Modulating Acidity and Bioactivities of Sauerkraut with *Proprionibacterium freudenreichii*

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Antonia Lüthi

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Health and nutrition are inextricably connected, and as consumer demand for health promoting products grows, the food industry is responding with the development of functional foods. Synthetic vitamins are commonly supplemented to maintain good health, particularly Vitamin B12, as it is an essential micronutrient obtained exclusively through the consumption of animal derived foods. This leaves vegans and plant based eaters at risk of deficiency. Studies have shown that *Propionibacterium freudenreichii* can be successfully co-fermented with Latic acid bacteria (LAB) to produce B12 in grain based substrates. In this study *P. freudenreichii* was inoculated into a traditional sauerkraut fermentation, resulting in synthesis of B12 at promising levels. Acidity was also modulated when compared with sauerkraut produced by spontaneous LAB fermentation, highlighting the potential for the wider organopleptic appeal of sauerkraut.

Keywords: Vitamin B12, cobalamin, vegan, vegetarian, plant based, sauerkraut, fermentation, *Propionibacteruim freudenreichii*, functional foods, food innovation, nutrition

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Chapter 1

Introduction

Functional and fortified foods are at the forefront of nutritional research as global health spans decline and health promoters look to modifiable risk factors, particularly diet for solutions. Functional foods recognise that food has a more advanced role than simply meeting energy requirements and strives to magnify the nutritional advantages of manufactured food products (Galanakis, 2021). Although used interchangeably, fortified foods either have low levels of specific nutrients, or they are not naturally present at all. The focus of both is largely on micronutrients and phytochemicals.

Cobalamin (B12) is a micronutrient that is commonly fortified in foods. As an essential vitamin that is only available from animal derived foods, vegans and predominantly plant-based eaters are at risk of deficiency (Santo et al., 2023). Meanwhile, plant based diets are becoming more popular as they are associated with a lower risk of metabolic disease compared with diets rich in animal derived foods. Environmental and ethical concerns around animal welfare further drive the trend to replace animal foods with plant based alternatives (FAO, 2022). This places an increasing amount of people at risk of low vitamin B12 intake and potential deficiency.

Vitamin B12 deficiency can lead to serious adverse health outcomes, impacting haematological, neurological, and psychiatric functions (Kumar et al., 2023). Sub clinical deficiency, although less serious is far more common and is estimated to affect around one quarter of some populations, notably those where animal food consumption is low, such as Southeast Asia (Devi et al., 2018). Supplementation and fortification with B12 can address deficiency, and in New Zealand foods fortified with synthetic B12 include plant milks and yeast extracts (Te Whatu Ora - Health New Zealand, 2023). Meanwhile, food producers face an increasing demand for natural and minimally processed foods, which presents challenges when formulating products to meet requirements of functional foods, particularly in plant based products.

Utilising microbial ability to produce B12 may provide a solution. *Propionibacterium freudenreichii* is a food safe species known for de novo B12 synthesis, most commonly in cheese (Thierry, Deutsch, et al., 2011). Introducing *P. freudenreichii* into plant based foods provides an opportunity for naturally derived B12 to be present in foods suited to vegan consumers. Furthermore, fermentation requires limited food processing and is associated with many health benefits.

Nutritionally dense, and with a long history of being fermented, cabbage is selected in this study as an ideal substrate to test this hypothesis. Sauerkraut is produced by spontaneous fermentation, relying on the lactic acid bacteria naturally present in cabbage (Medina-Pradas et al., 2017). Lactic acid bacteria and *P. freudenreichii* share commonalities in their environmental requirements for growth and metabolite production. Furthermore, preference for lactate as a carbon source, and low nutritional needs strengthen the potential for a plant based cross over fermentation of Lactic acid bacteria and *P. freudenreichii* (Dank et al., 2023).

Chapter 2

Literature Review

Sauerkraut (fermented cabbage)

The art of fermentation has been developed over many thousands of years. Empirically used to preserve and detoxify food, over time the practice became recognised for flavour enrichment properties and health benefits. Analysing stone mortars of eastern Mediterranean Nutufians has provided archaeological evidence of beer brewing some 13,000 years ago. Chemical analysis of pottery vessels points to fermented rice and fruits around 6,000 years ago in China and in ancient Egypt dietary staples included fermented bread, dairy products, and beer (Mannaa et al., 2021). Theirry et al., (2023) cites Kimchi (cabbage) as being the earliest fermented vegetable circa 2,000 BC. Others attribute the origin of fermented vegetables to China and workers building the Great Wall in the third century BC (Medina-Pradas et al., 2017). Nevertheless, fermented cabbage was introduced to Western countries in the 12th century by Genghis Khan. There is plenty of evidence for the fermentation of cabbage as a raw material over the ages, especially since kimchi is such an inherent part of the Korean diet. In more recent history, Captain James Cook, famous for his expeditions to New Zealand, Australia and the Pacific was known to protect his sailors from scurvy by instructing a diet which included sauerkraut, due to its vitamin C content (Thierry, Baty, et al., 2023).

Fermentation can be categorised into four categories: alcoholic, acetic, alkali and lactic fermentation. With alcoholic fermentation, sugars are converted to alcohol and carbon dioxide (CO^2), with yeast being the predominant microbe (beer, wine). In acetic fermentation, *Acetobacter* convert sugars and alcohols into acetic acid (kombucha, chocolate, vinegar). Alkali fermentation relies on the release of ammonia from amino acids and peptides, which elevates pH to 8-9, inhibiting microbes associated with spoilage. Lactic acid bacteria (LAB) are largely responsible for lactic fermentation, where sugars are converted to lactic acid (yoghurt, kefir, pickled vegetables) (Mannaa et al., 2021). Sauerkraut falls into the pickled vegetable category and is a product of lactic acid fermentation.

In lactic acid fermentation, lactic acid producing microbes (LAB) naturally present include, but are not limited to: *Lactobacillus brevis, Lactobacillus plantarum, Leuconostoc mesenteroides, Lactobacillus sakei, Lactobacillus pentosus, and Pediococcus pentosaceus* (Medina-Pradas et al., 2017; (Thierry, Baty, et al., 2023). In fresh vegetables the total bacterial population is expected to be 10^4 – 10^8 CFU/g, with LAB dominated by Gram negative species and Gram positive spore formers. On white cabbage tested over four growing seasons, Buckenhueskes (2015) found that LAB represented less than 1% of bacterium, and were dominated by aerobic bacteria and yeasts. Natural LAB habitation on living plants can be restricted by temperature, light and available nutrients. Maturity and presence of pesticides further influences LAB's presence (Medina-Pradas et al., 2017). The process of vegetable fermentation can provide an optimal environment for LAB to grow and is more important than initial counts. Low oxygen availability, high salt and moderate temperatures are favoured conditions (Yu et al., 2019).

Otherwise known as lacto-fermentation, essentially lactic acid and other metabolites produced by LAB result in decreased pH, inhibiting pathogenic and spoilage microorganisms. Thierry, Baty, et al., (2023) simply outline the stages of lacto-fermentation as follows:

Stage 1.	Fermentation is initiated in the first three days with an anaerobic atmosphere created by compacting vegetable matter. Fermentation of sugars into organic acids cause decrease in pH.
Stage 2.	During days 3-6 facultative anaerobic LAB (mainly *Leuconostoc* spp.) grow due to drop of pH and anaerobiosis. Lactic and acetic acid production by heterofermentative LAB leads to further pH drop. CO^2 produced, replacing residual air and increasing anaerobic conditions.
Stage 3.	At approximately one week, more acid tolerant homofermentative LAB such as *L. plantarum* dominate and LAB populations may reach 10^9 CFU/g. Lactic acid concentration of approximately 2% causes pH to decrease to around 4.1 – 3.8.

Stage 4. Heterofermentative species such as *L. brevis*, that can ferment pentoses grow. Lactic acid concentration rises to 2.5% resulting in a pH as low as 3.4. Complete fermentation can take up to several weeks.

These stages outline the significant changes in microbes during lacto-fermentation in response to the changing environmental conditions. The addition of salt at time of preparation creates osmotic pressure and withdraws water and nutrients from plant material, providing microorganisms with substrates needed for growth (Thierry, Madec, et al., 2023). Detrimental bacterium are inhibited with increasing salt concentration while supporting growth of LAB. Heterofermentative species favour low concentrations and are inhibited at around 3%, whereas homofermentative species are more adaptive to a more saline environment. Salt concentrations below 0.8% can result in softening of fermented vegetables due to plant and microbial enzymes (Buckenhueskes, 2015). The ideal salt concentration is generally accepted to be 1 – 2.5% (Medina-Pradas et al., 2017; Peñas, et al., 2016; Thierry, Madec, et al., 2023).

Cabbage is an ideal candidate for fermentation. Its firm texture can withstand the fermentation process, which, due to its microbial community can be spontaneous in ideal conditions (Thierry, Baty, et al., 2023). It has a relatively high sugar content for a vegetable (5%) comprising mostly glucose and fructose. Characteristic of the *Brassicaceae* family, cabbage contains glucosinolates, which contribute to the flavour and taste profile of sauerkraut. Sauerkraut by spontaneous fermentation is prepared by removing the outer leaves of white cabbage, shredding the usable parts, dry salting and compacting into airtight vessels and leaving at ambient temperature, commonly for seven days (Rahmat et al., 2019).

Figure 1 shows an excellent visual representation of significant reactions and interactions that occur during the making of sauerkraut. Here it can be seen that the reducing pH inhibits undesirable microorganisms, therefore supporting LAB dominance. The addition of salt and its various effects on microbes and organoleptic qualities are clearly shown (Buckenhueskes, 2015).

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Figure 1. Reactions taking place during lactic acid fermentation of cabbage to sauerkraut. *preservatives, excluding salt only permitted in few countries (Buckenhueskes, 2015)

In addition to salt content, microbial metabolites are responsible for organoleptic qualities of sauerkraut. Taste is influenced by organic acids as well as other metabolites produced, for instance glucosinolates which after being initially released by cutting, degrade from day two (Medina-Pradas et al., 2017). The products of sulphur containing glucosinolates, isothiocyanates, thiocyanates and nitriles contribute to pungency of cruciferous vegetables and their concentration in sauerkraut is related to pH and salt content (Thierry, Baty, et al., 2023).

