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Nourishing a growing population

Overcoming the challenge of B12 deficiency in an increasingly vegetarian world

Helen Thompson

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Att nära en ökande befolkning
Om utmaningen med vitamin B12 i en allt mer vegetarisk kost och hur den kan övervinnas

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Abstract

Increasing numbers of people are eliminating animal products from their diet in response to environmental concerns, as well as for human health reasons and in recognition of animal rights. Vitamin B12, an essential nutrient produced exclusively by bacteria and archaea, is present in animal products but is generally absent from unfortified plant foods. People adhering to strict vegetarian diets must consume a dietary supplement or sufficient fortified foods to prevent deficiency, and the rise of vegetarian eating patterns could cause increased deficiency rates if consumers are not aware of, or able to meet, their B12 needs. The purpose of this study was to gain insight into current knowledge and behaviour regarding B12 within a population in Southern Sweden, and to explore the potential for novel fermented foods to provide a viable source of B12. Three methods were employed: a survey, a literature review and an experimental study to examine the B12 production potential of *Lactobacillus plantarum* during the fermentation of white-beans and cauliflower; special focus is given to the inclusion of beans given the well establish health and environmental benefits of legumes. Knowledge relating to vitamin B12 was significantly higher among respondents who currently consume a vegan diet, but the majority of respondents were able to identify B12 as a necessary supplement for vegetarians (80%), and at least one symptom of B12 deficiency (63%). Consumption of fortified drinks and supplements containing B12 was reported by 75-97% of vegans and 40-63% of vegetarians, compared to 21-34% of meat eaters. Consumption of B12 fortified food was low among all respondents. Attitudes to B12 fortification did not vary significantly between demographic groups; most respondents disagreed that foods should not be fortified with B12 and agreed that there are potential health benefits. A number of bacteria spp. have been reported to produce B12 during the fermentation of a variety of plant-based foods, although the quantity and bioavailability varies widely. Following the fermentation of mixed white beans and cauliflower by *L. plantarum* 299 B12 content increased significantly, although the average concentration (0.048 µg/100g) was low relative to the daily recommended intake value of 2 to 4 µg. Combining white beans with cauliflower represents a novel approach to producing fermented legume-based products and warrants further investigation. Further research is also needed to understand consumer interest in fermented products that contain B12.

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Foreword

Before coming to study agroecology at Alnarp I was fortunate to spend five years working for wildlife conservation and rehabilitation projects in the tropical rainforests of South America and South East Asia. During this time, I saw first-hand the ongoing destruction of primary rainforest in the name of industry, and my understanding of the impact human society has on the world around us was forever changed. My previous knowledge, from textbooks and lectures, was extensive but abstract, and although I was greatly concerned about the destruction of the natural world, nothing I learned in a classroom had the same impact as driving for hours through an endless sea of ranches and plantations to find a pocket of wilderness that was only clinging to life thanks to the good will and charity of those who had fought to protect it. Returning to Europe, I no longer saw the environment I had grown up with through the same eyes; Scotland is considered rugged and wild, renowned for its beauty, but to me it is a barren landscape reduced to little more than grazing land and monocultural plantations. Our native forests and mega-fauna are all but lost, and those species that remain are persecuted or exploited wherever they come into contact with human endeavours, at the centre of which is inevitably agriculture.

Deciding to study agroecology was a step out of my comfort zone; I was not then, nor am I now, skilled in the art of growing and I have the utmost respect for those who produce the food I eat. It is easy to criticize farmers when looking at the world through the eyes of a biologist with a resounding love of the rainforest, but I know I must not lose sight of their humanity. We are all just trying to survive in a society that largely values economic growth above and beyond our own well-being and that of the environment we depend on. Having the opportunity to study alongside students with experience in agricultural and to connect with farmers and researchers has been a humbling experience, but also an encouraging one. Despite clashes of opinions and personal ethics, we were able to work productively together, and to ask difficult questions about the role each of has to play in shaping a better world.

I am strongly committed to the vision of a vegan future; beyond a change in diet I recognize a philosophy that gets to the very root of so many of our problems – our violent and utterly dominating relationship with nature and non-human animals. We can step back, give up some space and let the natural world flourish around us, but to do so we must reduce our ecological demands. Removing animals from our diets is not the only change needed to achieve this, but as far as I can see, it is an essential one. It is also the only solution I see that truly values the concept of justice; non-human animals are here with us, not for us, and they deserve freedom to live their own lives, not just ones that profit humanity. As a former wildlife rehabilitator, I know only too well the trauma they experience at our hands, but also their resilience and determination to keep on living.

