermentation of wheat bran (Study II)

Figure 5 shows the contents of 5 monosaccharides (glucose, fructose, xylose, galactose and arabinose) during fermentation of wheat bran (Study II). On Day 0, the sum of monosaccharides in the wheat bran was ca. 6.0 mg/g dw and glucose and fructose were the main monosaccharides. In the control batter, the sum of monosaccharides increased slightly during the first day, but then decreased to ca. 1.2 mg/g dw by Day 3. In the batter fermented with *P. freudenreichii* monoculture, the sum of monosaccharides decreased to ca. 5.4 mg/g dw on Day 1 but was still mainly composed of glucose and fructose. In the co-fermented batter with pH control, only ca. 3.6 mg/g dw of monosaccharides, composed mostly of xylose and arabinose, were detected on Day 1. After fermentation, the content of monosaccharides in 3 batters with pH control was lower than 1.0 mg/g dw. Without pH control, the content of all monosaccharides increased during fermentation and reached a sum of ca. 11.5 mg/g dw at the end of fermentation.

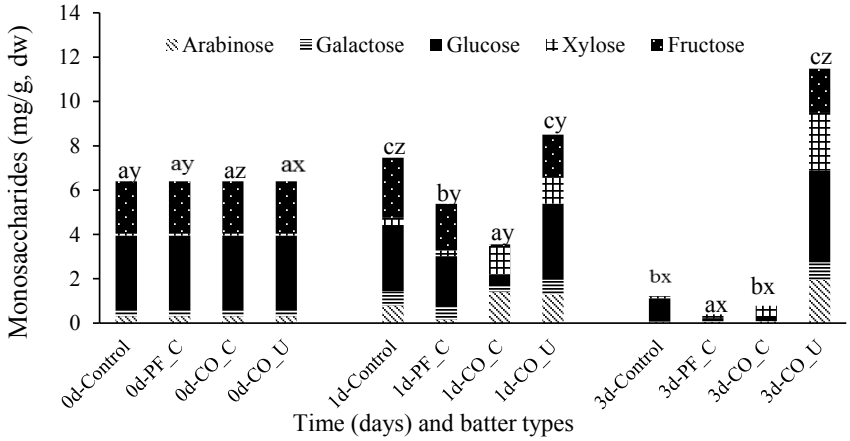

Figure 5. Contents (mg/g dry weight) of monosaccharides during co-fermentation.
Control, spontaneously fermented with pH control; PF_C, fermented with *P. freudenreichii* and pH control; CO_C, fermented with *P. freudenreichii* and *L. brevis* with pH control; CO_U, fermented with *P. freudenreichii* and *L. brevis* without pH control.
Values of total monosaccharide concentration from the same day (a–c) or the same batter type (x–z), bearing different superscripts, are significantly different ($p < 0.05$).

5.2.2 Change of pH and TTA during fermentation (Studies I-III)

The values of pH and TTA were determined during fermentation. In general, pH ranged from 5.7 to 6.8 and TTA ranged from 0.5 to 4.8 ml in all batters before fermentation (Figure 1 in Study I, Table 1 in Study II and Table 2 in Study III). Table 8 shows the pH and TTA of batters with inoculation on Day 3. In general, pH in all batters ranged from 3.6 to 5.3 on Day 3. Without pH control, only the oat bran, rice bran and sorghum flour batters had a pH higher than 5.0. The TTA of the batters differed greatly on Day 3. The oat bran and sorghum flour batters had a TTA ranging from 3.1 ml to 4.4 ml. In the other batters, TTA varied from ca. 11 ml to ca. 22 ml.

Table 8. Values of pH and TTA (ml) and contents (mg/g dw) of lactic acid (LA), acetic acid (AA) and propionic acid (PA), after 3 days of fermentation for selected batters

Batters	pH	TTA	LA	AA	PA	Study
Durum flour_P9	3.8	12.1±0.3	28.3±1.1	3.9±0.7	6.3±0.7	I
Whole-wheat flour_P9	3.9	14.5±0.3	48.3±2.7	1.0±0.3	0.6±0.0	I
Wheat bran I_P9	4.2	20.6±0.8	39.2±6.1	9.8±1.5	7.8±0.8	I
Wheat bran II_P9*	5.0	-**	212.1±6.1	40.3±1.6	9.3±1.1	II
Wheat bran II_P9L6*	5.0	-	239.8±32	32.6±2.1	7.9±1.0	II
Wheat bran II _P9L6	3.7	-	98.8±2.5	3.1±0.0	1.4±0.0	II
Rye bran_P9L6	3.6	17.4±0.6	98.1±4.4	2.7±0.1	7.2±2.9	III
Oat bran_P9L6	5.2	3.1±0.3	nd***	12.0±2.0	15.0±2.4	III
Rice bran_P9L6	5.0	12.5±0.4	nd	19.4±2.8	6.7±1.2	III
Sorghum flour_P9L6	5.3	4.4±0.5	nd	11.0±1.0	11.8±1.4	III
Millet flour_P9L6	3.6	17.3±0.5	49.8±1.1	1.4±0.2	1.6±0.4	III
Buckwheat bran_P9L6	4.9	18.5±1.1	nd	18.0±0.6	32.8±4.4	III
Quinoa flour_P9L6	3.6	21.9±1.6	58.2±0.9	1.6±0.2	3.0±0.4	III
Amaranth flour_P9L6	3.7	22.1±1.5	68.8±0.6	1.4±0.2	4.5±0.0	III
Fava bean flour_P9L6	4.3	19.5±1.1	40.0±1.8	4.8±0.8	10.4±1.4	III
Soybean flour_P9L6	4.7	13.5±1.1	8.9±2.1	13.6±1.5	22.4±0.8	III
Lupine flour_P9L6	4.9	11.3±0.8	nd	13.2±0.5	15.5±0.7	III

P9, P9L6, P9L7 and P9L8 means the batters fermented with ca. 10^9 CFU/g *P. freudenreichii*, with ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^6 CFU/g *L. brevis*, with ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^7 CFU/g *L. brevis* as well as with ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^8 CFU/g *L. brevis*. CT means the control batter, which fermented without starter culture

* means the batters with pH control

**Not studied ***nd = not detected

5.2.3 Production of acids (Studies I-III)

Prior to fermentation, acids were not detected in any of the batters. The lactic, acetic and propionic acid content produced during fermentation is shown in Table 8. In the durum flour, whole-wheat flour, wheat bran, rye bran, millet flour, quinoa flour, amaranth flour and fava bean flour batters, the main acid was lactic acid. In the oat bran, rice bran, sorghum flour, buckwheat bran and lupine flour batters, no lactic acid was found on Day 3 and the content of acetic and propionic acids varied.

5.2.4 Content of riboflavin during fermentation (Studies I and II)

The content of riboflavin in the durum flour, whole-wheat flour and wheat bran I batters fermented with *P. freudenreichii* monoculture are shown in Figure 6 (Study I). In the durum flour and whole-wheat flour batters, riboflavin content ranged from 0.18 µg/g dw to 0.45 µg/g dw on Day 0 and increased by ca. 0.3 µg/g dw during fermentation, In contrast, the riboflavin content in the wheat bran I batter decreased from ca. 1.93 µg/g dw on Day 0 to ca. 1.78 µg/g dw on Day 7 (Study I).

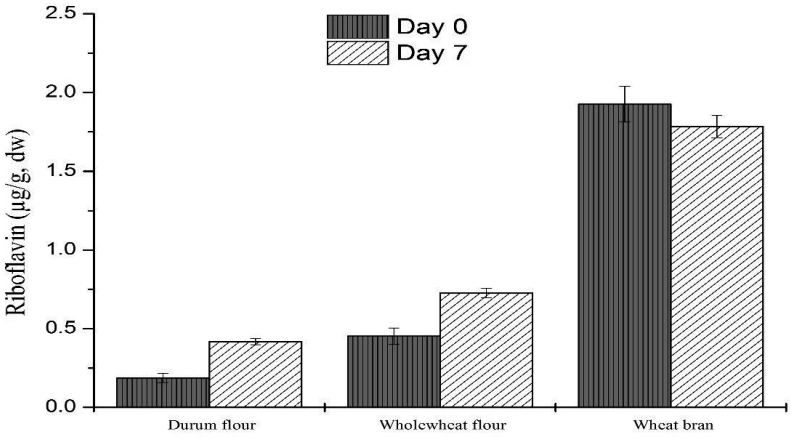

Figure. 6 Concentration (µg/g dry weight) of riboflavin in the inoculated wheat batters on Day 0 and Day 7. (Study I)

The wheat bran II batters contained ca. 4.0 µg/g dw of riboflavin on Day 0, and its concentration remained stable during fermentation without a starter culture (Figure 7; Study II). In the batters with inoculation, riboflavin content decreased during the first day and varied from ca. 3.2 µg/g dw to ca. 3.5 µg/g dw. From Day 1 to Day 3, the riboflavin content increased to ca. 4.6 µg/g dw in batters with pH control. Meanwhile, in the co-fermented batter without pH control, ca. 3.2 µg/g dw of riboflavin were detected on Day 3 (Study II).