It should be noted that the fermentation process can change the composition of foods. Figure 2 compares the nutritional profile of fresh cabbage and sauerkraut. As expected, due to microbial activity, there is substantially less carbohydrate available (mostly sugars) and slightly less vitamin C in fermented cabbage. Rahmat et al., (2019) found that this loss increased over longer fermentation, and explain it is because of vitamin C's water solubility as well as initial losses during slicing preparation. Kiczorowski et al., (2022) examined the effect fermentation has on the nutritional profile of selected vegetables. Cabbage was excluded, nevertheless the findings support the theory that micronutrient concentration may be less in fermented vegetables. When comparing fresh with lacto-fermented vegetables they found mineral, vitamin and phenolic content was reduced. An exception was an increase of vitamin A and precursor carotene in carrots and red peppers. The vitamin C loss of up to 50% was largely attributed to its instability as noted by Rahmat et al., (2019). Conversely, an increase in the vitamin C content of fermented cabbage was reported by Drašković Berger et al., (2020), who surmised it may have been due to microbial degradation of ascorbigen, which is formed by indole-3-carbinole and ascorbic acid. Overall, losses are associated with the metabolic needs of the growing LAB population during fermentation. This study also highlighted a favourable occurrence, where there was a reduction of heavy metals such as copper, lead and cadmium post fermentation. Kiczorowski et al., (2022) point out that it is otherwise difficult to extract these metallic elements from plant foods. Although there is a lack of literature around how fermentation affects specific micronutrient content, there is a consensus that vitamin and mineral concentrations are lower in fermented foods (Thierry, Baty, et al., 2023).

Cabbage, green drumhead, leaves, raw

Nutritional Information	Unit	Serving per 80g	% RDI* per serve	Quantity per 100g	Potential Claim
Energy	kJ	86	0.01	108	Energy - low
Protein	g	1	2	1.2	
Fat, total	g	0.2	0	0.2	
Fat, saturated	g	0.03	0	0.04	
Carbohydrate available	g	3.1	1	3.9	
Sugars	g	3.1	3	3.9	Sugar - low
Dietary fibre	g	1.4	5	1.8	
Sodium	mg	6	0	7	
Potential claimable					
Potassium	mg	200		250	Source
Vitamin C	mg	10.7	27	13.4	Good source

Sauerkraut, undrained, refridgerated

Nutritional Information	Unit	Serving per 80g	% RDI* per serve	Quantity per 100g	Potential Claim
Energy	kJ	43	0	54	Energy - low
Protein	g	0.9	2	1.1	
Fat, total	g	0.2	0	0.2	
Fat, saturated	g	0.02	0	0.03	
Carbohydrate available	g	0.4	0	0.5	
Sugars	g	0.3	0	0.4	Sugar - low, sugar % free
Dietary fibre	g	1.9	6	2.4	
Sodium	mg	648	28	810	
Potential claimable					
Folate	µg	27	14	34	Source
Vitamin C	mg	9.4	23	11.7	Source

Figure 2 General nutritional information panels as presented to New Zealand consumers. Comparison of fresh cabbage and sauerkraut. For vitamins and minerals excluding sodium and potassium, not less than 10% (source) and 25% (good source) of RDI respectively are required for nutrient content claims in accordance with Australia New Zealand Food Standards Code – Schedule 4 – Nutrition, health and related claims (New Zealand Food Composition Database, 2022).

Nutritional properties attached to raw cabbage include soluble sugars, fibre, vitamins and minerals. A characteristic of plants in general, cabbage also contains phytochemicals: phenolics, glucosinolates, polyphenols, and carotenoids (Zhao et al., 2020). Many epidemiological studies have shown that diets high in phytochemical containing plant foods, such as the Mediterranean Diet (MD), are correlated to lower incidences of NCD. . For instance, in their cross-sectional analysis of 3461 US participants in a longitudinal study, Iaccarino Idelson et al., (2017) linked adherence to (MD) to a negative association with inflammatory biomarkers and a positive association with blood biomarkers related to cardiovascular health. In a prospective study involving 1988 Greek men and women investigating the relationship between MD and cardiovascular disease (CVD), Georgoulis et al., (2024) found that participants with high adherence to MD had a seven-fold lower incidence of CVD after adjusting for age and lifestyle factors. The MD is characterised by high consumption of plant foods, low consumption of red meat and moderate amounts of fish and dairy (Mazza et al., 2021).

A January 2024 Google search returned a range of health benefits purportedly associated with sauerkraut and/or its probiotic properties: anticancer, anti-obesity, antiaging, a promoter of intestinal cardiovascular and brain health. A fundamental rationale is to attribute many of these, especially obesity, to vegetable consumption. The World Health Organization (WHO) states that low fruit and vegetable consumption is linked to increased risk of non-communicable disease, and in 2017 attributed almost four million global deaths to inadequate fruit and vegetable intake (WHO, 2023). In their recent systematic review on the health promoting components of fermented foods Melini et al., (2019) reiterate this association, citing The Food Agriculture Organization of the United Nations (FAO) and WHO's report stating low fruit and vegetable intake is in the top 10 contributors to the burden of disease and premature death in high income countries. They highlight that fermenting fruit and vegetables is an effective way to preserve these highly perishable, but nutrient dense foods.

In the 2018/19 New Zealand Health Survey, it was found that just 53% of New Zealand adults ate three or more serves of vegetables per day, the recommended amount at the time. Due to the vitamins, minerals, fibre and phytochemicals vegetables offer, and reflective of WHO's stance, the recommendation presently stands at five servings of vegetables and two servings of fruit per day for adults (Ministry of Health, 2020).

Insofar as anticancer, antiaging and brain health claims, the consumption of sauerkraut itself is not a "magic bullet". For instance, it is estimated that while 5-10% of cancer is genetically driven, major causes are environmental and lifestyle factors, which include the calorific value and nutrient density of the diet (Stromsnes et al., 2021). In turn, the diminishing physiological ability associated with poor lifestyle choices, including diet contribute to impaired metabolic functions and potentially non-communicable disease (cancers, CVD, diabetes and neurodegenerative conditions) (López-Otín et al., 2013). Essentially, there is an interplay between cellular health, ageing, and disease, in which diet plays a key role. The difficulty in making specific evidence-based claims lies in the challenges associated with clinical interventions and epidemiological studies due to lack of biomarkers associated with fermented food consumption. Research around the absorption of bioactive compounds provides better insight into heath promoting and disease preventing mechanisms. This is an important consideration because the bioactivity of compounds ex vivo does not necessarily translate into a benefit when stomach acidity and complex digestion processes are considered (Wilburn & Ryan, 2017).

Nevertheless, there is no shortage of studies investigating specific mechanisms whereby fermented foods might positively influence health beyond the attributes of the starting materials and basic nutrition. In this context mechanisms include health promoting fermentation metabolites, the conversion of compounds to biologically active metabolites and probiotic potential, with findings specific to sauerkraut where possible.

Antioxidant Capacity

Reactive oxygen species (ROS), otherwise known as free radicals, are produced via oxygen metabolism or from exogenous sources. When antioxidant capacity is compromised and there are high levels of oxidative stress, cellular function may be impaired and can lead to the development of non-communicable disease, including cancer, inflammatory bowel disease (IBD), and metabolic syndrome leading to CVD and diabetes (Maduro, Luís and Soares, 2021). Marco et al., (2017) study explains how the fermentation of vegetables is linked to antioxidant's capability by the conversion of compounds. As LAB grow during fermentation, phenolics are converted into bioactive metabolites. Some resultant alkyl catechols act as natural co-factors for the activation of nuclear factor erythroid 2-related factor 2 (Nrf2). A transcription protein that is a master regulator of oxidant stress response, Nrf2 offers protection against ROS and oxidative damage. It is worth pointing out that dietary alkyl catchols are less common these days due to modern food processing, highlighting a benefit of traditionally fermented sauerkraut in the context of antioxidant capacity (Senger et al., 2016). Sauerkraut also contacts vitamins C and E, known for their antioxidant capacity (Peñas, et al., 2016).

Anticarcinogenic properties

Recent studies on the prevention and inhibition of cancer and sauerkraut relate to the induction of detoxifying and antioxidant enzymes by metabolites formed during fermentation. The positive association between *Brassica* family vegetables and cancer risk is largely due to glucosinolates (GLS). Although not bioactive, GLS degrades to form various "anticancer compounds" (Peñas, et al., 2016). Glucosinolate breakdown begins when cabbage is shredded, then dramatic degradation is seen between days two and six of fermentation (Medina-Pradas et al., 2017). Breakdown products shown in Figure 3 are responsible for the taste and aroma of sauerkraut. From this group isothiocyanates, in particular have been studied for their antioxidant, anti-inflammatory and antibacterial properties. Isothiocynates sulforaphane and iberine (among other products) act as phase I and phase II enzymes, enhancing the body's ability to neutralise or eliminate carcinogenic matter (Palani et al., 2016). In their study on fermented cabbage, Palani et al., (2016) noted a sudden decrease in GLS coincided with the highest LAB count, supporting microbial involvement in this process.

Figure 3. Degradation of glucosinolates in cabbage into flavour compounds during fermentation. Isothiocyanates are phytochemicals of particular interest in human health. (Adapted from Thierry, Baty, et al., 2023)

In a study using rats, Krajka-Kuźniak et al., (2011) administered sauerkraut juice at 1.25ml/kg body weight for 28 days. The expression of two key detoxifying enzymes in the liver was increased due to the activation of Nrf2. Findings from subsequent rat studies by these researchers were that sauerkraut juice induced phase I enzymes involved in oestrogen metabolism. Of note was inhibited activity of CYP19 which encodes aromatase, an enzyme that synthesises oestrogen and is found in concentrated levels in cancerous breast tissue.