I also know from personal experience that pursuing this future doesn't mean sacrificing something that is essential to our physical and social well-being – good food. Since becoming vegan I have come to enjoy a vastly more diverse diet and found new joy in sharing creative and delicious food with the people in my community. Going forward I hope I can put these skills to good use by developing new products that inspire others to value the diversity and quality of plant-based food.

The following work represents an attempt to pursue my vision of the future without losing touch with reality. Radical change won't happen overnight, and philosophy alone isn't enough to guide us; the direction we take must be founded in sound science and reason. Taking a systems approach and developing solutions that are designed to drive positive systemic change, without creating too many new problems, will be central to building a more sustainable society.

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Abbreviations

AdCbl	Adenosylcobalamine
Cbl	Cyanocobalamin
dw	Dry weight
GHG	Greenhouse gas
GRAS	Generally regarded as safe
HCbl	Hydroxocobalamine
LAB	Lactic acid bacteria
LCA	Life cycle assessment
MeCbl	Methylcobalamine
MND	Micronutrient deficiency
MRS	de Man, Rogosa and Sharpe
RDI	Recommended Daily Intake
ww	Wet weight

Definitions

Plant-based – derived wholly from plant origins

Vegetarian* – excludes the consumption of all forms of meat, including poultry, fish and shellfish, but may include the consumption of eggs and/or dairy products.

Vegan*† – excludes the consumption of all products of animal origin, i.e. all meat, fish, dairy and egg products.

* For the purpose of this report, which is primarily focused on diet and nutrition, these definitions do not give consideration to the use of animal derived materials, such as wool and leather.

†There is some debate about the inclusion of honey in a vegan diet; in the present research the definition of vegan does not exclude the consumption of honey as it does not provide a source of vitamin B12.

1. Introduction

In recent years, there has been increased public awareness about the negative environmental impacts of animal agriculture, as well as the treatment and experiences of animals within farming systems, and a significant number of people have adopted vegan and vegetarian diets in response, reducing or entirely eliminating their consumption of animal products. Although the promotion of veganism is often met with resistance and even anger, it has become a significant area of research and shifting towards "plant-based" diets is increasingly advocated as a necessary step towards achieving sustainability (IPCC, 2019; Willett *et al.*, 2019). The American Academy of Nutrition and Dietetics and the British Dietetics Association both acknowledge that well planned vegetarian diets, including vegan diets, are healthful and nutritionally adequate at all stages of life, and may provide health benefits in the prevention, management and treatment of diseases such as type II diabetes and cardiovascular disease (Melina *et al.* 2016; British Dietics Association, 2020), however, there is concern that the reduced consumption of animal products could increase the risk of some micronutrient deficiencies if consumers are not aware of their nutritional needs, or pro-active in ensuring those needs are met (Strain *et al.*, 2017; Röös *et al.*, 2020). Vitamin B12 (B12), which is not naturally present in plant foods, is an essential nutrient that must be supplemented into vegan and vegetarian diets, either as vitamin tablets or through fortified foods (Pawlak *et al.*, 2013). Since B12 is produced by bacteria, fermentation offers an alternative method to create a source that is suitable for vegans and vegetarians. The purpose of the present research is threefold; to gain insights into consumer knowledge about B12 and existing attitudes towards dietary supplements and food fortification, to review the existing literature relating to the production of B12 during the fermentation of plant-based foods, and to experimentally test the B12 production potential of lactic acid bacteria during the fermentation of vegetables and legumes. The role of novel food products in the development of a sustainable food systems is considered, and special focus is given to the inclusion of legumes considering they are widely regarded to offer many benefits, both for agricultural systems and human health.

1.1 Background

1.1.1 The development of agri-food systems

Globally, agricultural activity covers as much as 43% of ice-free, non-dessert land and is a leading driver of land use change, fresh-water consumption, pollution and greenhouse gas (GHG) emissions (Poore and Nemecek, 2018; Ramankutty *et al.*, 2018). Modern farming practices have developed following the green revolution, which lead to a significant increase in available calories and had an overall positive impact on global hunger and food security (Ramankutty *et al.*, 2018). However, the industrialization of food production has also increased the environmental impact of agricultural systems and created a system that is heavily dependent on agri-chemical inputs and mechanization (Gliessman, 2015). More recently, the globalization of food markets and the expansion of production systems modelled on the economy of scale have encouraged the growth of monocultures, leading to decreased agricultural biodiversity and, consequently, decreased diet diversity (Allen *et al.*, 2014; Gliessman, 2015; Zander *et al.*, 2016). These changes are believed to negatively impact the resilience of agricultural systems and may contribute to a decline in human health outcomes, characterized by pervasive malnutrition and the rise of non-communicable diseases such as cardiovascular disease and diabetes (Allen *et al.*, 2014; Jones, 2017). Advocates of agroecology, which can be defined as the ecology of food systems (Francis *et al.*, 2003), argue that the continued growth and intensification of industrial agriculture and the globalized food market will further lead to decreased food security and environmental devastation, and that alternative strategies are necessary to achieve environmentally sustainable, nourishing and socially just agri-food systems (Hill, 1998; Gliessman, 2015). The future widespread adoption of vegan diets is one such strategy that would have transformative effects on the global agricultural landscape and is predicted to bring many benefits. However, it would also create a new set of challenges for the management of agricultural systems, and for public health and nutrition.