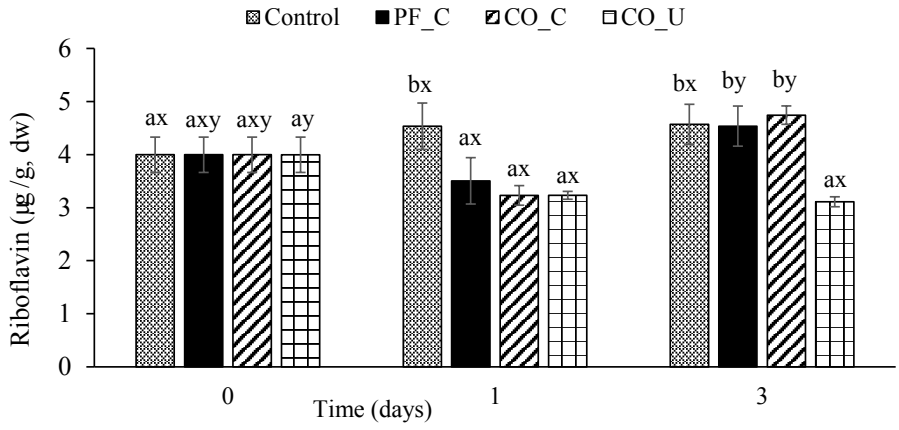

Figure 7. Concentration (µg/g, dry weight) of riboflavin during fermentation. (Study II)

Control = spontaneously fermented with pH control; PF_C = fermented with *P. freudenreichii* and pH control; CO_C = fermented with *P. freudenreichii* and *L. brevis* with pH control; CO_U = fermented with *P. freudenreichii* and *L. brevis* without pH control. Values from the same day, bearing different superscripts (a–b) are significantly different ($p < 0.05$). Values from the same batter type bearing different superscripts (x–y) are significantly different ($p < 0.05$).

5.3 Vitamin B12 production (Studies I-III)

Table 9 shows the vitamin B12 content of the batters with inoculation of *P. freudenreichii*. In general, the vitamin B12 content in all batters ranged from 17-47 ng/g dw on Day 0 and increased to different levels after fermentation. In the fermentation of durum flour, wholewheat flour and wheat bran with *P. freudenreichii* monoculture, the vitamin B12 contents after a 7-day fermentation were ca. 33 ng/g dw, ca. 87 ng/g dw and ca. 155 ng/g dw, respectively (Study I). Notably, the addition of cobalt chloride (0.6 μg/g dw) in durum batter increased the vitamin B12 content to ca. 203 ng/g dw at the end of fermentation (Study I).

In Study II, after fermentation of wheat bran II with pH control, the highest content of vitamin B12 was found in the batter with *P. freudenreichii* monoculture both on Day 1 (ca. 358 ng/g dw) and Day 3 (ca. 385 ng/g dw). In the co-fermented batters, the vitamin B12 contents on Day 1 were ca. 255 ng/g dw with pH control and ca. 214 ng/g dw without pH control, respectively. On Day 3, the vitamin B12 content in the co-fermented batter with pH control increased to ca. 332 ng/g dw. Without pH control, the vitamin B12 content decreased to ca. 183 ng/g dw.

In Study III, the highest content of vitamin B12 on Day 1 was found in the buckwheat bran (ca. 335 ng/g dw) and contents in other grain materials ranged from 58 to 246 ng/g dw. On Day 3, the highest content of vitamin B12 was found in the rice bran (ca. 742 ng/g dw), followed by the buckwheat bran (ca. 631 ng/g dw) and the soybean flour (ca. 407 ng/g dw). With fermented oat bran, sorghum, lupine and fava bean flour, the vitamin B12 content ranged from 265 to 343 ng/g dw on Day 3. When the initial inoculum of *L. brevis* increased from 6 log CFU/g to 8 log CFU/g, the vitamin B12 content on Day 3 in the fermented sorghum flour decreased from ca. 265 ng/g dw to ca. 67 ng/g dw. With fermented oat, rice and buckwheat bran, the content of vitamin B12 decreased by about 100 ng/g dw during fermentation, when the addition of *L. brevis* increased from 6 log CFU/g to 8 log CFU/g.

Table 9. Vitamin B12 content (ng/g dw) during fermentation in different grain materials

Batters	Day 0	Day 1	Day 3	Day 7	Study
Durum flour_P9	19±2	37±1	40±3	33±4	I
Durum flour_P9 + Co*	19±2	-***	-	203±24	I
Whole-wheat flour_P9	17±2	34±3	78±8	87±10	I
Wheat bran I_P9**	47±6	81±8	114±5	155±17	I
Wheat bran II_P9**	38±9	358±9	385±32	-	II
Wheat bran II_P9L6**	41±10	251±24	332±44	-	II
Wheat bran_P9L6	41±7	215±35	183±5	-	II
Rye bran_P9L6	38±5	103±7	104±29	-	III
Oat bran_P9L6	36±4	128±3	332±24	-	III
Oat bran_P9L7	38±5	104±18	312±3	-	III
Oat bran_P9L8	38±5	128±12	298±10	-	III
Rice bran_P9L6	37±5	133±16	742±18	-	III
Rice bran_P9L7	38±5	119±24	717±10	-	III
Rice bran_P9L8	38±5	122±3	675±30	-	III
Sorghum flour_P9L6	20±3	149±9	265±13	-	III
Sorghum flour_P9L7	19±3	88±9	125±27	-	III
Sorghum flour_P9L8	19±3	68±6	67±14	-	III
Millet flour_P9L6	19±3	58±1	51±6	-	III
Buckwheat bran _P9L6	29±4	335±76	631±61	-	III
Buckwheat bran _P9L7	29±4	237±38	572±58	-	III
Buckwheat bran _P9L8	29±4	256±28	508±26	-	III
Quinoa flour _P9L6	32±4	60±1	65±5	-	III
Amaranth flour_P9L6	31±4	81±13	78±4	-	III
Fava bean flour_P9L6	20±5	155±23	298±51	-	III
Soybean flour_P9L6	29±6	154±2	407±5	-	III
Lupine flour_P9L6	28±4	246±15	343±71	-	III

Abbreviations: Batters fermented with ca. 10^9 CFU/g *P. freudenreichii* (P9), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^6 CFU/g *L. brevis (*P9L6), ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^7 CFU/g *L. brevis (*P9L7), and ca. 10^9 CFU/g *P. freudenreichii* and ca. 10^8 CFU/g *L. brevis (*P9L8).

*Co means the batter with addition of 0.6 µg/g dw cobalt chloride

** Batters fermented with pH control

*** Not studied

5.4 Raw material and fermentation factors predicting B12 production (Study III)

A PLS modelling was used to investigate how the nutrient composition, cobalt content and acidification properties (pH, TTA, and production of acids) correlated with vitamin B12 production on Day 1 (Figure 8 A) and Day 3 (Figure 8 B). The projection of x variables, on the line drawn via the y variable and the origin of the plot, describes the correlation between the two variables. The distance of x variables from the origin indicates their influence on the y variable. On both Day 1 and Day 3, the vitamin B12 content was positively correlated with pH, the content of protein, fat and dietary fiber and the production of propionic acid and acetic acid. On the other hand, vitamin B12 production was negatively correlated with TTA, the content of available carbohydrate and lactic acid. Cobalt content had only a very limited influence on vitamin B12 production, both on Day 1 and Day 3.