While there have been human studies on the anticancer associations of brassica vegetables, and cabbage, trials for sauerkraut specifically are limited. In terms of the link with breast cancer, researchers looking for an explanation of varying rates around the world looked to the influence of diet on breast cancer expression. The fact that rates of breast cancer change in migrant women led to an investigation of Polish women in the United States, where breast cancer incidence increased an astounding threefold in one generation. In a case control study researchers found that more than three weekly serves of raw, short cook cabbage and sauerkraut during adolescence and adulthood was associated with a 72% decreased breast cancer risk when compared with those who consumed half or less that amount. Although the risk was least in those who consumed these foods from adolescence, the higher consumption during adulthood was also beneficial. Other traditionally cooked foods appeared to have no bearing and researchers noted the benefits of brassica consumption, pointing out the anticariogenic properties derived from GLS (Nelson, 2006). By nature, as an epidemiological study, there were limitations, however at the least, these observations highlight the health promoting outcomes from high vegetable consumption. Furthermore, studies such as these add to the body of research around why specific populations, such as those in “Blue Zones” experience health and longevity.

Immunological Effects

Acute inflammation is a protective and targeted response to infection or injured tissue. Macrophages are immune cells involved in this response, producing inflammatory mediators such as nitric oxide (NO) and cytokines (Leonardi et al., 2018). Young individuals have low cytokine levels which become elevated with physiological stresses. In older adults several cytokines have been seen to increase with age in the absence of acute physiological stress, therefore serve as predictors of disease and healthy ageing (Singh & Newman, 2011). The literature indicates that derivatives of GSL, indole-3-carbinole and sulforaphane downregulate cytokines, supporting the anti-inflammatory claims of sauerkraut consumption

(Peñas, et al., 2016). In a study comparing fresh cabbage with sauerkraut, both substrates showed dose dependent ability to suppress NO production. Sauerkraut had a stronger potency for NO inhibition, and although in vitro, this work points to sauerkraut's potential to address inflammation with in vivo research needed (Martinez-Villaluenga et al., 2012).

Probiotic Potential

Attributing positive health outcomes to sauerkraut's probiotics must firstly lay in WHO's definition of probiotics as being "live microorganisms which when administered in adequate amounts confer a health benefit on the host". It is well established in human health, that gut microbiome influences physiological pathways involved in the development of non-communicable disease (Peñas, et al., 2016). Gut microbiome status is influenced by factors such as antibiotic use, lack of exercise, and poor diet. Diet is a modifiable factor, therefore, using probiotics and prebiotics in a nutritional context is a potential way to improve health outcomes (Crowder et al., 2023). While research is scarce compared with probiotics in dairy foods, evidence suggests LAB isolated from sauerkraut has probiotic potential. Beganovic´ et al., (2014) demonstrated this capability in their in vitro study of autochthonous sauerkraut LAB. Probiotic testing criteria included survival through a simulated gastrointestinal tract, capability to adhere to epithelial cells and antibacterial activity. *Lactobacillus paraplantarum* and *Lactobacillus brevis* strains were able to meet these three criterium. (Marco et al., (2017) point out the emerging view that major health benefits of LAB probiotic cultures can be credited to bacterium species rather than being strain specific. They argue that if sauerkraut is sufficiently populated with live species such *as L. paraplantarum* and *L. brevis* there should be similar health benefits to probiotic lactobacilli of the same species.

Epidemiological evidence for probiotics in sauerkraut specifically is sparse. In a clinical trial (n=39) of people with irritable bowel there was no significant difference in symptoms or gut microbiota between the control and intervention group was found. After six weeks of consuming 75g of pasteurised or unpasteurised sauerkraut, both groups reported a reduction in severity of symptoms, implying benefit from simply consuming cabbage and possibly the metabolites of fermentation, independent of live microbes (Nielsen et al., 2018).

The number of viable microorganisms, to have survived GI transit need to remain high to obtain a clinical effect: 10^6 CFU/mL in small intestine, 10^8 CFU/mL (Wendel, 2022). Sauerkraut may contain relatively high numbers at 10^6 – 10^9 CFU/mL, and since a high proportion can survive digestion, consumption increases the number of microbes in the diet at the very least. While introducing transient microbes to the gut flora by consuming sauerkraut has potential benefits, there is insufficient evidence to claim that it is a dietary source of probiotics in the context of the WHO's definition.

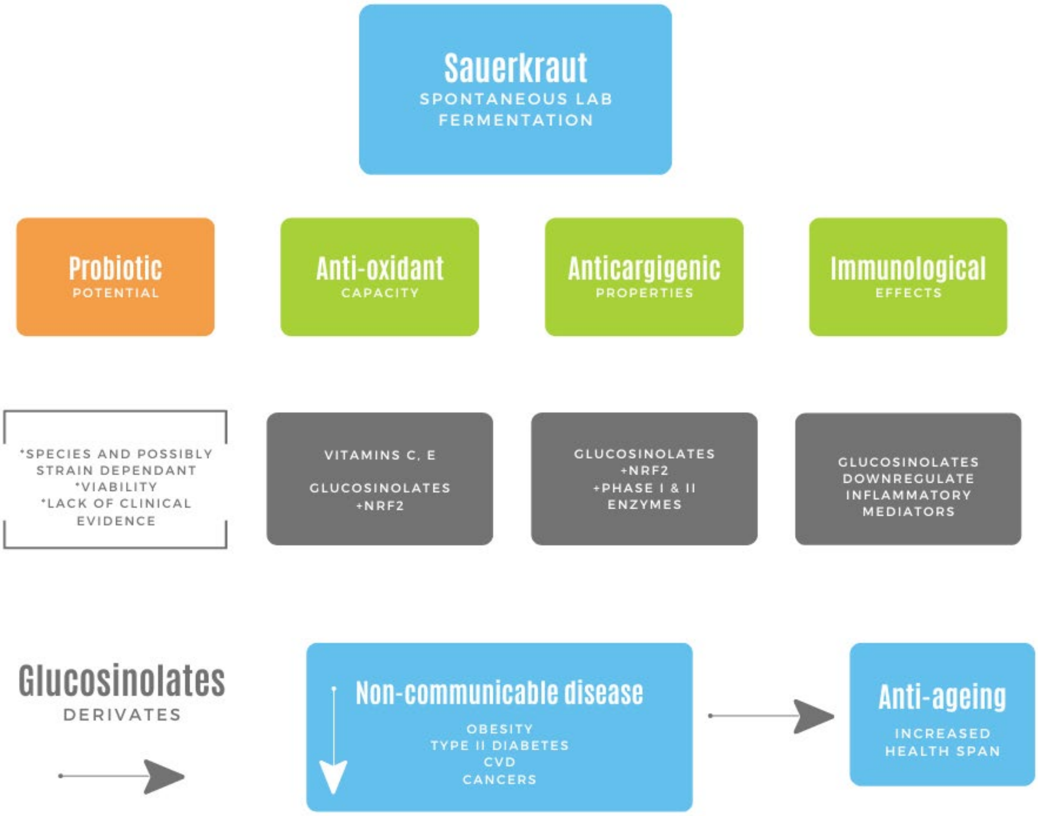

Figure 4. Summary of findings for health benefits of Sauerkraut

Vitamin B12

The term "vitamin" derived from the words "vital" and "amine" describes a group of organic compounds required for metabolic reactions and cellular processes. Typically, they are unable to be synthesised by humans, except for Vitamin D, therefore considered essential micronutrients to be sourced from the diet (Santos et al., 2023). These single molecule, low weight compounds are classified into two main groups based on their solubility characteristics: fat-soluble and water-soluble vitamins. The B-group vitamins comprise the majority of water-soluble vitamins. This family of eight substances includes thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9) and cobalamin (B12) (Mateeva et al., 2023).

Folic acid, or folate when sourced from foods, and cobalamin are dependent on each other for activation. Cobalamin removes and keeps a methyl group from folate, which activates the pair's enzymes (Whitney et al., 2017). Folate is essential for new cell formation and in New Zealand commercial flour is fortified with folic acid to prevent prenatal neural tube defects. High intakes of naturally occurring folate poses no issue, however high folic acid intake can mask B12 deficiency. Given that anaemia is a symptom of both folate and B12 deficiency, supplementation with folic acid instead of B12 is possible. This is problematic, due to B12's critical role in neurological health (Ministry for Primary Industries, 2019).

Figure 5. Vitamin B12 activating folate. Both co-enzymes become active.

Vitamin B12 is known as cobalamin because it contains cobalt ions and is the sole vitamin that has a metal ion associated with it (Leyssens et al., 2017). The intricate molecular structure exists in four variations: cyanocobalamin, hydroxocobalamin, methylcobalamin, adenosylcobalamin (Santos et al., 2023). Cobalamins contain a central corrinoid ring and are surrounded by four pyrrole nitrogen atoms. Upper and lower ligands complete the B12 molecule, with characteristics of the former primarily differentiating the four variations. Methylcobalamin and adenosylcobalamin are naturally occurring, whereas cyanocobalamin and hydroxocobalamin are manufactured versions. Active B12 is differentiated from the pseudo form by the presence of 5,6-dimethylbenzimidazole (DBMI) at the lower ligand position (Piwowarek et al., 2017). The most stable form is cyanocobalamin, used in supplements and fortified foods, and hydroxocobalamin is a medical grade injectable form (Kumar et al., 2023).