1.1.2 The positive potential of dietary change

Dietary change has become a hot topic. It is increasingly recognised that the most significant factor affecting the environmental impact of our food choices is the extent to which animal products are included in our diets (Aleksandrowicz *et al.*, 2016; Poore and Nemecek, 2018). In 2010 the UN advised that a global shift to vegetarian diets would be necessary to prevent the worst effects of climate change, and in the ten years since research has continued to highlight the role of dietary change as an important component of sustainable development, (Bajželj *et al.*, 2014; Aleksandrowicz *et al.*, 2016; Bryngelsson *et al.*, 2016), particularly in industrialized countries where the “western” diet, characterized by high meat, dairy and egg consumption, is associated with particularly high environmental impacts and increased rates of non-communicable chronic diseases (Tilman and Clark, 2014; Westhoek *et al.*, 2014).

Much attention has been given to the concept of food miles as an important factor in the carbon food print of food, and ‘eat local’ has become a common mantra. However, farm to table LCA assessment has shown that transport typically accounts for little more than 10% of any given products impact, and that the production system is the most significant factor, accounting for approximately 80% of GHG emissions (Weber and Matthews, 2008; Sandström *et al.*, 2018). A comprehensive review of dietary change studies found that the calculated GHG emission reduction associated with vegan diets ranges from 20-70% with a median of 45%, the largest decrease of any dietary change (Aleksandrowicz *et al.*, 2016). A subsequent review of >38,000 farms, 1600 food processors, packaging types and retailers reported that vegan diets could reduce agricultural GHG emissions by as much as 49% (Poore and Nemecek, 2018). Agricultural emissions have been notably absent from most international climate change conferences and attempts to include agriculture in future policy have been more focused on climate adaptation and future food security than mitigation efforts (CCAFS, 2016). The potential reductions achieved by dietary change are significant, especially considering that recent analysis of mitigation policies has found that current measures are unlikely to successfully limit global warming to 2°C. For example, a recent modelling experiment, published in the journal Nature, predicted that global temperature increase is likely to fall between 2.0°C and 4.9 °C, and that there is only a 5% chance that the target of >2°C will be achieved. These results were not calculated under a 'business as usual' scenario, but were based on observed data from emission mitigation policies that are already in place (Raftery *et al.*, 2017).

Further to the reduction of GHG emissions, Poore and Nemecek (2018) estimated that (relative to 2010 reference levels) the wide-spread adoption of vegan diets would reduce global agricultural land use by up to 76%, eutrophication and acidification by approximately 50%, and freshwater withdrawals by 19%. Global uptake of vegan diets could also have many positive consequences for human health; compared to non-vegan diets they are typically higher in protective nutrients and phytochemicals, for example, dietary fibre, folic acid, vitamin C and E, magnesium and iron, and lower in dietary factors associated with chronic diseases, such as saturated fat and cholesterol (Craig, 2009). Vegan diets are correlated with a lower risk of cardiovascular disease (CVD), type 2 diabetes, and some cancers (Craig, 2009; Orlich *et al.*, 2013; Esselstyn, 2017); diseases that are affecting a growing number of people in all but the poorest nations (Tilman and Clark, 2014).

1.1.3 Legumes: healthy food, healthy soil

Increasing the consumption of legumes has been identified as a key strategy for reducing meat consumption and achieving sustainability in the food system (Foyer *et al.*, 2016; Röös *et al.*, 2020). Legumes constitute a broad variety of plants belonging to the plant family Fabaceae, many of which are grown for their edible seeds - green beans and peas, which are classified as vegetables, and dry beans, peas and lentils, referred to as grain legumes or pulses (FAO, 2017). Legumes are rich in dietary fibre, protein and micronutrients (Messina, 2014), and there are several recognized health benefits associated with regular legume consumption – including reduced risk of cardiovascular disease and diabetes (Messina, 2014; Mudryj *et al.*, 2014). There is also ongoing research into other potential benefits, such as reduced risk of certain cancers (Zhu *et al.*, 2015; Rungruangmaitree and Jiraungkoorskul, 2017) and improved gut health (Borresen *et al.*, 2017).