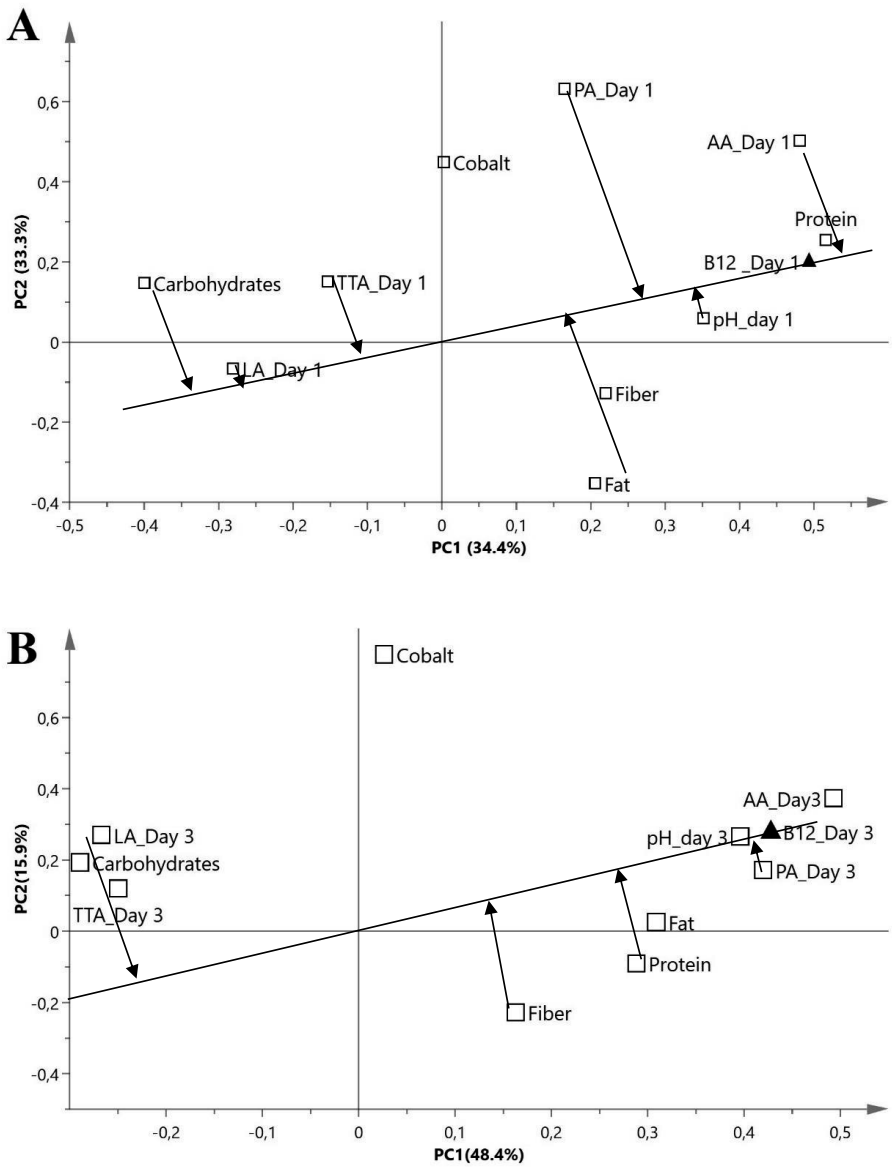

Figure 8. PLS loading plots, showing the distribution of x variables and y variables along the first and second principal components (PC) on Day 1 (A) and Day 3 (B). In each plot, the x variables are pH, total titratable acid (TTA) and content of protein, fat, fiber, available carbohydrates, cobalt, lactic acid (LA), acetic acid (AA) and propionic acid (PA). The y variable is the content of vitamin B12.

6 Discussion

6.1 *In situ* fortification of vitamin B12 in grain materials is a promising way to provide vitamin B12

This study has clearly shown that it is possible to produce vitamin B12 in different native grain raw materials by fermenting them with *P. freudenreichii*. During fermentation, no vitamin B12 was detected in the control batters (Table 3 in Study I, Figure 3 in Study II and Table 7 in Study III), showing that the increase of vitamin B12 content in the inoculated batters was due to biosynthesis by the inoculated *P. freudenreichii*. The grain materials provided different vitamin B12 levels, however, depending on the properties of raw materials and fermentation factors. The grain materials used in this thesis were of varying chemical composition, which led to different acidification kinetics and a greatly diverging production of vitamin B12 by *P. freudenreichii* during fermentation. In general, brans and legume flours demonstrated a higher production of vitamin B12 than did cereal and pseudocereal flours.

Brans are the main side stream of the cereal milling process and utilization of these abundant ingredients in food products can contribute to a more resilient food chain. After the 3-day fermentation with *P. freudenreichii* and *L. brevis* without any chemical additive or pH control, the vitamin B12 production in the oat bran was about 330 ng/g dw. In the rice bran and the buckwheat bran, the production rose as high as ca. 742 ng/g dw and ca. 631 ng/g dw, respectively. Moreover, synthesized vitamin B12 during fermentation is accumulated in the cells of *P. freudenreichii* and was shown to be as stable as cyanocobalamin, with a maximum loss of 23% during different baking processes (Edelmann et al., 2016). Therefore, the addition of fermented brans in food products can be a promising way to provide vitamin B12 to consumers. For instance, by replacing 15% of wheat flour with fermented oat bran, 2 slices of bread (40-60 g) per day can provide a sufficient amount of vitamin B12 (2.4 μg; Institute of medicine, 1998).

Some traditional fermented legume products, such as tempeh and natto, are known to contain small amounts of vitamin B12 (12 to 134 ng/g dw) produced by the contaminated bacteria (Nout and Rombouts, 1990; Watanabe et al., 2014). Recently, some studies have shown that using *P. freudenreichii* could significantly increase the production of vitamin B12 (up to 1230 ng/g dw) during fermentation

in the lupine tempeh (Signorini et al., 2018; Wolkers-Rooijackers et al., 2018). In the present study, the production of vitamin B12 during the fermentation of three legume flours (300-400 ng/g dw) was not as high as in the lupine tempeh. However, these fermented legumes can be used as ingredients in various food products, which may provide a new alternative for the utilization of legume materials as a source of vitamin B12.

The production of vitamin B12 in cereal and pseudocereal flours was not as high as in brans and legume flours. A relatively high level of vitamin B12 was produced in the sorghum flour (ca. 265 ng/g dw on Day 3). This was probably due to the high pH during fermentation, owing to the poor growth of LAB in this material. Sorghum products are important foods in some parts of Asia and Africa (Nout, 2009) and people in these areas have a higher prevalence of vitamin B12 deficiency than on other continents (Hunt et al., 2014). Therefore, the *in situ* fortification of sorghum fermented with *P. freudenreichii* can be an important way to increase the overall dietary intake of vitamin B12, in the developing countries of Asia and Africa.

6.2 Controlling pH and increasing availability of cobalt can enhance *in situ* production of vitamin B12 during fermentation

The production of vitamin B12 by *P. freudenreichii* has been shown to be strongly dependent on pH during fermentation (Quesada-Chanto et al., 1994). In Study III, a significant positive correlation between pH and vitamin B12 production was observed in the PLS analysis (Figure 8). Meanwhile, the contents of compounds with high pH buffering capacity, such as protein (Lević et al., 2005) and dietary fiber (Tadesse, 1986) were also found to be positively correlated to vitamin B12 production (Figure 8). On the other hand, the content of available carbohydrates was found to be negatively correlated with vitamin B12 production (Figure 8), probably due to the more intense acidification in these materials. These results can provide useful information for the selection of new materials for vitamin B12 *in situ* fortification, in different food categories.

Controlling pH during fermentation was shown to be an efficient way to enhance vitamin B12 production. Although the optimal pH for the production of vitamin B12 was around 7, Study II showed that maintaining pH at 5 can increase the production by about 80% during a 3-day fermentation of wheat bran.

Meanwhile, more than 600 ng/g of vitamin B12 were produced in buckwheat bran batter (Study III), which had a pH of 4.9 during fermentation. Therefore, controlling pH at a moderately acidic level (e.g. pH 5.0) can be a practical method to enhance the *in situ* production of vitamin B12, while simultaneously controlling the propagation of potential pathogens, as shown in Study II.

Cobalt content has been shown to influence the production of vitamin B12 during fermentation with *P. freudenreichii* in different matrices (Berry and Bullerman, 1966; Hugenschmidt et al., 2011; Chamlagain et al., 2017). In Study I, the production of vitamin B12 increased from ca. 33 ng/g dw to ca. 200 ng/g dw when 600 ng/g dw of cobalt chloride was supplied to durum flour (originally containing 2.5 ng/g dw cobalt) batter. On the other hand, no significant correlation was found between cobalt content and vitamin B12 production (Figure 8) among different grain materials during fermentation. Interestingly, in Study III, a comparable production of vitamin B12 was found between the oat bran (ca. 332 ng/g dw) and the fava bean flour (ca. 298 ng/g dw) even though the cobalt content in fava bean flour (ca. 696 ng/g dw) was much higher than in the oat bran (ca. 17 ng/g dw). Meanwhile, the production of vitamin B12 in wheat bran II, which only contained 10 ng/g dw of cobalt, also reached ca. 330 ng/g dw when pH was maintained at 5 during fermentation (Study II). These results suggest that, during *in situ* production of vitamin B12 in grain materials, a certain amount of cobalt content is required. However, when it goes over a threshold, the influence of cobalt content on vitamin B12 production is less significant than pH.