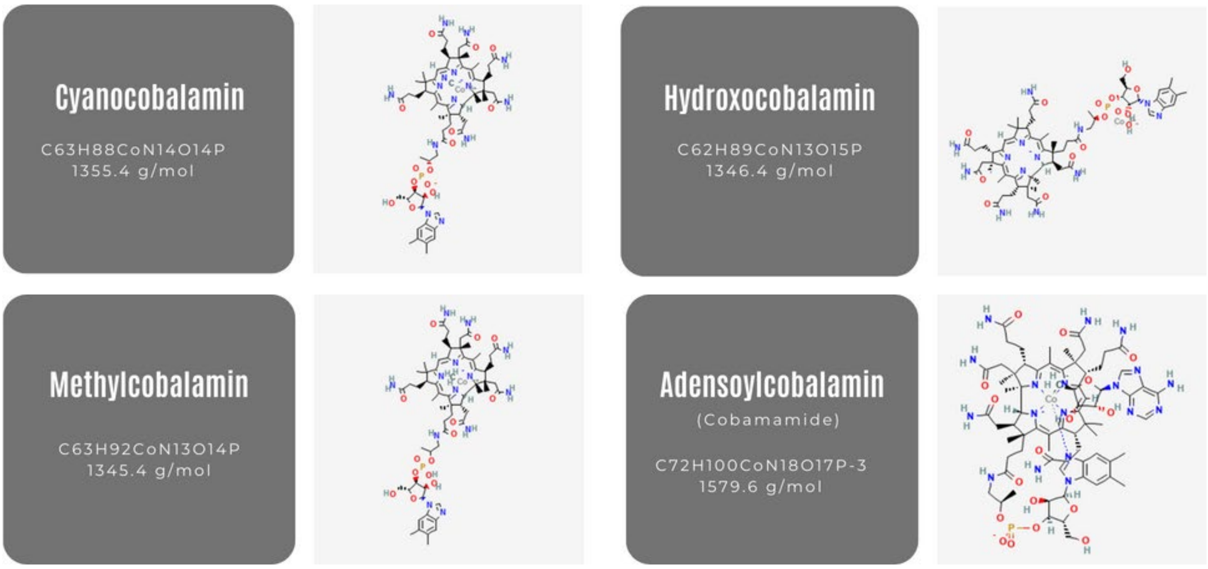

Figure 6. Chemical structures of cobalamin variations

The estimated average requirement (EAR) for adults 19 – 70+ years is 2.0µg and the recommended dietary intake (RDI) is 2.4µg daily. The EAR is estimated to meet requirements of 50 percent of a healthy population based on sex and life stage, whereas the RDI is sufficient to meet with up to 98 percent of healthy individuals. Pregnant and lactating women have higher needs, with the RDI being 2.6µg and 2.8µg respectively. Maternal RDIs consider foetal and placental needs and secretion of B12 in breastmilk (National Health and Medical Research Council [NHMRC], Australian Government Department of Health and Ageing [AGDHA], New Zealand Ministry of Health [NZMOH], 2006). New Zealand's most recent available adult nutrition survey reported an estimated 7.9% of the population as having an inadequate intake of B12 when compared with the EAR. This inadequacy was far more prevalent amongst females at 14.1% when compared with males at 1.3%. The female age groups most likely to have inadequate intakes were 19 – 30 years and > 70 years (University of Otago [UO] and Ministry of Health [MOH], 2011). Māori and Pasifika peoples were found to have lower rates of inadequacy on average, and maintained serum levels longer, showing decline at >75 years (Devi et al., 2018).

Vitamin B12 is a critical hematopoietic and neurotropic micronutrient. Deficiency can lead to several conditions, including anaemia (megaloblastic or pernicious) and de-myelinated neurons (Kumar et al., 2023). As a co-factor for homocysteine methyltransferase (methionine synthase), it has an essential role in maintaining the protective myelin sheath covering nerve cells and facilitating purine base production, building blocks for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA processes may be compromised in a B12 deficit, contributing to cellular abnormalities. Vitamin B12 is also involved in energy metabolism as a co-factor for the enzyme methylmalonyl-CoA mutase, and in osteocyte activity. Lack of B12, along with several other micronutrients, plays a role in inefficient erythropoiesis, a contributing factor to onset of anaemia (Guerra & Lopez-Gonzalez, 2023). Age related diseases, including cognitive decline and CVD are also associated with B12 deficiency (Kumar et al., 2023).

Deficiency can be caused by malabsorption related to digestive diseases (Devi et al., 2018). Inflammatory bowel disease (IBD) and coeliac disease (CD) are two such conditions, being autoimmune diseases marked by chronic intestinal inflammation. There is thought to be a complex interplay with commensal bacterial and the environment in IBD, whereas higher prevalence CD involves an immune response to dietary gluten (Pascual, 2014). In Aotearoa, New Zealand the incidence of clinically diagnosed CD stands at approximately 1% and increasing, in line with global trends (Coppell et al., 2017). Vitamin B12 deficiency is thought to be uncommon in CD as the terminal ileum (where absorption takes place) is not largely affected. Additionally, levels may normalise when a gluten free diet is followed. In their small prospective study of 39 patients untreated for CD, 41% were found to be B12 deficient (Dahele & Ghosh, 2001). In Akay et al., (2020) more recent study 50 patients with low serum iron and B12 deficiency were diagnosed as having CD, although only 30% had displayed typical symptoms such as abdominal pain and bloating, constipation, and weight loss. These studies highlight issues around lack of diagnosis and the role B12 has as an indicator for CD.

The absorption of B12 is a complex process involving the pancreas and gastrointestinal tract. Two carrier proteins, haptocorrin (HC) and intrinsic factor (IF) mediate the absorption process. In the stomach, gastric acid and pepsin releases B12 from the food matrix, where it binds to HC. Pancreatic proteases then degrade HC, allowing B12 to bind to IF. In the distal ileum, the IF-B12 complex enters mucosal cells via receptor-mediated endocytosis, then exits into circulation where B12 binds to transcobalamin (TC). The TC-B12 complex subsequently enters cells (Kozyraki & Cases, 2013). Gastric atrophy, where the stomach lining is compromised, can lead to reduced gastric acidity and lower IF production (NHMRC, AGDHA, NZMOH, 2006). Kozyraki and Cases (2013) highlight the prevalence of gastric atrophy in the aged, with chronic poor absorption explaining the lower B12 status commonly seen in persons over 60 years.

In the human diet B12 is obtained from animal derived sources such as meat, fish, eggs, and dairy products (Santo et al., 2023). Vitamin B12 is present in animal derived foods due to microbial interactions and accumulation in animal tissue. For instance, ruminants such as cows and sheep acquire B12 from bacteria due to their ability to extract nutrients from plant foods by fermentation in the reticulorumen, the primary site of microbial activity (Kumar et al., 2023). As the only vitamin absent from plant sources, a plant centric diet is a further risk factor for deficiency. Whereas lacto-ovo vegetarians can consume eggs and dairy in a “plant-based” diet, vegan eaters exclude all foods of animal origin (Watanabe et al., 2014).

Due to demand from the food and pharmaceutical industries, there in an interest in using bacteria to commercially produce B12. In large scale production *Pseudomonas denitrificans, Propionibacterium shermanni* and *Sinorhizobium melitoti* are used in fermentation to produce B12, albeit with limitations (Fang et al., 2017). Genetic and metabolic engineering of bacterium offers opportunities for industrial B12 production as Fang et al., (2018) have demonstrated with *Escherichia coli. Propionibacterium freudenreichii* has long been known for its natural ability to produce active vitamin B12. This bacterium has obtained Generally Recognised as Safe (GRAS) status from the US Food and Drug Administration (FDA).

Propionibacterium freudenreichii

Propionibacterium (PAB), of the Actinobacteria class are Gram positive, non-motile, non-spore forming bacilli, 1 – 5µg in length. Anaerobic, or close to these small microbes take the form of cocci in the absence of oxygen, but exhibit pleomorphism, forming club, V or Y shaped cells with oxygen exposure. Their optimal pH is 7.0 with a range of 4.5 – 8.0, and they are known to grow, even in the presence of 6.5% NaCl when at their optimal pH. Mostly mesophiles, their optimal growth temperature is 30 °C. Inhibitory factors to this genus include high acidity and salt concentrations, high and low temperatures, and water activity (Piwowarek et al., 2017). The ability to biofilm is a further characteristic of PAB (Bücher et al., 2021).

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Figure 7. Microscopy image showing morphological aspect of *P. freudenreichii* (de Rezende Rodovalho et al., 2021)

Propionibacterium can be divided into two groups according to their habitat, being skin (acne) or classical (dairy). The former, also known as cutaneous are pathogenic microorganisms found in humans on skin and mucosa, whereas the classical species have been traditionally isolated from dairy products (Dank et al., 2023). The classical group includes subspecies of *Propionibacterium freudenreichii,* which have been traditionally characterised by their ability to metabolise lactose (lac$^+$) or reduce nitrate (nit$^+$). Strains from subsp*. shermanii* can metabolise lactose and are unable to reduce nitrate, whereas subsp. *freudenreichii* has the opposing abilities (Piwowarek et al., 2017). The ability to ferment lactose during the cheese ripening can be important in some varieties, justifying the use of strains of subspecies *P. freudenreichii subsp. shermanii* (de Freitas et al., 2015). However, subspecies classification is now considered redundant with the discovery of strains that are both lactose and nitrate reductase positive or negative, removing justification for the subspecies level of classification ((Thierry, Deutsch, et al., 2011). This implies that strains whose phenotype lac+ /nit+ could be used in cheese ripening (de Freitas et al., 2015). All classical PAB have the ability for fermentation, producing valuable metabolites like namesake propionic acid, vitamin B12, bacteriocins and disaccharide trehalose (Piwowarek et al., 2017).

Propionibacterium freudenreichii was first described in 1906 by Orla-Jensen and von Freudenriech, who demonstrated the connection between the microbes producing propionic acid and the formation of holes in Emmentaler cheese (Thierry, Deutsch, et al., 2011). Today *P. freudenreichii* is the most widely studied PAB species and is commonly used in the ripening of Swiss cheese. The holes are formed due to the microbe's consumption of lactic acid and subsequent formation of carbon dioxide which is trapped within the cheese. These voids then become the "eyes" characteristic of cheeses such as Emmentaler or Gruyère. The aroma of these cheeses is also attributed, in part to *P. freudenreichii* through this lactic acid fermentation, along with the breakdown of lipids and amino acids and conversion to aromatic compounds (Yee et al., 2014).