In addition to providing a rich source of dietary fibre and human-edible proteins, legumes can make a significant contribution towards maintaining soil fertility; as nitrogen fixers they contribute nitrogen to cropping systems, reducing the need for the addition of other nitrogen sources (Jensen *et al.*, 2012). In the current system, animal manure and bone meal are widely used as fertilizers, but as demand for animal products decreases alternative strategies will be required as fewer animals are farmed and these inputs become scarce. Integrating legumes into crop rotations will go some way to addressing this need and avoiding increased dependence on synthetic nitrogen, which is energetically demanding to produce and associated with a high carbon footprint (Jensen *et al.*, 2012). Other benefits attributed to cultivating legumes include enhanced soil carbon content and improved soil structure (Jensen *et al.*, 2012; Stagnari *et al.*, 2017), improved biodiversity of cereal dominated landscapes, in turn supporting increased insect biodiversity, and improved resilience to pests and disease (Köpke and Nemecek, 2010; Ebert, 2014).

Despite these benefits, the production of legumes in Europe is minimal – in 2016 the area of arable land dedicated to grain legumes in Europe was only 1.5%, compared to 14.5% globally (Watson *et al.*, 2017). Increasing the area under production is therefore an area of research interest; a recent study of the production potential in Sweden concluded that, under the scenario whereby Swedish meat consumption is reduced by 50% and replaced with grain legumes, increasing the total area of grain legume cultivation from 2.2% to 3.2% would be feasible (Röös *et al.*, 2020). Improving the market value of legumes is a recognized strategy that could promote their production by European farmers (Preissel *et al.*, 2015). Food processors and product developers are uniquely placed to influence the production and consumption of sustainable products, but to do so they must take ecological and human health goals into consideration during product development (Spieldenner and Matope, 2017). The development of new legume-based products could increase consumer demand, increasing both their cultivation and consumption.

1.1.4 Micronutrient deficiencies: an existing challenge in the western world

Micronutrient deficiency (MND) often referred to as "hidden hunger", can be defined as "an inadequate intake [of nutrients] beyond deficiency or without typical clinical signs and symptoms" (Biesalski, 2017). It is a recognized problem throughout the world, including within affluent countries where there is an apparent abundance of food available (Biesalski, 2017; Troesch, 2017). Currently, the nutrients of highest concern are vitamins E, D and A and folate (Troesch, 2017). Low income, poor knowledge of nutrition and over consumption of nutrient poor food are probable causes of MND in affluent countries, which is of particular concern to children under 5 and pregnant women who have elevated nutrient requirements (Biesalski, 2017).

A variety of different strategies have been employed to improve nutritional health, including campaigns to promote the adoption of healthy diets, the promotion of vitamin supplements, and the fortification of food products, with mixed results. While public information campaigns, dietary guidelines and improved nutritional labelling offer consumers the ability to make informed choices, such interventions are designed around the principle of 'the rational consumer', whereby it is assumed that the right information will have a positive influence on consumer choices and long-term health outcomes (Réquillart and Soler, 2014). Assessment of these interventions has found them to have limited value (Brambila-Macias *et al.*, 2011; Troesch, 2017), and several other influencing factors have been identified, including "taste cost" – the trade-off between immediate pleasure over long-term health benefits (Irz *et al.*, 2015), conflicting messaging about the healthfulness of certain foods, and the role played by market forces in providing (or restricting) access to healthy food choices (Réquillart and Soler, 2014).

The development of dietary supplements as a consumer product has created a multi-million-dollar, global industry, however the widespread availability of supplements has not solved the problem of MND. Research has shown that the consumption of dietary supplements is highest among those who have the lowest need as they already derive sufficient nutrition from their diet, a paradox that has been named the "inverse supplement hypothesis" (Pajor *et al.*, 2017). These consumers are typically women in their 40s, 50s and 60s with above average income (Conner *et al.*, 2001). Supplement users typically report using them to promote overall good-health and as a protective measure against illness, despite a lack of evidence to support these claims (Conner *et al.*, 2001; Bailey *et al.*, 2013). Both users and non-users recognize media as a significant influencing factor on supplement consumption (Conner *et al.*, 2003), and research in the US has found that less than 1 in 4 supplements were consumed following instruction from a medical professional (Bailey *et al.*, 2013) suggesting that marketing campaigns are driving consumers to take supplements they do not need. This is perhaps not surprising if we consider that people with higher disposable income are more likely to consume sufficient nutrition from their diet, but also more likely to be the target of marketing campaigns by companies seeking to profit from dietary supplements. Among vegan populations dietary supplement use is varied. Research that examined the relationship between dietary motives and health behaviours found that vegans who were motivated by health reasons were less likely to consume dietary supplements than those who were motivated by ethical reasons (Radnitz, *et al.*, 2015). The report provided no data to explain why, however the authors hypothesized that due to their focus on obtaining their nutritional needs from food, they may believe they do not need to take dietary supplements. A survey of American "raw" vegans - a subgroup of the vegan community that are highly motivated by beliefs surround health and nutrition, and that largely adhere to diets based on raw fruits, vegetables, nuts, seeds and sprouts - found that only 6% of the sample population consumed a B12 supplement (Hobbs, 2005). Ironically these reports indicate that people who adhere to vegan diets for health reasons may be at increased risk of developing B12 deficiency.