6.3 Levels of riboflavin and oxygen were sufficient to synthesize active vitamin B12 during the fermentation of grain materials

In the present work, no DMBI was added during fermentation, so all the DMBI in the synthesized vitamin B12 was from *de novo* biosynthesis by *P. freudenreichii*. Riboflavin is the precursor for the *de novo* biosynthesis of DMBI in *P. freudenreichii* (Hollriegl et al., 1982). In Study II, the observation that riboflavin content in batters with vitamin B12 production was significantly ($p < 0.05$) lower than in the control batter after Day 1 indicates that DMBI was synthesized using riboflavin. However, the riboflavin content decreased only slightly in wheat bran batter (less than 20%) and it even increased in the durum flour and whole-wheat flour batters during fermentation (Study I). In Study II, higher riboflavin contents

were also observed during the fermentation of wheat bran batters with pH control. This was probably due to biosynthesis by inoculated *P. freudenreichii* or by some LAB commonly retrieved from cereal microflora, such as *Lactobacillus plantarum, Lactobacillus lactis* and *Lactobacillus fermentum* (Burgess et al., 2006; Capozzi et al., 2011; Russo et al., 2014). Therefore, it can be assumed that the level of riboflavin in the grain materials is high enough for the fortification of vitamin B12 by *P. freudenreichii*.

Oxygen is required during the biosynthesis of DMBI in *P. freudenreichii* (Iida et al., 2007) and pseudovitamin B12 will be produced under anaerobic conditions (Deptula et al., 2015). On the other hand, high level of oxygen can also inhibit the cell growth and vitamin B12 production of *P. freudenreichii* (Quesada-Chanto et al., 1998). Therefore, during the industrial production of vitamin B12, a two-stage fermentation is generally used for a high yield (Quesada-Chanto et al., 1994). In the first stage, the bacteria are anaerobically grown to produce cobamide, a vitamin B12 intermediate lacking the DMBI moiety. In the second stage, vitamin B12 formation is completed via gentle aeration, allowing *P. freudenreichii* to undertake the oxygen-dependent synthesis of the DMBI and to link it to cobamide (Burgess et al., 2009).

All the batters in the present work were fermented in tubes or bottles, with shaking/stirring to achieve a microaerobic condition. The presence of pseudovitamin B12 was followed during the analysis, based on the retention times and absorption spectra of the vitamin B12 compounds. No detectable amount of pseudovitamin B12 was present in any of the batters, except in the rice bran batter on Day 3. Notably, by opening the tubes and then closing them once on Day 1 to allow air in, the production of vitamin B12 increased from ca. 731 ng/g dw to ca. 900 ng/g dw on Day 3 in the rice bran batter fermented with ca. 9.0 log CFU/g *P. freudenreichii* and ca. 6.0 log CFU/g *L. brevis* (data not shown). In addition, by employing UHPLC-MS, it was confirmed that the increased content of vitamin B12 came from the transfer of pseudovitamin B12 to the active form.

6.4 Fermentation of native vs. heat-treated raw materials

Native grain materials have a varying content of indigenous microbes and, in general, LAB play a central role in their fermentation by dominating the microflora

(De Vuyst and Neysens, 2005). Therefore, one of the most limiting factors, when using *P. freudenreichii* in grain fermentations, is its intrinsic property of slow growth rate compared to LAB (Babuchowski et al., 1999). To date, studies about producing vitamin B12 in grain materials have been focusing on fermentation of autoclaved or comparably heat-treated raw materials with *P. freudenreichii* to ensure the production of vitamin B12 and competitiveness of the *P. freudenreichii* (Chamlagain et al., 2017; Signorini et al., 2018; Wolkers – Rooijackers et al., 2018).

However, the heat treatment of grain materials diminishes the economic feasibility of the process. It creates additional energy costs and is not feasible everywhere, for example, in the rural areas of developing countries. Many cereal processes, such as baking, are also heavily dependent on the activity of endogenous enzymes during processing (Gänzle, 2014). The denaturation of endogenous enzymes by heat treatment can also severely influence the technological or even the nutritional functionality of grain materials, limiting their usability over a wide application area.

This thesis has focused on developing a fermentation process for vitamin B12 production in native grain materials, via fermentation with *P. freudenreichii*. In Study I, endogenous LAB dominated the fermentation of whole-wheat flour and inhibited the propagation and acid production of *P. freudenreichii*. However, *P. freudenreichii* was still able to produce a nutritionally relevant amount of vitamin B12 during fermentation. The wider applicability of *P. freudenreichii* for vitamin B12 fortification was demonstrated by fermenting in various different native grain materials in Study III. Therefore, these results suggest that the process developed for fermentation with *P. freudenreichii* is economically feasible and may be utilized everywhere in the world, without need for costly equipment or energy for heat-treatment.

6.5 The benefits of co-fermentation with LAB during the *in situ* fortification of native grain materials

There are some potential pathogens existing in native grain materials. In the present study, high levels of *Enterobacteriaceae* were found in some materials, such as wheat bran and sorghum flour. Inoculating *P. freudenreichii* with indigenous LAB could not effectively inhibit their propagation. Furthermore, the indigenous microbiota of grains varies from crop to crop and from year to year,

which does not allow for controlled and repeatable fermentations. *Enterobacteriaceae* is a large family of Gram-negative bacteria, some of which are pathogens that cause gastrointestinal tract infections or produce various endotoxins (Singh et al., 2015). Therefore, a co-fermentation of *P. freudenreichii* with a strain exerting a highly competitive and antagonistic effect on *Enterobacteriaceae* was required to maintain the consistency of the fermentation and improve the microbial safety of the ferments.

The co-fermentation of PAB and LAB is commonly used in cheese fermentation, because the lactic acid produced by LAB is the preferential carbon source for PAB (Smid and Lacroix, 2013). Moreover, the co-fermentation of LAB and PAB in cereal materials has been studied for its preservative effect, texture-building and anti-staling ability, due to the synergistic production of exopolysaccharides and acids (Javanainen and Linko, 1993b; Javanainen and Linko, 1993a; Tinzl-Malang et al., 2015). In Study II, the screening process showed that all LAB strains tested could inhibit the growth of *Enterobacteriaceae* and *L. brevis* showed the strongest inhibition on *Enterobacteriaceae* among all the tested strains. In the fermentation of wheat bran II, the cell density of *Enterobacteriaceae* began to decrease on Day 1, when *L. brevis* was inoculated. This inhibitory effect in the early stage of fermentation can reduce the microbial risks, such as the potential production of endotoxins, to increase the overall safety of wheat bran. In Study III, *L. brevis* was also shown to effectively inhibit the propagation of *Enterobacteriaceae* in the fermentation of four materials (oat bran, rice bran, buckwheat bran and sorghum flour). The results thus show that co-fermentation of *P. freudenreichii* and *L. brevis* can be a promising alternative, to fortify vitamin B12 in a wide variety of plant-based foods.

6.6 Limitations of the study

This thesis has demonstrated the possibility of vitamin B12 *in situ* fortification in grain materials, via fermentation with *P. freudenreichii.* However, a significant variation in vitamin B12 production among different raw materials was observed. A major limitation of the present study is that only one type of material was studied, from each of the different grains. The composition of both macro- and micro-nutrients, as well as indigenous microflora, varies within the

same grain type (e.g. due to different varieties or growth location). This may result in different vitamin B12 productions during fermentation. More in-depth studies of grain varieties from different growing conditions and origins are therefore needed, for a more comprehensive understanding of their full potential in vitamin B12 production.

The strain *P. freudenreichii* DSM 20271, which is the type of strain isolated from cheese, was used in the current study for fortification of vitamin B12. Previous studies have shown that strains of *P. freudenreichii* differ greatly in their ability to synthesize vitamin B12 (Hugenschmidt et al., 2010; Chamlagain et al., 2016). Therefore, fermentation with different strains of *P. freudenreichii*, especially strains isolated from plant materials, should be studied, to fully exploit the potential of *P. freudenreichii* in fortifying grain materials with vitamin B12.

In the present study, at least 3 days of fermentation were applied to ensure a high production of vitamin B12 in grain materials. However, during a long fermentation time under an acidic pH, the activity of microorganisms and enzymes may lead to profound changes in the technological and nutritional properties of grain materials by affecting protein, starch and dietary fiber. For instance, a significant loss of firmness and elasticity in wheat bread was observed, when acidic sourdough with intensive proteolytic activity was utilized in baking (Clarke et al., 2004). Meanwhile, the content of starch and the solubility and molecular weight of dietary fibers, such as β-glucan and arabinoxylan, are likely to be influenced by pH, as the optimum pH for fiber hydrolyzing enzymes varies from 3.5 to 6.5 (Courtin and Delcour, 2002; Lambo et al., 2005; Egwim and Oloyede, 2006). On the other hand, various antinutrients, such as phytic acid and tannins, and even some toxins, such as vicine and convicine, may also be degraded during acidic fermentation (Rizzello et al., 2016; Popova and Mihaylova, 2019). The changes in these components during fermentation, especially in the co-fermented batters, should therefore be studied, and optimized if needed, for the future utilization of these fermented grain materials.