Health promoting properties add to the desirability of *P. freudenreichii*, with research focus moving to probiotic properties in recent years. Broadly, classical PAB are thought to positively influence gut microbiota and are valued as probiotics for their favourable metabolites (Thierry, Deutsch, et al., 2011). Probiotic prospective in the realm of health includes anti-cancer potential, anti-pathogenic activity, modulation of microbial composition and immunomodulatory properties (de Rezende Rodovalho et al., 2021).

Anti-cancer potential associated with the microbe's production of organic acids propionate and acetate has been reported (Piwowarek et al., 2017). Based on the premise that short chain fatty acids (SCFA) are cytotoxic to cancer cells, Cousin et al., (2016) showed that *P. freudenreichii* induced intrinsic apoptosis of cancerous colorectal cells via the mitochondrial pathway. These findings support previous research by Jan et al., (2002) who identified propionate and acetate as the major cytotoxic components produced by *P. freudenreichii* and investigated the mechanisms underlying cell death. This research highlighted the potential of probiotics for preventing digestive cancers.

Although not indigenous to the human gastrointestinal tract, *P. freudenreichii* can survive digestion and enhance the bifidobacteria population in human organism (D. Bouglé, N. Roland, F. Lebeurrier, 1999). The ability to adhere to host cells is important for digestive survival of probiotics. *P. freudenreichii* can adhere to glycoproteins and the mucus lining the intestinal tract of humans (de Rezende Rodovalho et al., 2021). Pathological bacteria such as opportunistic *Staphylococcus aureus (S. aureus)* also adheres to human intestinal mucus but can be displaced by probiotics. Vesterland (2006) showed that some lactic acid bacteria (LAB) and *P. freudenreichii* were able to reduce the adhesion and colonisation of *S. aureus* on colonic mucus. A role in the defence against pathogen *Heliobacter pylori* has been investigated in a number of studies as reviewed by (Thierry, Deutsch, et al., 2011). The authors surmised *P. freudenreichii* may be useful in the clinical treatment of *H. plylori* due to adhesion properties and by inhibiting inflammatory mediators (interleukins and prostaglandin E2) released by mucosal cells in response to the pathogen. Clinical studies have shown that P.

freudenreichii containing supplements have led to improved mucosal lining in patients, increasing their tolerance to treatment (Thierry, Deutsch, et al., 2011).

It is well known that *P. freudenreichii* has a positive effect on gut health by modulating and increasing *Bifidobacterium spp.*, a commensal bacterium which colonises the gastrointestinal tract early in the human life span. Further beneficial microbes such as *Enterococcus faecalis, Bacteroides spp.* and *Enterobactor spp.* have also been shown to be enhanced by the Bifidogenic growth stimulator (BGS) produced by *P. freudenreichii (Dank et al., 2023).* Furthermore, BGS has been shown to have a bactericidal action on *H. pylori* by inhibiting cellular respiration (Nagata et al., 2010).

Immunomodulatory probiotic bacteria influence inflammation of the intestine and may be useful for people with gastrointestinal disorders such as inflammatory bowel syndrome (IBS) and inflammatory bowel diseases (IBD): Crohn's and ulcerative colitis (Benoît Foligné et al., 2010). New Zealand has one of the highest rates of diagnosed IBD cases worldwide, with a steep rise seen in paediatric cases in recent years (Lopez et al., 2018). Prevalence is approximately six-fold higher in urban populations compared with rural areas, which is hypothesised to be due to a more hygienic urban environment. In a regional study, Coad et al., (2023) found IBD instances to be significantly lower in the Māori population at 1.1 cases per 100,000 compared with 24.8 per 100,000 in Europeans.

Propionibacterium freudenreichii is a promising immunomodulatory probiotic. However, this is dependent on strains and is associated with the presence of specific surface proteins, which play a role in *P. freudenreichii's* ability to induce Interluekin-10 (IL-10) (Mantel et al., 2023). One of the most important cytokines with anti-inflammatory properties, the production of IL-10 is a key aspect of immunomodulation (Sabat et al., 2010). Mantel et al., (2023) found *P. freudenreichii* CIRM-BIA129 fermented in milk products attenuated symptoms of induced colitis in mice by increasing goblet cell count, restoring colon crypt depth, and decreasing gut permeability. It is worth noting that the effect was evident in fat containing milk, but not in skim milk, leading to the hypothesis that fats and proteins in dairy protect probiotics from digestive secretions.

Whilst there is a wide body of research on IBD, altered gut microbiome and treatment with probiotics, evidence for their efficacy in this area is limited. In a recent systematic review, the American Gastroenterological Association found no evidence to support probiotics ability in inducement or remission of Crohn's. When combined with other therapies there is some evidence that in mild to moderate cases disease activity is reduced, however the effect in severe cases was unknown. Probiotic formulations were cited as a significant limiting factor, as well as small sample populations in studies (Preidis et al., 2020). That withstanding, there is a high level of research interest due to the clear associations between dysbiosis of gut microbiota and non-communicable disease.

Characteristic of all PAB is the production of organic acids propionate and acetate via the Wood-Werkman cycle (Bücher et al., 2021). Substrates are oxidised to pyruvate by glycolysis or via the pentose phosphate pathway, generating adenosine triphosphate (ATP) and reducing enzymes. Pyruvate is then oxidised to acetate and CO^2 or reduced to propionate (Thierry, Falentin, et al., 2011). Propionic acid is a three carbon SCFA, which serves as an energy source for epithelial cells in the colon, and as previously mentioned is associated with anti-inflammatory effects in the gut. It also serves as a precursor for gluconeogenesis in the liver after being cleared from circulation. Acetic acid is a two carbon SCFA, which also provides energy to colonic cells. It is estimated that 60-70% of colonocytes energy requirements are satisfied by SCFA oxidation. SCFA are associated with an increase in activated protein kinase (AMPK) an energy sensing enzyme that initiates the restoration of cellular energy balance, promoting metabolic health in addition to bowel health (den Besten et al., 2013).

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Figure 8. Wood-Werkman cycle showing pyruvate catabolism in *Propionibacterium.* (Thierry, Falentin, et al., 2011)

In addition to its relevance to human and animal health, *P. freudenreichii* has nutritional and functional significance to the food industry. For example, products of lactate fermentation propionate and acetate are considered flavour compounds in cheese. Propionibacterium also catabolises branched chain amino acids into volatile compounds and has a role in lipolysis into free fatty acids, which are also important flavour compounds in many cheeses (Thierry, Deutsch, et al., 2011). Furthermore, propionic and acetic acids act as mould inhibitors, preventing or delaying fungi growth and the subsequent degradation of foods. This avoids the development of unacceptable flavours as well as growth of potentially harmful toxins (Babuchowski et al., 1999). The secretion of bacteriocins is an additional food protection mechanism. Bacteriocins are antimicrobial peptides which offer protection against generally similar bacterium, and those produced by *P. freudenreichii* are particularly attractive due to its GRAS status (Faye et al., 2010).

Animal studies have shown that it is possible to retain the immunomodulating properties of *P. freudenreichii* when incorporated into food matrices such as cheese. Cousin et al., (2012) found that when cheese fermented with *P. freudenreichii* was fed to piglets, improvements were seen in their food intake and growth. Furthermore, substantially less cytokines (molecules involved in the immune system's inflammatory response) were secreted from colonic mucosa of the treated animals, while gut structure and function remained uncompromised. Comparable findings were seen in mice when Plé et al., (2015) developed an anti-inflammatory cheese using *P. freudenreichii* and *Lactobacillus deleuckii.* These studies unveil promise of the use of dairy bacterium in the development of novel functional foods using fermentation.

As a de novo producer of human active B12, *P. freudenreichii* is a vehicle for providing this essential nutrient in various food products. Its prevalence in dairy is attributed to its low proteolytic (enzymic protein metabolism) activity and preference for lactate over glucose, therefore being suited to environments where proteolytic lactic acid bacteria are present *(Dank et al., 2023).* This is a form of cross over, or co-fermentation, where bacterium is introduced to a microbial partner with a view to enhance organoleptic and/or nutritional properties of a product. As implied by their name, LAB produce lactic acid via the fermentation of carbohydrate. Glucose undergoes glycolysis to become a carbon source to produce pyruvate, which is metabolised with enzyme lactate dehydrogenase to produce lactate at a 2:1 ratio (Wang et al., 2021). In turn, *P. freudenreichii* requires lactic acid as its carbon source to produce its metabolites, including active vitamin B12 (Thierry, Falentin, et al., 2011). However, too much lactic acid can result in a steep decline in environmental pH and this can inhibit PAB growth due to their preference for neutral pH. At pH < 4.5 PAB cease producing organic acids.

Xie et al., (2020) found that higher rates of B12 production were correlated with more propionic and acetic acid in their study on in situ fortification of plant foods using *P. freudenreichii* and *Levilactobacillus brevis*. This work confirmed ideal co-fermentation conditions, where preparations with high lactic acid content produced low yields of both organic acids and B12. It also found that cobalt was not a limiting factor in B12 production under ideal conditions, contrary to previous cereal based studies. For instance, when investigating in situ production of B12 in aqueous cereal matrices Chamlagain et al., (2017) introduced cobalt resulting in a 10-fold increase in B12. Substantially higher levels of active B12 were evidenced with co-supplementation of cobalt and DMBI, however it is not possible to include the cobalamin's axial ligand in foods. Additionally, cobalt compounds have not been approved for food use. Yeast extract is a natural food grade cobalt rich supplement, which can potentially enhance B12 production. During their two-step fermentation in solubilised bioprocessed wheat bran Chamlagain et al., (2024) assessed the viability of yeast extract to optimise B12. A natural supplementation of 1% w/v was found to enhance B12 production as effectively as the 10ng/mL of cobalt. The results of this study confirmed that an acidic environment was not favourable for *P. freudenreichii* to synthesise for B12. It also provided insight into optimal fermentation time, with most B12 being produced in the first three days and associated this with a decrease from 6.4 pH to 5.3 pH after one day, with no further decrease after seven days.