Food fortification has been identified as one of the most cost-effective health interventions, and a significant factor in the reduction of MND in the industrialized world (Darnton-Hill and Nalubola, 2002). A variety of fortification products have been used to address a range of specific health conditions, such as iodized salt to treat goitre, vitamin D fortified milk for rickets, and folic-acid enriched cereals and flours to prevent neural-tube defects arising during foetal development (Darnton-Hill and Nalubola, 2002; Molster *et al.*, 2009; Troesch, 2017). It has been suggested that food fortification is a successful strategy because it requires little change in consumer behaviour and habits, however it is important to recognise that knowledge and attitudes still play an important role and the benefits of fortification will not be felt if consumers and policy makers are not convinced of its efficacy and safety (Darnton-Hill and Nalubola, 2002). Research into the consumption of so called "functional foods" (products that are marketed as having enhanced health value, such as nutrient fortification or probiotic properties) has found that attitudes and behaviour vary significantly across Europe. While a high percentage of Swedish, Finnish and Dutch consumers report consuming functional foods, Danish, Belgian and Italian consumers report lower levels of consumption and are more sceptical of their reported benefits (Özen *et al.*, 2014).

Over-coming the challenge of malnutrition will require the actions of multiple stakeholders. Much focus has been put on the importance of responsible consumption, but the available evidence indicates that educational campaigns and product labelling alone have limited and inconsistent effects on consumer behaviour. Governing bodies, research institutes and actors within the food industry must also recognise their roles in achieving public health and sustainability goals.

1.1.5 Vitamin B12 deficiency: a challenge for dietary change

Although vegan diets confer many benefits, it is well documented that people who consume little or no animal products have an increased risk of suffering from B12 deficiency, a serious problem that can cause irreversible damage to the body (Pawlak, Lester and Babatunde, 2014). B12 deficiency is not currently a major public health concern as there are few people in the population who don't consume enough, and deficiency is largely limited to people adhering to vegan or vegetarian diets and older adults with malabsorption (Strain et al 2014). The increased up-take of vegan and vegetarian diets however, could lead to increase B12 deficiency if measures are not taken to prevent it (Strain *et al.*, 2017; Röös *et al.*, 2020).

The term "Vitamin B12" is used to describe a variety of water-soluble compounds of the cobalamin group (Kumar, Chouhan and Thakur, 2010), which are biosynthesized exclusively by bacteria and archaea, commonly *Salmonella typhimurium, Escherichia coli and several Thermotoga spp. (Fang et al.,, 2017; Nakos et al., 2017).* There are currently four recognised analogues of B12 that can be administered to prevent or treat deficiency in humans; methylcobalamin (MeCbl), adenosylcobalamin (AdCbl), hydroxocobalamin (HCbl) and cyanocobalamin (Cbl) (Thakkar and Billa, 2015). In humans and non-human animals, B12 is essential to the maintenance of myelin, the formation of red blood cells and the rapid synthesis of DNA during cell division (Kumar *et al*., 2010; Pawlak *et al.,* 2014). Deficiency can lead to megaloblastic anaemia and neurological damage due to irregular development of the myelin sheath surround nerve cells. It can present with a variety of clinical symptoms including fatigue, constipation, the sensation of numbness and tingling in the hands and feet, mental confusion and memory problems, headaches, mouth-sores and a smooth tongue (Pawlak et al., 2014). The recommended daily intake (RDI) for adults ranges from 2µg – 4 µg per day (EFSA, 2015; Livsmedelsverket, 2019). In contrast, plants do not metabolize B12 and it is generally accepted there are no naturally occurring plant-based dietary sources (Pawlak, Lester and Babatunde, 2014). There is therefore a consensus among the medical community that people adhering to strict vegetarian diets must consume B12 supplements, either in vitamin tablets or fortified foods (Pawlak *et al.*, 2013). However, as has already been established, consumer behaviour is influenced by a number of factors, and it is not safe to assume that information campaigns will lead to improved health outcomes; despite the importance of B12 supplementation being well understood by the medical community, studies frequently report low levels of B12 in vegan and vegetarians. For example, a 2013 literature review found 18 reports on B12 deficiency in vegans and vegetarians, ranging from 62% among pregnant women, 25-86% among children, 21-41% among adolescents and 11-90% among the elderly (Pawlak *et al.*, 2013).