6.7 Future prospects

Grain materials are a staple food in global nutrition, and thus a promising option to provide vitamin B12 for people whose intake of animal-based food is limited. The current trend is toward replacing animal products with plant-based food, due to the need for a healthy diet, food security and environmental sustainability (Willett et al., 2019). Grains fermented by *P. freudenreichii* can be a good way to compensate for the global decrease of dietary vitamin B12 intake, due to the reduced intake of animal food products. In the present study, cereal brans and legumes were found to be the most promising materials for vitamin B12 fortification.

Brans have been known as a good source of dietary fiber, minerals and phytochemicals. Recent studies have shown that bioprocessing technologies, such as enzymatic processing and fermentation, can greatly enhance the technological functionality and protein bioavailability of brans (Arte et al., 2015; Coda et al., 2015; Arte et al., 2016). Therefore, the combined use of starter cultures, including *P. freudenreichii* and LAB, and cell-wall-degrading enzymes could be studied to convert brans into a feasible yet versatile food material, providing various nutrients in a number of food products.

Legumes are good sources of protein and recently have been widely studied as the material to produce meat or dairy analogues (Malav et al., 2015; Maroma, 2015). Since vitamin B12 does not naturally exist in legumes, the utilization of legume materials fermented with *P. freudenreichii* can be beneficial for developing meat or dairy analogues, from the nutritional angle.

To fully exploit the potential of the proposed co-fermentation technology for *in situ* fortification of vitamin B12, different strains of *P. freudenreichii* should be studied with a wide variety of most potential raw materials. Moreover, the fermentation of the same material in different conditions (e.g. pH and oxygen content) and with the addition of different ingredients (e.g. protein or materials with good availability of cobalt) should be applied in the future study, to deeply investigate the factors influencing vitamin B12 production in grain materials. Differences in gene expression and protein synthesis related to vitamin B12 production under different conditions, especially under different pH, should also

be studied to illustrate the mechanism and regulation of vitamin B12 synthesis of *P. freudenreichii* in different grain materials.

In addition, the application of fermented grains in different foods should be studied in future, to guarantee their application potential. The stability of synthesized vitamin B12 in different processes, and its bioavailability to the human body, should also be studied.

7 Conclusions

Animal-origin food products are the main dietary source of vitamin B12. Thus, developing plant-origin food products that are fortified with vitamin B12 can provide not just a way to increase dietary intake of the vitamin in people with limited access to animal products, but also help meet growing nutritional needs, caused by the current trend of replacing animal food products with plant-based alternatives.

The present thesis has demonstrated that fermentation with *P. freudenreichii* can produce a nutritionally significant level of vitamin B12 in various native grain materials. Several grain materials, such as oat bran, rice bran, buckwheat bran, soy bean flour and lupine flour were found to be the most promising substrates for *in situ* fortification of vitamin B12 (more than 300 ng/g dw on Day 3). Meanwhile, PLS analysis suggested that the production of vitamin B12 during fermentation was greatly influenced by the chemical composition of the raw materials. Furthermore, maintaining pH at a level higher than 5.0, especially by utilising raw materials with a good buffering capability, was shown to be an effective method for enhancing the production of vitamin B12 during fermentation.

On the other hand, the growth of potential pathogens, such as *Enterobacteriaceae*, in the batters fermented with *P. freudenreichii* monoculture suggested that a co-culture of *P. freudenreichii* with LAB was required to control the microbial safety of the fermentation. After a screening process, *L. brevis* ATCC 14869 was selected as the co-culture with *P. freudenreichii*. It showed an excellent antagonistic effect on *Enterobacteriaceae,* during fermentation in different grain materials.

In conclusion, the present thesis has proven the feasible technology of *in situ* fortification of vitamin B12, by fermentation with *P. freudenreichii* in different native grain materials. Furthermore, a co-fermentation with *P. freudenreichii* and *L. brevis* was established for vitamin B12 *in situ* fortification in different grain materials, ensuring the microbial safety of the process.

References

Allen L, d. B. B., Dary O, Hurrell RF,. (2006). *Guidelines on Food Fortification With Micronutrients*. Geneva: World Health Organization and Food and Agriculture Organization

Allen, L. H. (2009). How common is vitamin B-12 deficiency? *The American Journal of Clinical Nutrition, 89*(2), 693-696.

Allen, L. H., Rosenberg, I. H., Oakley, G. P., & Omenn, G. S. (2010). Considering the case for vitamin B12 fortification of flour. *Food and Nutrition Bulletin, 31*, 36-46.

Allen, R. H., Seetharam, B., Podell, E., & Alpers, D. H. (1978). Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *Journal of Clinical Investigation, 61*(1), 47-54.

Alsberg, T., Minten, J., Haglund, J., & Törnqvist, M. (2001). Determination of hydroxyalkyl derivatives of cobalamin (vitamin B12) using reversed phase high performance liquid chromatography with electrospray tandem mass spectrometry and ultraviolet diode array detection. *Rapid Commun Mass Spectrom, 15*(24), 2438-2445.

Alvarez-Jubete, L., Arendt, E. K., & Gallagher, E. (2009). Nutritive value and chemical composition of pseudocereals as gluten-free ingredients. *International Journal of Food Science and Nutrition, 60 Suppl 4*, 240-257.

Alvarez-Jubete, L., Arendt, E. K., & Gallagher, E. (2010). Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. *Trends in Food Science & Technology, 21*(2), 106-113.

Arte, E., Katina, K., Holopainen-Mantila, U., & Nordlund, E. (2016). Effect of Hydrolyzing Enzymes on Wheat Bran Cell Wall Integrity and Protein Solubility. *Cereal Chemistry*, *93*(2), 162-171.

Arte, E., Rizzello, C. G., Verni, M., Nordlund, E., Katina, K., & Coda, R. (2015). Impact of Enzymatic and Microbial Bioprocessing on Protein Modification and Nutritional Properties of Wheat Bran. *Journal of Agricultural and Food Chemistry, 63*(39), 8685-8693.

Babuchowski, A., Laniewska-Moroz, L., & Warminska-Radyko, I. J. L. (1999). Propionibacteria in fermented vegetables. *Le Lait, 79*(1), 113-124.

Berry, E. C., & Bullerman, L. B. (1966). Use of cheese whey for vitamin b12 production .2. Cobalt precursor and aeration levels. *Applied Microbiology, 14*(3), 356-357.

Bito, T., Bito, M., Asai, Y., Takenaka, S., Yabuta, Y., Tago, K., . . . Watanabe, F. (2016). Characterization and Quantitation of Vitamin B12 Compounds in Various Chlorella Supplements. *Journal of Agricultural and Food Chemistry, 64*(45), 8516-8524.

Burgess, C. M., Smid, E. J., Rutten, G., & van Sinderen, D. (2006). A general method for selection of riboflavin-overproducing food grade micro-organisms. *Microbial Cell Factories, 5*, 24.

Burgess, C. M., Smid, E. J., & van Sinderen, D. (2009). Bacterial vitamin B2, B11 and B12 overproduction: An overview. *International Journal of Food Microbiology, 133*(1-2), 1-7.

Calinoiu, L. F., & Vodnar, D. C. (2018). Whole Grains and Phenolic Acids: A Review on Bioactivity, Functionality, Health Benefits and Bioavailability. *Nutrients, 10*(11).

Campaniello, D., Bevilacqua, A., Sinigaglia, M., & Altieri, C. (2015). Screening of Propionibacterium spp. for potential probiotic properties. *Anaerobe, 34*, 169-173.

Campbell-Platt, G. (1994). Fermented foods — a world perspective. *Food Research International, 27*(3), 253-257.

Campbell, A. K., Miller, J. W., Green, R., Haan, M. N., & Allen, L. H. (2003). Plasma vitamin B-12 concentrations in an elderly latino population are predicted by serum gastrin concentrations and crystalline vitamin B-12 intake. *The Journal of Nutrition, 133*(9), 2770-2776.

Campos-Gimenez, E., Fontannaz, P., Trisconi, M. J., Kilinc, T., Gimenez, C., Andrieux, P., & Nelson, M. (2012). Determination of vitamin B12 in infant formula and adult nutritionals by liquid chromatography/UV detection with immunoaffinity extraction: First Action 2011.08. *Journal AOAC International, 95*(2), 307-312.