Biosynthesis of B12 by *P. freudenreichii* is a complicated process which starts with glutamic acid and requires multiple reactions (Bücher et al., 2021). Although *P. freudenreichii* is an anaerobe, its biosynthesis of active B12 requires oxygen as the lower ligand (DMBI) responsible for the active form cannot be made under anaerobic conditions. If oxygen is not available, and DMBI is not attached to the molecule, the product is pseudo cobalamin. Deptula et al., (2017) observed both the active and pseudo forms of B12 produced by *P. freudinreichii* in a medium resembling cheese, without cobalt or DMBI supplementation. Contrary to what Chamlagain et al., (2017) found in wheat bran, the largest increases in cobalamin levels were observed later, between days three and seven of incubation. While pseudo B12 was measured during fermentation, it was barely detectable at the end of the seven-day incubation period, indicating the production of DMBI and subsequent conversion to the active form of B12 (Deptula et al., 2017).

While the manufacture of B12 is dependent on the addition of cobalt and DMBI, neither is permitted in foods, highlighting the desirability of alternative fortification methods in functional foods. *Propionibacterium freudenreichii* is the only known bacterium capable of synthesising DMBI, highlighting its potential use for in-situ B12 food production. Cabbage is a promising substrate as a plant food with a rich nutritional profile and ability to supply cobalt (Leyssens et al., 2017).

Chapter 3

Materials and methods

Sauerkraut production

Two fermentations were performed using separate fresh cabbage heads (*Brassica oleracea var. capitata* L.) sourced from a local supermarket. Outer leaves were removed, and the edible parts of the cabbage were shredded into approximately 2mm strips using a domestic hand shredder. Five hundred grams of shredded matter from each cabbage was placed into a zip lock bag. To each bag, 13.75g (2.5%w/w) sea salt was added and massaged into the cabbage until salt was homogenised and generous amount of brine was produced. At this point *Propionibacterium freudenreichii subsp. globosum* was added to one recipe (1.9g in 10mL RO water). Contents from each recipe were compressed into separate glass jars to capacity, then sealed with a screw top lid. Both samples were placed in an incubator set at 20^{O}C for 14 days. The *P. freudenreichii* inoculum was a dairy culture sourced from The Urban Cheese Company, New Zealand. The control fermentation is traditional sauerkraut recipe, “Control A”, the P. freudenreichii fermentation is referred to as “Culture B”.

Microbial enumeration

On days zero and eight, 3 mL of brine from each fermentation was collected and refrigerated. On day nine microbial enumeration was performed by quantification of colony forming units (CFU) per mL. Serial dilutions were prepared as shown in Figure 9.

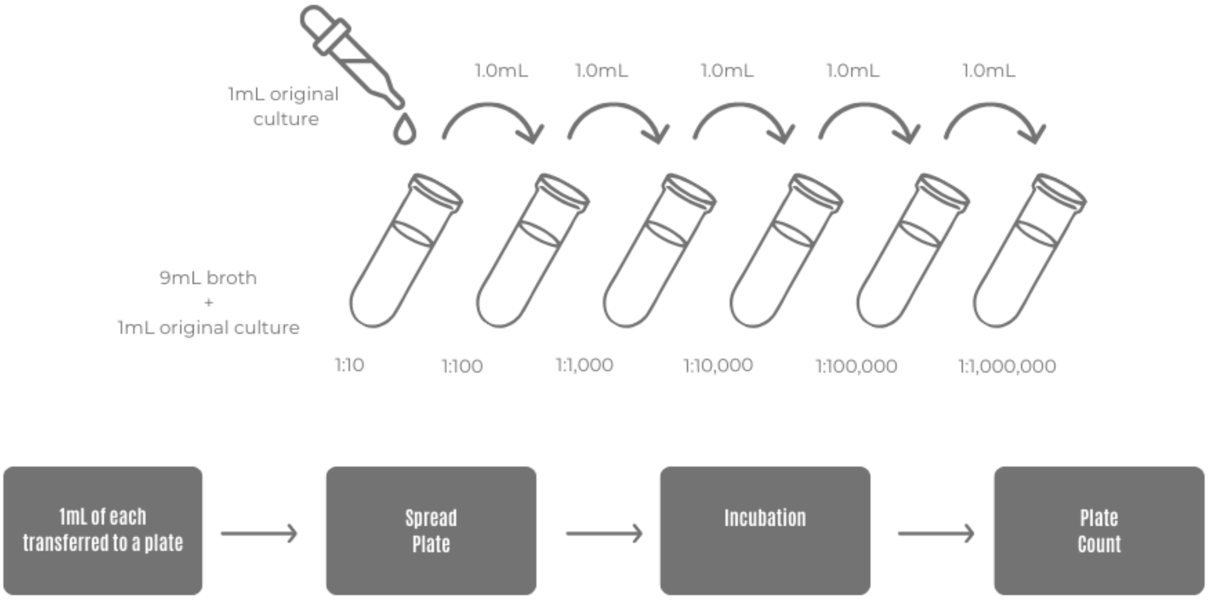
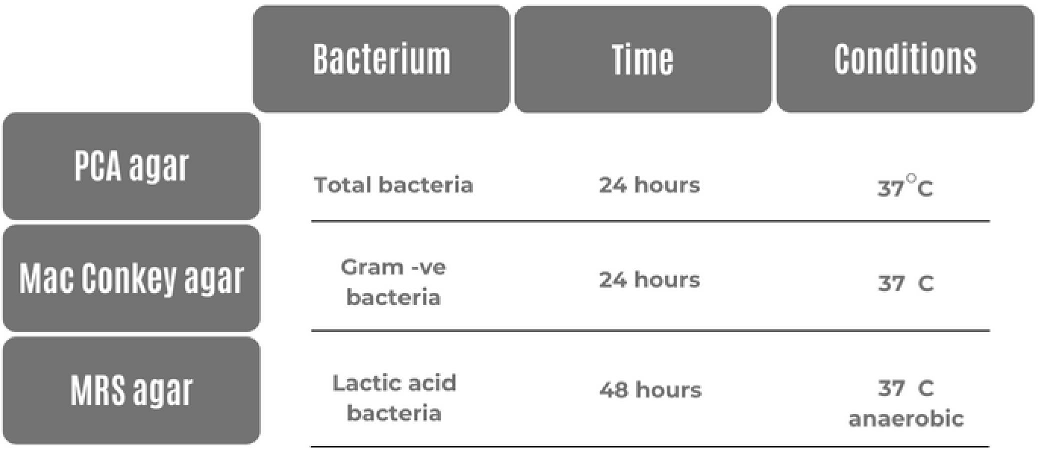


Figure 9. Schematic diagram of serial dilution and enumeration process

Six samples of each fermentation was prepared for each agar plate type: , 10^{-2}, 10^{-4}, 10^{-6} in duplicate, then incubated under conditions shown in Figure 10. Visible colonies on each plate were counted, then average CFU calculated based on dilution, for each fermentation and media type. The bacterial counts are reported as log CFU/mL.



	Bacterium	Time	Conditions
PCA agar	Total bacteria	24 hours	37°C
Mac Conkey agar	Gram -ve bacteria	24 hours	37 C
MRS agar	Lactic acid bacteria	48 hours	37 C anaerobic

Figure 10. Media and conditions used for enumerating bacteria

Vitamin B12 content assessment

Brine from each fermentation was collected at day 14 and was sent for vitamin B12 cyanocobalamin analysis by high pressure liquid chromatography. This was performed by Assure Quality Limited, Auckland, New Zealand, using method AOAC 2014.02 (modified).

Short chain fatty acid quantification

Brine samples collected at day 14 were tested for acetic acid, propionic acid and butyric at Lincoln University, New Zealand, using gas chromatography with flame ionisation detection (GC-FID), Shimadazu, Japan. Filtered, diluted brine was injected into a SGE-BP21 column. In each fermentation selected acids were quantified with a flame ionisation detector using external standards.

Determination of titratable acidy and pH

A manual titration was performed on brine collected at days two and eight and stored frozen. Brine was titrated with 0.1M NaOH to equivalence point of 8.2pH. Two titrations were performed for each sample, and titrate values recorded were used in following the equation to determine titratable acidity.

$$\underset{\text{(as gL}^{-1-}\text{ lactic acid)}}{\text{Titratable Acidity}} = \frac{90}{1} \times \frac{\text{Molarity of NaOH}}{1} \times \frac{\text{Titre Value of NaOH(mL)}}{\text{Volume of Brine (mL)}}$$

A bench top pH meter was used to determine pH of the same samples collected at days 0, 2 and 8.

Chapter 4

Results

Microbial analysis

No bacterium was detectable in the Control A fermentation at day zero. On day eight the total bacterial count was log 7.2 CFU/mL, and Gram negative -was slightly less at log 6.7 CFU/mL. At day eight, LAB was not detectable, indicating a failed spontaneous fermentation. Visual inspection of this fermentation at day two showed signs of contamination with black mould appearing on the inner lid of the jar. The *P. freudienreichii* Culture B's total population increased two-fold after eight days fermentation. Gram negative bacteria was not detected at any stage, and as can be seen in Table 1. the PCA and MRS agar readings at day eight for total bacteria and LAB are comparable.

Table 1. Changes in microbial cell counts during fermentation at 20°C, for Control A and *P. freudenreichii* Culture B expressed as CFU/mL. Raw data = absolute counts, ND = not detected

	Total (PCA)				**Gram -ve (MacConkey)**				**LAB (MRS)**			
	Control A		**Culture B**		**Control A**		**Culture B**		**Control A**		**Culture B**	
	Raw	Log CFU/mL	Raw	Log CFU/mL	Raw	Log CFU/mL	Raw	Log CFU/mL	Raw	Log CFU/mL	Raw	Log CFU/mL
Day 0	ND	-	2.55 x 10^4	4.4	ND	-	ND	-	ND	-	1.4 x 10^4	4.1
Day 8	1.5 x 10^7	7.2	9.25 x 10^8	8.9	7.3 x 10^6	6.7	ND	-	ND	-	7.5 x 10^8	8.9

Acidity and pH analysis

As can be seen in Figure 11. there was no significant change in acidity for the Control A fermentation between day two (3.96 gL^-) and day eight(4.38 gL^-) where there was 0.43% acidity. Similarly, the pH is largely unchanged at day eight at pH 5.0 (Figure 12). These results support the microbial analysis and lack of LAB present. In a successful LAB fermentation, a pH as low as 3.4 could be expected and acidity of approximately 2.5% (Thierry, Baty, et al., 2023). Culture B had similarly low acidity at day two (2.7 gL^-), however there was a steep increase to day eight (14.76 gL^-) when 1.48% acidity was reached.