1.1.6 Novel fermented foods: a possible source of vitamin B12

Fermentation is a simple and effective biotechnological method that has been exploited for countless generations without knowledge of the microbial processes involved (Di Cagno *et al.*, 2013; Melini *et al.*, 2019). During fermentation carbohydrates are broken down to produce energy. During this process a variety of micronutrients can be produced, including B vitamins (Waters *et al.*, 2015). The contribution made by fermented foods to meeting B12 requirements is controversial, although research from South Korea provides some evidence that diets rich in fermented foods protect against B12 deficiency in elderly women (Kwak, *et al.*, 2010).

Lactic acid fermentation is one method that has traditionally been used around the world to enhance the organoleptic properties of food, and as a method of preservation; in Europe, it is associated with the preservation of vegetable products, such as capers and cabbage (sauerkraut), and also in the production of breads and dairy products. Lactic acid bacteria (LAB) refers to a diverse group of gram-positive bacteria that share certain morphological, metabolic and physiological characteristics, notably the production of lactic acid during the fermentation of carbohydrates (von Wright and Axelsson, 2012). Members of the genus *Lactobacillus* are subject to generally regarded as safe (GRAS) status, and are widely used as starter cultures in the food industry, and as probiotics (Barrangou *et al.*, 2012). It has also been observed that lactic acid fermentation can result in enhanced nutrient content of foods, as vitamins are created during the metabolic processes within the fermenting bacteria. *L. reuteri* has been shown to produce vitamin B12 during the co-fermentation of glycerol and fructose in soy-yoghurt (Gu *et al.*, 2015b). Strains of *L. coryniformis* and *L. plantarum* isolated from Japanese pickles have also been recorded to produce vitamin B12, though not in significant quantities (Masuda *et al.*, 2012).

Fermented bean products, predominately made from soya, are traditional to East and South East Asia and make an important contribution to Asian diets (Xu, Cai and Xu, 2017). Bean consumption in northern Europe is comparatively low, possibly due to a negative culinary perception as well as digestive discomfort arising from the high oligosaccharide content of beans (Messina, 2014). Fermentation has been identified as a possible method to reduce digestive problems association with bean consumption (Granito *et al.*, 2005). Tofu, miso and tempeh have recently become more popular in Europe and are now widely available in supermarkets and specialist food stores. Fermented bean products based on European legume varieties, however, remain limited. One example is lupin tempeh, which has been developed in southern Sweden and commercially available since 2019 (Lupinta, 2020).

Cauliflower (*Brassica oleracea var. botrytis*) is an annual cool-weather crop that is grown for its edible flower structures. More than two thirds of global cauliflower production occurs in China and India, however it is cultivated all over the world and grows most successful within the latitudinal range 11 – 60^{o} N (Singh *et al.*, 2018). This range covers most of Europe, including the southern regions of Sweden. It is reported as a good source of antioxidants, including vitamin C (24.8 mg/100 g), as well as micronutrients such as phosphorus (61.35 mg/100g) and calcium (41.16 mg /100g) (Singh *et al.*, 2007; Baloch *et al.,* 2015). Between 2002 and 2014 cauliflower production in Sweden increased from 4.9 – 6.7 thousand tonnes per year (Karlsson, 2015). Cauliflower has becoming increasingly popular following the recent trend of low-carb and gluten free diets; it is promoted as an alternative to grains with products such as cauliflower “rice” and pizza bases entering the market (O’Connor, 2018). Despite being a close relative of cabbage, cauliflower is not traditionally prepared as a fermented product in northern Europe, however it’s healthy nutritional profile and current popularity make it an excellent candidate for the development of a novel fermented food with high consumer appeal. It’s cultivation poses a unique set of challenges as it is highly sensitive to environmental conditions, and it requires significantly more attention than other members of the *Brassica* family (Ray and Mishra, 2017), but it has also been identified as a valuable addition to vegetable intercrops, contributing to both increased yield and profitability (Yildirim and Guvenc, 2005).

1.2 Aims and objectives

The development of future agricultural systems will require innovations that support ecological sustainability without compromising human health outcomes. In recognition of the increased risk of B12 deficiency associated with the adoption of ecologically sustainable dietary patterns, the aim of the present research is to gain insight into consumer knowledge of B12 while simultaneously exploring fermentation as a tool for increasing levels of B12 in plant-based food. The following research question is considered: can novel fermented foods provide a viable source of vitamin B12 and contribute to the development of sustainable food systems in northern Europe?