Capozzi, V., Menga, V., Digesu, A. M., De Vita, P., van Sinderen, D., Cattivelli, L., . . . Spano, G. (2011). Biotechnological production of vitamin B2-enriched bread and pasta. *Journal of Agricultural and Food Chemistry, 59*(14), 8013-8020.

Carmel, R. (2011). Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *The American Journal of Clinical Nutrition, 94*(1), 348-358.

Chamlagain, B., Deptula, P., Edelmann, M., Kariluoto, S., Grattepanche, F., Lacroix, C., . . . Piironen, V. (2016). Effect of the lower ligand precursors on vitamin B12 production by food-grade Propionibacteria. *LWT - Food Science and Technology, 72*, 117-124.

Chamlagain, B., Edelmann, M., Kariluoto, S., Ollilainen, V., & Piironen, V. (2015). Ultra-high performance liquid chromatographic and mass spectrometric analysis of active vitamin B12 in cells of Propionibacterium and fermented cereal matrices. *Food Chemistry, 166*, 630-638.

Chamlagain, B., Sugito, T., Deptula, P., Edelmann, M., Kariluoto, S., Varmanen, P., & Piironen, V. (2017). *In situ* production of active vitamin B12 in cereal matrices using Propionibacterium freudenreichii. *Food Science & Nutrition, 00*, 1-10.

Clarke, C. I., Schober, T. J., Dockery, P., O'Sullivan, K., & Arendt, E. K. (2004). Wheat sourdough fermentation: effects of time and acidification on fundamental rheological properties. *Cereal Chemistry, 81*(3), 409-417.

Coda, R., Katina, K., & Rizzello, C. G. (2015). Bran bioprocessing for enhanced functional properties. *Current Opinion in Food Science, 1*, 50-55.

Combs, G. F., & McClung, J. P. (2017). Chapter 18 - Vitamin B12. In G. F. Combs & J. P. McClung (Eds.), *The Vitamins (Fifth Edition)* (pp. 431-452): Academic Press.

Courtin, C. M., & Delcour, J. A. (2002). Arabinoxylans and Endoxylanases in Wheat Flour Bread-making. *Journal of Cereal Science, 35*(3), 225-243.

Cousin, F. J., Mater, D. D. G., Foligné, B., & Jan, G. (2011). Dairy propionibacteria as human probiotics: A review of recent evidence. *Dairy Science and Technology, 91*(1), 1-26.

De Angelis, M., Bottacini, F., Fosso, B., Kelleher, P., Calasso, M., Di Cagno, R., . . . Gobbetti, M. (2014). *Lactobacillus rossiae*, a vitamin B12 producer, represents a metabolically versatile species within the Genus Lactobacillus. *PLoS One, 9*(9), e107232.

De Vuyst, L., & Neysens, P. (2005). The sourdough microflora: biodiversity and metabolic interactions. *Trends in Food Science & Technology, 16*(1-3), 43-56.

Deptula, P., Kylli, P., Chamlagain, B., Holm, L., Kostiainen, R., Piironen, V., . . . Varmanen, P. (2015). BluB/CobT2 fusion enzyme activity reveals mechanisms responsible for production of active form of vitamin B12 by Propionibacterium freudenreichii. *Microbial Cell Factories 14*(1), 186.

Edelmann, M., Aalto, S., Chamlagain, B., Kariluoto, S., & Piironen, V. (2019). Riboflavin, niacin, folate and vitamin B12 in commercial microalgae powders. *Journal of Food Composition and Analysis, 82*.

Edelmann, M., Chamlagain, B., Santin, M., Kariluoto, S., & Piironen, V. (2016). Stability of added and in situ-produced vitamin B12 in breadmaking. *Food Chemistry, 204*, 21-28.

Egwim, E. C., & Oloyede, O. B. (2006). Comparison of a-amylase activity in some sprouting Nigerian cereals. *Biokemistri, 18*(1).

Ekholm, P., Reinivuo, H., Mattila, P., Pakkala, H., Koponen, J., Happonen, A., . . . Ovaskainen, M.-L. (2007). Changes in the mineral and trace element contents of cereals, fruits and vegetables in Finland. *Journal of Food Composition and Analysis, 20*(6), 487-495.

F Waer, H., & A Helmy, S. (2017). Cytological and Histochemical Studies in Rat Liver and Pancreas during Progression of Streptozotocin Induced Diabetes and Possible Protection of Certain Natural Antioxidants. *Journal of Nutrition & Food Sciences, 07*(04).

Fletcher, R. J. (2016). Pseudocereals, Overview. *Encyclopedia of Food Grains*: Second Edition. United Kingdom: Elsevier.274-279.

Fröhlich-Wyder, M.-T., Bachmann, H.-P., & Lait, M. G. C. J. (2002). Interaction between propionibacteria and starter/non-starter lactic acid bacteria in Swiss-type cheeses. *Le Lait, 82*(1), 1-15.

Gänzle, M. G. (2014). Enzymatic and bacterial conversions during sourdough fermentation. *Food Microbiology, 37*, 2-10.

Gilsing, A. M. J., Crowe, F. L., Lloyd-Wright, Z., Sanders, T. A. B., Appleby, P. N., Allen, N. E., & Key, T. J. (2010). Serum concentrations of vitamin B12 and folate in British male omnivores, vegetarians and vegans: results from a cross-sectional analysis of the EPIC-Oxford cohort study. *European journal of clinical nutrition, 64*(9), 933-939.

Gimenez, E. C. (2014). Improved AOAC First Action 2011.08 for the Analysis of Vitamin B(1)(2) in Infant Formula and Adult/Pediatric Formulas: First Action 2014.02. *Journal of AOAC International, 97*(5), 1397-1402.

Gobbetti, M., De Angelis, M., Di Cagno, R., Polo, A., & Rizzello, C. G. (2019). The sourdough fermentation is the powerful process to exploit the potential of legumes, pseudo-cereals and milling by-products in baking industry. *Critical Reviews in Food Science and Nutrition*, 1-16.

Graybosch, R. A. (2016). The Grain Crops: An Overview. *Encyclopedia of Food Grains:* Second Edition United Kingdom: Elsevier. 16-21

Green, R., Allen, L. H., Bjorke-Monsen, A. L., Brito, A., Gueant, J. L., Miller, J. W., . . . Yajnik, C. (2017). Vitamin B12 deficiency. *Nature Reviews Disease Primers, 3*, 17040.

Green, R., & Miller, J. (2013). Vitamin B12. *Handbook of Vitamins, Fifth Edition*: CRC Press. 447-490

Haard, N. F. (1999). *Fermented cereals: a global perspective,* (No. 138), Food & Agriculture Org.

Hammes, W. P., Brandt, M. J., Francis, K. L., Rosenheim, J., Seitter, M. F. H., & Vogelmann, S. A. (2005). Microbial ecology of cereal fermentations. *Trends in Food Science & Technology, 16*(1), 4-11.

Hammes, W. P., & Gänzle, M. (1998). Sourdough breads and related products. In *Microbiology of fermented foods* (pp. 199-216): Springer.

Heiniö, R. L., Noort, M. W. J., Katina, K., Alam, S. A., Sozer, N., de Kock, H. L., . . . Poutanen, K. (2016). Sensory characteristics of wholegrain and bran-rich cereal foods – A review. *Trends in Food Science & Technology, 47*, 25-38.

Herbert, V. (1988). Vitamin B-12: plant sources, requirements, and assay. *The American journal of clinical nutrition, 48*(3), 852-858.

Herrmann, W., Schorr, H., Obeid, R., & Geisel, J. (2003). Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. *The American journal of clinical nutrition, 78*(1), 131-136.

Hosseinpour-Niazi, S., Mirmiran, P., Hedayati, M., & Azizi, F. (2015). Substitution of red meat with legumes in the therapeutic lifestyle change diet based on dietary advice improves cardiometabolic risk factors in overweight type 2 diabetes patients: a cross-over randomized clinical trial. *European journal of clinical nutrition, 69*(5), 592-597.

Hugenholtz, J., & Smid, E. J. (2002). Nutraceutical production with food-grade microorganisms. *Current Opinion in Biotechnology, 13*(5), 497-507.

Hugenschmidt, S., Schwenninger, S. M., Gnehm, N., & Lacroix, C. (2010). Screening of a natural biodiversity of lactic and propionic acid bacteria for folate and vitamin B12 production in supplemented whey permeate. *International Dairy Journal, 20*(12), 852-857.

Hugenschmidt, S., Schwenninger, S. M., & Lacroix, C. (2011). Concurrent high production of natural folate and vitamin B12 using a co-culture process with *Lactobacillus plantarum* SM39 and *Propionibacterium freudenreichii* DF13. *Process Biochemistry, 46*(5), 1063-1070.