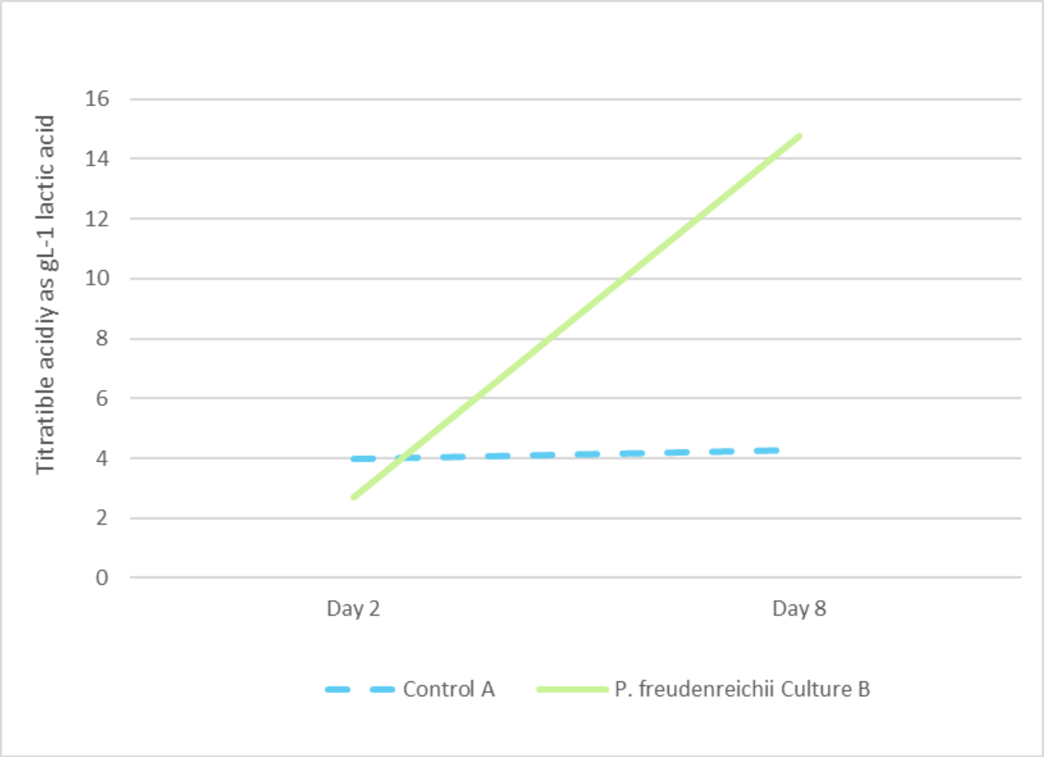


Figure 11. Titratable acidity of sauerkraut brine from Control A and P. *freudenreichii* culture B fermentations

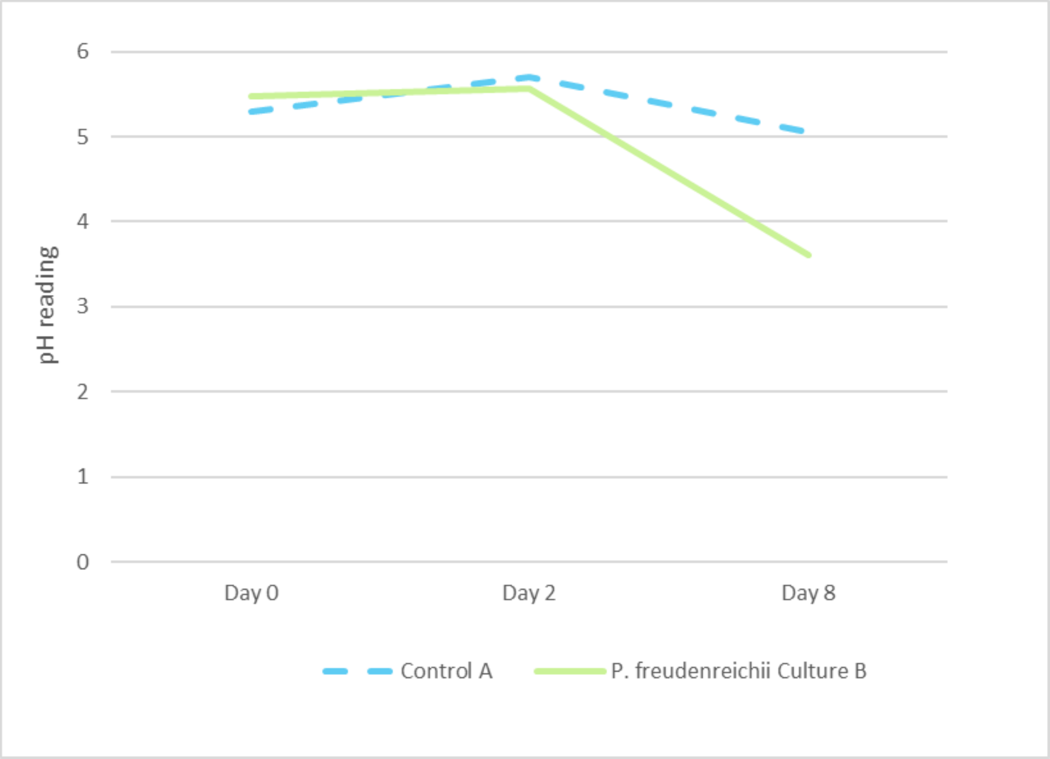


Figure 12. pH of sauerkraut brine from Control A and *P. freudenreichii* Culture B fermentations.

Vitamin B12 content

Vitamin B12 was not detected in the Control A fermentation. This is in line with traditional sauerkraut's B12 content (*New Zealand Food Composition Database*, 2022). At 14 days B an average measure of 1.62 µg per 100grams was reported for Culture B.

Table 2. Vitamin B12 content of sauerkraut brine taken at day 14 from Control A and *P. freudenreichii* Culture B fermentations (duplicate). Expressed as µg/100g.

	Replicate 1	**Replicate 2**
Control A	**< 0.0100 µg**	**< 0.0100 µg**
Culture B	**1.69 µg**	**1.55 µg**

Short chain fatty acids

Control A contain less than 1% acetic acid, where approximately 2% could be expected (Satora et al., 2021). This is further evidence of a failed fermentation as acetic acid is a product of lactic acid, and the acidity remained low during Control A fermentation (Thierry, Baty, et al., 2023). Butyric acid was typically low for both fermentations. Propionic Acid was present in both fermentations, and notably there was approximately seven times more measured in Culture B.

Table 3. Short chain fatty acid content of sauerkraut brine taken at day 14 from Control A and *P. freudenreichii* Culture B fermentations. (millimolar / L)

SCFA expressed in mM

	Acetic Acid	**Propionic Acid**	**Butyric Acid**
Control A	0.92	1.63	0.02
Culture B	7.10	12.29	0.01

Chapter 5

Discussion

The inoculation of *P. freudenreichii* into traditionally fermented sauerkraut recipe resulted in biofortification of vitamin B12. The concentration of 1.62µg/100g measured after a 14-day fermentation equates to a single 80 gram serving supplying 54% of the RDI for an adult. Unfortunately, due to the failure of the control sauerkraut to ferment it cannot be confirmed in this study that the B12 found in the inoculated sauerkraut can be attributed to *P. freudenreichii*, however in a similar study sauerkraut inoculated with only LAB did not result B12 synthesis (Babuchowski et al., 1999). Furthermore, it is widely published that B12 is absent from the nutritional profile of traditional sauerkraut (New Zealand Food Composition Database, 2022; US Department of Agriculture, 2020).

The results of this study suggest that the fermentation conditions were adequate for B12 synthesis in sauerkraut. During the fermentation changes in the microbial population were observed, with total bacteria more than doubling to 10^8 CFU/g at day eight in the *P. freudenreichii* culture. At day zero microbial analysis indicated total bacterial population of 10^4 CFU/g, which corresponds with the expected LAB presence previously reported in raw cabbage (10^2 – 10^3 CFU/g), given the addition of *P. freudenreichii* (Peñas, et al., 2016). Furthermore, a spontaneous LAB fermentation can be initiated with very slow starting levels if appropriate conditions are met to promote growth (Zabat et al., 2018). Lactic acid bacteria dominance during fermentation is essential to reach sufficiently low pH levels to inhibit harmful bacteria. Additionally, in this experiment, the production of lactic acid, a byproduct of LAB was needed as the preferred carbon source of *P. freudenreichii*, which allowed for growth and metabolic activity of the introduced microbes.

The co-fermentation of LAB and *P. freudenreichii* appears to have modulated acidity, which is attributed to *P. freudenreichii's* consumption of lactic acid. The final pH was 3.6, which is close to the lowest expected pH of 3.4 when heterofermentative species such as *L. brevis* complete fermentation, and when lactic acid concentration is anticipated to rise to 2.5% (Thierry, Baty, et al., 2023). At day 14, the titratable acidity of *P. freudenreichii* enriched sauerkraut was somewhat less at 1.48%, suggesting that the consumption of lactic acid during fermentation, coupled with production of organic acids produced by *P. freudenreichii* may influence the organoleptic qualities of sauerkraut. While pH is important for microbial stability, the titratable acidity defines the sum of organic acids and because it is measured as a percentage of weight, is an indicator of the intensity of acidic flavour

(Hill & Ferrer, 2021). Therefore, an adjustment in overall flavour is possible by modulating acidity while maintaining safe fermentation conditions.