The objectives are:

1. to assess consumer knowledge, behaviour and attitudes relating to B12 consumption within the Swedish population

2. to review current research on different fermentation methods, with specific focus on lactic acid fermentation and increasing B12 content

3. to explore the production of B12 during lactic acid fermentation of white beans and cauliflower, as potential ingredients for a novel fermented food product.

2. Methods

2.1 Survey

2.1.1 Survey design and distribution

A cross-sectional questionnaire was developed using Netigate online survey platform to gather the following information:

- demographic information
- animal product consumption frequency
- diet identification and motivation
- dietary supplementation behaviour
- fortified food consumption
- attitude toward dietary supplementation
- attitude toward food fortification
- knowledge pertaining to vitamin B12.

Question design was based on previously published research on dietary choices, nutritional knowledge and attitudes and behaviour relating to folic acid consumption and food fortification (Molster *et al.*, 2009; Janssen *et al.*, 2016). To assess animal product consumption frequency, respondents were asked to score how often they consume the following animal products: beef, lamb, pork, chicken, fish and/or shellfish, other meats, eggs and/or egg products, milk and/or dairy products, and honey. Possible answers were 'Never', 'Rarely (once or twice a year)', 'Sometimes (once or twice a month)', 'Often (once or twice a week)' and 'Everyday'. Later, respondents were asked if they identify as vegan or vegetarian, followed by an open text question asking for three factors that motivate dietary choice. A seven-point likert scale was employed for all questions designed to measure attitudes. To assess B12 consumption, respondents were asked to identify from a list of 14 dietary supplements which (if any) they had consumed within the last two weeks. Similarly, they were also asked if they had consumed any foods or drinks that were fortified with B12 within the past two weeks, and whether fortified products were consumed specifically because they were fortified.

The survey was open to responses for a 3-week period in February 2020 and was distributed via email to students at the Alnarp campus of the Swedish University of Agriculture, and via the facebook group "Vegan i Malmö".

2.1.2 Demographic grouping

Gender was divided into three categories, female, male and non-binary. Age was divided into five brackets: 21-25, 26-30, 31-35, 36-40 and 40+. Occupation and nationality were both divided into two groups: students vs. non-students and Swedish vs. non-Swedish.

2.1.3 Dietary grouping

Respondents were classified as belonging to one of four dietary groups, Vegan (V), Vegetarian (Vg), Low Meat (LM) and High Meat (HM), depending on their response to the food consumption frequency questions. Vegans answered “Never” to all animal products, with the exception of honey; although there is debate about the inclusion of honey in a vegan diet, honey is not a source of vitamin B12, so from a nutritional perspective those who consume honey are at the same level of risk as those who don’t. Vegetarians answered “Never” to all meat products but included dairy and/or eggs in their diet at any frequency. Low meat eaters answered “Rarely…” or “Sometimes…” to one or more kind of meat, and high meat eaters answered “Frequently…” or “Everyday” to one or more kind of meat.

2.1.4 Dietary motivation

Respondents were asked to provide up to three motivating factors that influenced their dietary choices in an open format question. Answers were coded into the following categories: self-related motives (e.g. health, well-being, taste, pleasure), ethical (concern for animals, moral concerns about harming others), environmental (e.g. climate change, biodiversity loss, deforestation), economic, local sourcing and miscellaneous (e.g. tradition, accessibility, social factors).

2.1.4 Vitamin B12 Knowledge

Respondent’s knowledge was assessed based on three factors: their ability to identify B12 as a necessary supplement for vegans and vegetarians, their knowledge of the Recommended Daily Intake, and their ability to name symptoms of B12 deficiency. The dependent variable “B12 knowledge” was derived from the aggregate score of these three questions:

1) From a list of 14 dietary supplements, participants were asked to identify which (if any) were recommended for people following a strictly vegetarian diet. Respondents were scored 0 if they did not select B12 and 1 if they did, regardless of which other supplements were selected.

2) From the question “Approximately, what is the recommended daily intake of vitamin B12 for children over 10 and adults?” respondents were scored 1 if they answered 2µg, and 0 if they answered 200µg, 2 mg or don’t know. This intake value was based on information presented on the Swedish Food Agency website (Livsmedelsverket, 2019).

3) Knowledge of B12 symptoms was assessed based on the number of symptoms that were correctly named in response to the question “Can you name any symptoms associated with vitamin B12 deficiency? (List as many as you know or leave the answer blank)”. Respondents were given a score of 0, 1, 2 or 3, in correspondence with the number of correct answers provided (a score of three represented 3 or more correct answers). B12 deficiency symptoms were established based on information provided by the Swedish Health system website (1177.se), and other web-based references (WebMD, Wikipedia). The following answers were accepted as correct:

- Weakness, tiredness, lethargy
- Heart palpitations, shortness of breath
- Trouble concentrating, confusion
- Smooth tongue, mouth sores
- Problems with digestion, i.e. constipation, gas, loss of appetite, nausea
- Anaemia
- Nerve problems; pins and needles, loss of feeling in arms and legs
- Vision loss
- Headache, earache
- Dizziness
- Depression, memory loss, behavioural change

2.1.5 Attitude measures (likert scales)

In order to assess respondent’s attitudes towards dietary supplements and food fortification, a series of questions were designed using the likert method. A seven-point scale was used for each likert item, with scores designated from 1-7 as strongly agree, agree, somewhat agree, don’t know, somewhat disagree, disagree and strongly disagree.