Hunt, A., Harrington, D., & Robinson, S. (2014). Vitamin B12 deficiency. *BMJ, 349*, g5226.

Iida, K., & Kajiwara, M. (2000). Evaluation of Biosynthetic Pathways to δ-Aminolevulinic Acid in Propionibacterium shermanii Based on Biosynthesis of Vitamin B12 from d-[1-13C]Glucose. *Biochemistry, 39*(13), 3666-3670.

Iida, K., Ohtaka, K., & Kajiwara, M. (2007). Mechanism of the ring contraction process in vitamin B12 biosynthesis by the anaerobe *Propionibacterium shermanii* under aerobic conditions. *The FEBS Journal, 274*(13), 3475-3481.

Javanainen, P., & Linko, Y. Y. (1993). Factors affecting rye sourdough fermentation with mixed-culture pre-ferment of lactic and propionic acid bacteria. *Journal of Cereal Science, 18*(2), 171-185.

Javanainen, P., & Linko, Y. Y. (1993). Utilization of sprout-damaged wheat as raw material for sour dough pre-ferments with mixed cultures of lactic and propionic acid bacteria. *Food Biotechnology, 7*(2), 99-113.

Jenkins, D. J., Kendall, C. W., Augustin, L. S., Mitchell, S., Sahye-Pudaruth, S., Blanco Mejia, S., . . . Josse, R. G. (2012). Effect of legumes as part of a low glycemic index diet on glycemic control and cardiovascular risk factors in type 2 diabetes mellitus: a randomized controlled trial. *Archives of internal medicine, 172*(21), 1653-1660.

Kariluoto, S., Edelmann, M., Nystrom, L., Sontag-Strohm, T., Salovaara, H., Kivela, R., . . . Piironen, V. (2014). *In situ* enrichment of folate by microorganisms in beta-glucan rich oat and barley matrices. *International Journal of Food Microbiology, 176*, 38-48.

Katina, K., Laitila, A., Juvonen, R., Liukkonen, K. H., Kariluoto, S., Piironen, V., . . . Poutanen, K. (2007). Bran fermentation as a means to enhance technological properties and bioactivity of rye. *Food Microbiology, 24*(2), 175-186.

Kelleher, B. P., & Broin, S. D. (1991). Microbiological assay for vitamin B12 performed in 96-well microtitre plates. *Journal of clinical pathology, 44*(7), 592-595.

Koskinen, P., Deptula, P., Smolander, O. P., Tamene, F., Kammonen, J., Savijoki, K., . . . Varmanen, P. (2015). Complete genome sequence of *Propionibacterium freudenreichii* DSM 20271(T). *Stand Genomic Sci, 10*, 83.

Kumar, S. S., Chouhan, R. S., & Thakur, M. S. (2010). Trends in analysis of vitamin B12. *Analytical Biochemistry, 398*(2), 139-149.

Lambert, D., Adjalla, C., Felden, F., Benhayoun, S., Nicolas, J. P., & Guéant, J. L. (1992). Identification of vitamin B12 and analogues by high- performance capillary electrophoresis and comparison with high-performance liquid chromatography. *Journal of Chromatography A, 608*(1), 311-315.

Langsrud, T., Reinbold, G. J. J. o. m., & technology, f. (1973). Flavor development and microbiology of Swiss cheese-A review: III. Ripening and flavor production. *Journal of milk and food technology 36*(12), 593-609.

Lević, J., Prodanović, O., & Sredanović, S. J. B. i. A. H. (2005). Understanding the buffering capacity in feedstuffs. *Biotechnology in Animal Husbandry*, *21*(5-6), 309-313.

Malav, O. P., Talukder, S., Gokulakrishnan, P., & Chand, S. (2015). Meat analog: a review. *Critical Reviews in Food Science and Nutrition, 55*(9), 1241-1245.

Malik, A. C., Reinbold, G. W., & Vedamuthu, E. R. (1968). An evaluation of taxonomy of propionibacterium. *Canadian Journal of Microbiology, 14*(11), 1185-1191.

Mann, J., Schiedt, B., Baumann, A., Conde-Petit, B., & Vilgis, T. A. (2013). Effect of heat treatment on wheat dough rheology and wheat protein solubility. *Food Science and Technology International, 20*(5), 341-351.

Maroma, D. P. (2015). Meat Analogue Derived from Common Legumes. *Open Access Library Journal*. *2(01), 1.*

Marsh, K., Zeuschner, C., & Saunders, A. (2011). Health Implications of a Vegetarian Diet. *American Journal of Lifestyle Medicine, 6*(3), 250-267.

Martens, J. H., Barg, H., Warren, M. J., & Jahn, D. (2002). Microbial production of vitamin B12. *Applied Microbiology and Biotechnology, 58*(3), 275-285.

Mogensen, G., Salminen, S., O'Brien, J., Ouwehand, A., Holzapfel, W., Shortt, C., . . . Zink, R. (2002). Inventory of microorganisms with a documented history of use in food. *Bulletin of the International Dairy Federation, 377*, 10-19.

Moore, S. J., Lawrence, A. D., Biedendieck, R., Deery, E., Frank, S., Howard, M. J., . . . Warren, M. J. (2013). Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B12). *Proceedings of the National Academy of Sciences of the United States of America, 110*(37), 14906-14911.

Morkbak, A. L., Poulsen, S. S., & Nexo, E. (2007). Haptocorrin in humans. *Clinical Chemistry and Laboratory Medicine, 45*(12), 1751-1759.

Nielsen, M. J., Rasmussen, M. R., Andersen, C. B., Nexo, E., & Moestrup, S. K. (2012). Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. *Nature Reviews Gastroenterology & Hepatology, 9*(6), 345-354.

Nkhata, S. G., Ayua, E., Kamau, E. H., & Shingiro, J. B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Science and Nutrition, 6*(8), 2446-2458.

Nóbrega, J. A., Pirola, C., Fialho, L. L., Rota, G., de Campos Jordão, C. E. K. M. A., & Pollo, F. (2012). Microwave-assisted digestion of organic samples: How simple can it become? *Talanta, 98*, 272-276.

Noort, M. W. J., van Haaster, D., Hemery, Y., Schols, H. A., & Hamer, R. J. (2010). The effect of particle size of wheat bran fractions on bread quality – Evidence for fibre–protein interactions. *Journal of Cereal Science, 52*(1), 59-64.

Nout, M. J. (2009). Rich nutrition from the poorest-cereal fermentations in Africa and Asia. *Food Microbiology, 26*(7), 685-692.

Nout, M. J. R., & Rombouts, F. M. (1990). Recent developments in tempe research. *in tempe research. Journal of Applied Bacteriology, 69*(5), 609-633.

Obeid, R., Fedosov, S. N., & Nexo, E. (2015). Cobalamin coenzyme forms are not likely to be superior to cyano- and hydroxyl-cobalamin in prevention or treatment of cobalamin deficiency. *Molecular Nutrition & Food Research, 59*(7), 1364-1372.

Pawlak, R., James, P. S., Raj, S., Cullum-Dugan, D., & Lucus, D. (2012). Understanding Vitamin B12. *American Journal of Lifestyle Medicine, 7*(1), 60-65.

Pawlak, R., Parrott, S. J., Raj, S., Cullum-Dugan, D., & Lucus, D. (2013). How prevalent is vitamin B(12) deficiency among vegetarians? *Nutrition Reviews, 71*(2), 110-117.

Peña, R. J. (2002). *Wheat for bread and other foods*. Rome: Food and Agriculture Organization of the United Nations.

Piao, Y., Kiatpapan, P., Yamashita, M., & Murooka, Y. (2004). Effects of expression of hemA and hemB genes on production of porphyrin in *Propionibacterium freudenreichii*. *Applied and Environmental Microbiology, 70*(12), 7561-7566.

Piwowarek, K., Lipinska, E., Hac-Szymanczuk, E., Kieliszek, M., & Scibisz, I. (2018). Propionibacterium spp.-source of propionic acid, vitamin B12, and other metabolites important for the industry. *Applied Microbiology and Biotechnology, 102*(2), 515-538.

Popova, A., & Mihaylova, D. (2019). Antinutrients in Plant-based Foods: A Review. *The Open Biotechnology Journal, 13*(1).

Quadros, E. V., Regec, A. L., Khan, K. M. F., Quadros, E., & Rothenberg, S. P. (1999). Transcobalamin II synthesized in the intestinal villi facilitates transfer of cobalamin to the portal blood. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 277*(1), 161-166.