B12 synthesis and acidity modulation appeared to be mutually possible in this study. Previous studies indicate that this could in part be due to commonalities between LAB and PAB. Both species are anaerobic and salt tolerant. The optimal pH for PAB is 7.0, and rapid decreases in pH caused by lactic acid accumulation can inhibit growth. However, PAB can adapt to stresses during fermentation. This can be seen in cheese production where despite an optimal growth temperature of 30 °C they can survive temperatures from 4 °C - 50 °C, and 5.2 pH during cheesemaking (Thierry, Deutsch, et al., 2011). Others have suggested *P. freudenreichii* has a pH tolerance of 4.5 pH and agree that organic acids are not metabolised from this point (Piwowarek et al., 2017, Xie et al.,2020). The *P. freudenreichii* sauerkraut was 5.5 pH at day two, then experienced a steep drop to 3.6 pH by day eight. This leads to the premise that B12 was produced early in the fermentation, before the more acid tolerant homofermentative LAB populations dominated and peaked. This association was demonstrated in the fermentation of cereals and legumes where Xie et al., (2020) found B12 yield to be inversely correlated with low pH batters, which in turn was due to higher inoculation of LAB.

Propionibacterium freudenreichii influenced the organic acid profile of sauerkraut. The presence of 0.91% propionic acid points to the reduction of pyruvate to propionate via the Werkman cycle. At day eight, 0.46% acetic acid was detected, a common metabolite of LAB and PAB. Acetic acid is the second predominant acid in sauerkraut, typically seen at 1%, about half of the lactic acid content (Peñas, et al., 2016). These outcomes can vary based cabbage's sugar content and subsequent amount of lactic acid produced because acetic acid is a by- product of lactic acid (Satora et al., 2021). This study's results suggest that the flavour profile in the *P. freudenreichii* sauerkraut has likely been influenced by the types and quantities of acids present. Further to the lower overall acidity, taste profiles associated with acids differ. Lactic acid has a mild sourness, while acetic acid has a sharp and pungent flavour; however, its high olfactory threshold prevents it from adversely affecting overall flavour (Wang et al., 2021). Piwowarek et al., (2017) describes propionic acid as having an unpleasant and pungent odour, recommending a concentration of no more than 0.3%, which was exceeded in the *P. freudenreichii* sauerkraut. Investigating the "tastes of fermentation" a small study found that when acetic acid, propionic acid and butyric acid were mixed into a mildly sweet solution and served with citric acid as control, the propionic acid and glucose was the most preferred (Hanselman & Breslin, 2021). Ideally a sensory analysis would have been performed in this study to understand how modulating acidity might impact consumer acceptance.

These results suggest that fortification of B12 in sauerkraut utilising *P. freudenreichii* is possible. This has been confirmed in several recent studies focusing on grain and legume matrixes. Xie et al., (2018) fermented various wheat grains with *P.* freudenreichii, finding that the least processed wheat bran yielded higher amounts of B12 (15.5µg per 100g) than durum wheat due to the lack of cobalt in the most processed grain. This concentration is substantially higher than found in *P. freudenreichii* sauerkraut, however, a 20% supplementation of fortified wheat bran into bread would require the daily consumption four slices. A study followed using other cereals and legumes, where between 5.1 – 74.2µg/100g of B12 was produced. The variances between substrates were explained by pH levels and nutrient differences, including cobalt levels (Xie et al., 2020). It is noted grain and spontaneous vegetable fermentations differ in nature, and that in both studies selected LAB was inoculated with *P. freudenreichii.* Vitamin B12 has also been successfully produced by co-fermentation in sourdough bread, with a yield of 1.39 µg/100g (Zhang et al., 2023). While this is a similar amount to the *P. freudenreichii* fortified sauerkraut, it is substantially less than yields in the unbaked wheat and cereals studies described by Xie et al., (2018, 2020). In part this is likely due to a considerably lower cobalt content in the processed whole-meal flour, and the added ingredients, particularly the sugar as a carbon source for LAB. This study investigated sensory aspects of *P. freudenreichii* enriched sourdough bread, and although there were no changes in pH or acidity, participants noted more sourness in the treated sourdough. This may have been because of SCFA acid profile, particularly the addition propionic acid, however this was not measured (Zhang et al., 2023).

These studies conducted within the last three years have contributed to our understanding of in-situ B12 synthesis using *P. freudenreichii*, highlighting the potential for large scale fortification into processed foods. No recent literature could be found pointing to this work being carried out on vegetable substrates for human consumption, however over 20 years ago Babuchowski et al., (1999) investigated a "new" use of *P. freudenreichii* to develop fermented vegetable products with enhanced nutritional value. Similar results to the current study were achieved for sauerkraut (1.8 – 2.2 µg/100g), depending on the composition of the starter culture used, which included specifically selected LAB. A modest increase in folacin (B9) was also observed. Participants in sensory testing described the *P. freudenreichii* sauerkraut as having a pleasant flavour and slight acidic and salty taste. The lack of development in this area using vegetables supports the current study and review, which is underpinned by the nutritional value of cabbage as a substrate.

The concentrations of B12 produced in this study may be sufficient to provide a health benefit. As a plant food, B12 containing sauerkraut offers a non-animal derived source to plant based eaters who are vulnerable to deficiency (Kumar et al., 2023). Research is needed into the bioaccessibilty of B12 delivered in this way, however studies indicate that bioaccessibility of B12 synthesised by *P. freudenreichii* is promising. Using in-vitro methods, Chamlagain et al., (2021) found that in bread fortified with enriched wheat bran extract 98.9% of B12 was bioaccessible. Interestingly, this was substantially higher than for fresh broth (53.2%). Heat treated broth rose to 72.7%, suggesting heat treatment leads to the release of B12 from cells. Generally water-soluble vitamins are vulnerable to degradation when heated, however this is not the case with B12 (Whitney et al., 2017). This suggests that the pasteurisation of sauerkraut would be beneficial insofar as B12 bioaccessibilty, although modest losses (7%) are known to occur in milk pasteurisation (Yaman et al., 2021).

The B12 enriched sauerkraut contained propionic, acetic butyric acids, major SCFA subtypes that contribute to human health. There is little evidence to suggest that dietary SCFA provide the same benefits as those produced by gut microbes from fermenting fibre and resistant starch. In fact, an in-vitro study examining links between gut microbiota, dietary intake and serum/faecal SFCA found dietary SCFA did not influence and serum/faecal levels (Yamamura et al., 2019). Furthermore, SCFA taken orally is reported to be rapidly assimilated in the stomach or small intestine (Chambers et al., 2015). Regardless of absorption, the trace amounts per serving of sauerkraut would be unlikely to confer intestinal health benefits considering human gut microbiota produce 20g of SCFA daily (Otsuka et al., 2015). Indirectly, recent research points to the potential of dietary propionic acid which binds to fatty acid taste receptors. It is hypothesised that these receptors send signals to the brain's taste centre, encouraging the consumption of foods containing healthful bacteria (Hanselman & Breslin, 2021).

Conclusion

In this experiment introducing *P. freudenreichii* to a traditional sauerkraut recipe resulted in the synthesis of vitamin B12. Although pH at the end of fermentation was at an expected level for sauerkraut, the treated sauerkraut was less acidic, therefore may have a wider organoleptic appeal. This work complements recent research into B12 biofortification with *P. freudenreichii* in grain and cereal products, which are suited to plant-based eaters. Compared with grain foods, the successful B12 enrichment of sauerkraut has the additional benefit of the cabbage's rich nutritional profile, which is largely enhanced through fermentation. Research is needed to assess the bioaccessibility of B12 delivered in sauerkraut, and sensory analysis would test the acidity modulation findings.

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Appendix

Certificate of Analysis for Vitamin B12 (Assure Quality Ltd)

AsureQuality Limited | 131 Boundary Road | Blockhouse Bay | Auckland 0600 | New Zealand
PO Box 41 | Auckland 1140 | New Zealand
t. 00 64 9 626 8200 | e. VLabAuckland@asurequality.com | w. www.asurequality.com

Certificate of Analysis

Final Report

Luca Serventi
Lincoln University
PO Box 84
Lincoln
Christchurch 8140
New Zealand

Report Issued: 13-Dec-2023 **AsureQuality Reference: 23-326506** **Sample(s) Received:** 06-Dec-2023 15:25

Testing Period: 07-Dec-2023 to 13-Dec-2023
Date of analysis is available on request.

Sampled By: Luca Serventi

Results

The tests were performed on the samples as received.

Customer Sample Name: C1 Control, Fermented, Replicate 1 **Lab ID:** 23-326506-1

Sample Description: Sauerkraut brine

Sample Condition: Acceptable

Test	Result	Unit	Method Reference
Vitamin B12	<0.0100	µg/100 g	AOAC 2014.02 (modified)

Customer Sample Name: C2 Control, Fermented, Replicate 2 **Lab ID:** 23-326506-2

Sample Description: Sauerkraut brine

Sample Condition: Acceptable

Test	Result	Unit	Method Reference
Vitamin B12	<0.0100	µg/100 g	AOAC 2014.02 (modified)

Customer Sample Name: PF1 P.f. Fermented Replicate 1 **Lab ID:** 23-326506-3

Sample Description: Sauerkraut brine

Sample Condition: Acceptable

Test	Result	Unit	Method Reference
Vitamin B12	1.69	µg/100 g	AOAC 2014.02 (modified)

Customer Sample Name: PF2 P.f. Fermented Replicate 2 **Lab ID:** 23-326506-4

Sample Description: Sauerkraut brine

Sample Condition: Acceptable

Test	Result	Unit	Method Reference
Vitamin B12	1.55	µg/100 g	AOAC 2014.02 (modified)

Analysis Summary

Auckland Laboratory

Analysis	Method	Accreditation	Authorised by
Vitamin B12 MN-VITB31, 02-FOOD_OTHER	AOAC 2014.02 (modified)	IANZ	Matthew Lockwood

Results that are prefixed with '<' indicate the lowest level at which the analyte can be reported, and that in this case the analyte was not observed above this limit.

Matthew Lockwood

Analyst

Accreditation