Quesada-Chanto, A., S.-Afschar, A., & Wagner, F. (1994). Microbial production of propionic acid and vitamin B12 using molasses or sugar. *Applied Microbiology and Biotechnology, 41*(4), 378-383.

Quesada-Chanto, A., Schmid-Meyer, A., Schroeder, A., Carvalho-Jonas, M., Blanco, I., & Jonas, R. (1998). Effect of oxygen supply on biomass, organic acids and vitamin B12 production by Propionibacterium shermanii. *World Journal of Microbiology and Biotechnology, 14*(6), 843-846.

Rizzello, C. G., Losito, I., Facchini, L., Katina, K., Palmisano, F., Gobbetti, M., & Coda, R. (2016). Degradation of vicine, convicine and their aglycones during fermentation of faba bean flour. *Scientific Reports, 6*(1), 32452.

Roy, F., Boye, J. I., & Simpson, B. K. (2010). Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. *Food Research International, 43*(2), 432-442.

Russo, P., Capozzi, V., Arena, M. P., Spadaccino, G., Dueñas, M. T., López, P., . . . Spano, G. (2014). Riboflavin-overproducing strains of *Lactobacillus fermentum* for riboflavin-enriched bread. *Applied Microbiology and Biotechnology, 98*(8), 3691-3700.

Salovaara, H., & Simonson, L. (2004). Fermented cereal-based functional foods. In *Handbook of food and beverage fermentation technology,* 736-743: CRC Press.

Sanz Penella, J. M., Collar, C., & Haros, M. (2008). Effect of wheat bran and enzyme addition on dough functional performance and phytic acid levels in bread. *Journal of Cereal Science, 48*(3), 715-721.

Sekwati-Monang, B., Valcheva, R., & Gänzle, M. G. (2012). Microbial ecology of sorghum sourdoughs: Effect of substrate supply and phenolic compounds on composition of fermentation microbiota. *International Journal of Food Microbiology, 159*(3), 240-246.

Selhub, J., & Paul, L. (2011). Folic acid fortification: why not vitamin B12 also? *Biofactors, 37*(4), 269-271.

Shemin, D. (1969). Mechanism and Control of Pyrrole Synthesis. In *Vitamins & Hormones* (Vol. 26, pp. 357-373): Academic Press.

Signorini, C., Carpen, A., Coletto, L., Borgonovo, G., Galanti, E., Capraro, J., . . . Scarafoni, A. (2018). Enhanced vitamin B12 production in an innovative lupin tempeh is due to synergic effects of Rhizopus and Propionibacterium in cofermentation. *International Journal of Food Science and Nutrition, 69*(4), 451-457.

Singh, J., Sharma, S., & Nara, S. (2015). Evaluation of gold nanoparticle based lateral flow assays for diagnosis of enterobacteriaceae members in food and water. *Food Chemistry, 170*, 470-483.

Smid, E. J., & Lacroix, C. (2013). Microbe–microbe interactions in mixed culture food fermentations. *Current Opinion in Biotechnology, 24*(2), 148-154.

Smith, A. D., Warren, M. J., & Refsum, H. (2018). Vitamin B12. *Advances in Food and Nutrition Research, 83*, 215-279.

Stabler, S. P., & Allen, R. H. (2004). Vitamin B12 deficiency as a worldwide problem. *Annual Review of Nutrition, 24*, 299-326.

Stupperich, E., & Nexo, E. (1991). Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B12 binding proteins intrinsic factor, transcobalamin and haptocorrin. *European Journal of Biochemistry, 199*(2), 299-303.

Sundin, D. P., & Allen, R. H. (1992). Analysis of the nucleoside moiety of cobalamin and cobalamin analogues using gas chromatography-mass spectrometry. *Archives of Biochemistry and Biophysics, 298*(2), 658-666.

Svensson, L., Sekwati-Monang, B., Lutz, D. L., Schieber, A., & Ganzle, M. G. (2010). Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor (L.) Moench*). *Journal of Agricultural and Food Chemistry, 58*(16), 9214-9220.

Tadesse, K. (1986). The effect of dietary fibre isolates on gastric secretion, acidity and emptying. *British Journal of Nutrition, 55*(3), 507-513.

Taga, M. E., Larsen, N. A., Howard-Jones, A. R., Walsh, C. T., & Walker, G. C. (2007). BluB cannibalizes flavin to form the lower ligand of vitamin B12. *Nature, 446*(7134), 449-453.

Takahashi-Iniguez, T., Garcia-Hernandez, E., Arreguin-Espinosa, R., & Flores, M. E. (2012). Role of vitamin B12 on methylmalonyl-CoA mutase activity. *Journal of Zhejiang University Science B, 13*(6), 423-437.

Taranto, M. P., Vera, J. L., Hugenholtz, J., De Valdez, G. F., & Sesma, F. (2003). *Lactobacillus reuteri* CRL1098 produces cobalamin. *Journal of Bacteriology, 185*(18), 5643-5647.

Thielecke, F., & Nugent, A. P. (2018). Contaminants in grain-a major risk for whole grain safety? *Nutrients, 10*(9).

Thierry, A., Deutsch, S. M., Falentin, H., Dalmasso, M., Cousin, F. J., & Jan, G. (2011). New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *International Journal of Food Microbiology, 149*(1), 19-27.

Tinzl-Malang, S. K., Rast, P., Grattepanche, F., Sych, J., & Lacroix, C. (2015). Exopolysaccharides from co-cultures of *Weissella con*fusa 11GU-1 and *Propionibacterium freudenreichii* JS15 act synergistically on wheat dough and bread texture. *International Journal of Food Microbiology, 214*, 91-101.

Trzebiatowski, J. R., O'Toole, G. A., & Escalante-Semerena, J. C. (1994). The cobT gene of Salmonella typhimurium encodes the NaMN: 5,6-dimethylbenzimidazole phosphoribosyltransferase responsible for the synthesis of N1-(5-phospho-alpha-D-ribosyl)-5,6-dimethylbenzimidazole, an intermediate in the synthesis of the nucleotide loop of cobalamin. *Journal of Bacteriology, 176*(12), 3568-3575.

Van Wyk, J., Witthuhn, R. C., & Britz, T. J. (2011). Optimisation of vitamin B12 and folate production by *Propionibacterium freudenreichii* strains in kefir. *International Dairy Journal, 21*(2), 69-74.

Warren, M. J., Raux, E., Schubert, H. L., & Escalante-Semerena, J. C. (2002). The biosynthesis of adenosylcobalamin (vitamin B12). *Natural Product Reports, 19*(4), 390-412.

Watanabe, F. (2007). Vitamin B12 sources and bioavailability. *Experimental Biology and Medicine, 232*(10), 1266-1274.

Watanabe, F., & Bito, T. (2018). Determination of Cobalamin and Related Compounds in Foods. *Journal of AOAC International, 101*(5), 1308-1313.

Watanabe, F., Takenaka, S., Kittaka-Katsura, H., Ebara, S., & Miyamoto, E. (2002). Characterization and bioavailability of vitamin B12-compounds from edible algae. *Journal of Nutritional Science and Vitaminology, 48*(5), 325-331.

Watanabe, F., Yabuta, Y., Bito, T., & Teng, F. (2014). Vitamin B12-containing plant food sources for vegetarians. *Nutrients, 6*(5), 1861-1873.

Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., . . . Murray, C. J. L. (2019). Food in the Anthropocene: the EAT–Lancet Commission on healthy diets from sustainable food systems. *The Lancet, 393*(10170), 447-492.

Wolkers – Rooijackers, J. C. M., Endika, M. F., & Smid, E. J. (2018). Enhancing vitamin B 12 in lupin tempeh by *in situ* fortification. *Lwt, 96*, 513-518.

Wrigley, C. W. (2016). An overview of the family of cereal grains prominent in world agriculture. *Encyclopedia of Food Grains:* Second Edition. United Kingdom: Elsevier.73-85.

Xu, Y., Wang, Y., Coda, R., Sade, E., Tuomainen, P., Tenkanen, M., & Katina, K. (2017). *In situ* synthesis of exopolysaccharides by Leuconostoc spp. and Weissella spp. and their rheological impacts in fava bean flour. *International Journal of Food Microbiology, 248*, 63-71.

Zironi, E., Gazzotti, T., Barbarossa, A., Farabegoli, F., Serraino, A., & Pagliuca, G. (2014). Determination of vitamin B(12) in dairy products by Ultra performance liquid chromatography-tandem mass spectrometry. *Italian journal of food safety, 3*(4), 4513-4513.

ISBN 978-951-51-6355-4 (PAPERBACK)
ISBN 978-951-51-6356-1 (PDF, HTTP://ETHESIS.HELSINKI.FI)
ISSN 0355-1180
UNIGRAFIA
HELSINKI 2020