2.1.6 Statistical analysis of survey

All analysis was performed using IBM SPSS Statistics version 26.

Principle component analysis was performed with varimax rotation to group likert items, and reliability analysis was used to confirm internal consistency. New likert scale variables were created by calculating the mean of all items in a group. A likert scale value <4 indicated a positive average response, and a value >4 indicated a negative average response.

Where the assumptions were met, the effect of dietary group, age, gender, nationality, occupation and motivation on dependent variables was tested using ANOVA analysis, followed by Tukey's multiple comparison test at significance level $p = 0.05$. In two instances the assumption of homogeneity of variance was not met ($p<0.05$) and non-parametric Kruskal-Wallis and Mann-Whitney tests were performed.

The factors Age, Gender, Nationality, Occupation and Diet were treated as independent variables in all binary logistic analysis. Age was a continuous variable. Gender was treated as a categorical factor with "female" as the reference factor. Nationality and Occupation were coded as binary factors with a score of 1 for Swedish and student, and a score of 0 for all non-Swedish and non-student respondents. Diet was treated as a categorical factor with HM as the reference factor. Multicollinearity was assessed using regression analysis.

2.2 Literature review

A search was performed on the citation database "Web of Science" using the following key words: B12, cobalamin, lactic acid bacteria, fermentation and fermented food. The resulting manuscripts were assessed for their relevance by examination of their titles and abstracts. Papers were included for review if they represented original research pertaining to the production of vitamin B12 by lactic acid bacteria, the production of vitamin B12 during the fermentation of plant-based foods, or the assessment of vitamin B12 content of fermented plant-based foods. Two subsequent screening processes were then followed to search for additional papers that might have been missed from the original search; firstly the reference lists of manuscripts were checked, and secondly the Web of Science "times cited" tool was used to search for more recent research that had referenced papers identified by the original search criteria. One review paper was included as it provided a summary of results that were otherwise not available in English, and one paper, which detailed an experiment, performed using mice, to test the bioavailability of vitamin B12 produced by *L. reuteri* during the fermentation of soymilk, was omitted due to concern over the nature of the research methods employed.

Papers were divided into three categories and are discussed accordingly: the B12 content of fermented foods, the production of vitamin B12 during fermentation by *Propionibacterium freudenreichii,* and the production of vitamin B12 during fermentation by lactic acid bacteria.

For the purpose of comparison, B12 concentrations have been reported as µg/100g throughout this report, except where otherwise stated, as well as whether they represent dry weight (dw) or wet weight (ww) values. A variety of methods have been developed to analyse B12 concentrations, including microbiological assay, polarographic, spectrophotometric, radio-ligand binding and various chromatographic techniques (Lawrance, 2015). Microbiological assay, which utilizes the known requirement of certain bacterial organisms (e.g. *Lactobacillus delbreueckii*) for vitamin B12 to enable their growth in a supporting medium, has been the most commonly reported method used for the analysis of food (Lawrance, 2015) This method, as well as spectrophotometric methods, cannot differentiate between the different B12 analogues present in a sample. High performance liquid chromatography (HPLC) methods are more effective at differentiating the levels of different analogues (Quesada-Chanto *et al.*, 1998), however the use of HPLC to test un-fortified food samples is limited due to the relatively low levels present, compared to the high detection limits (Lawrance, 2015). In this review, reported methods have been defined only as microbiological assay or HPLC, and B12 analogues have been reported whenever possible.

2.3 LAB Fermentation of cauliflower and white beans

Two separate fermentation experiments were conducted. Experiment 1 was performed to test the rate of fermentation of a single *L. plantarum* strain using three different preparations of cauliflower: raw, cooked and a 50:50 raw-cooked mixture. Experiment 2 was performed to test the rate of fermentation, and the vitamin B12 production, of four different *L. plantarum* strains using three different substrates: raw cauliflower, cooked white beans and a 50:50 raw cauliflower/cooked white bean mixture.

2.3.1 Bacterial cultures

Starter cultures from four strains of *L. plantarum* were provided by Probi AB (Lund, Sweden): 299v, Lp900, 299 and Heal19. The strains were stored at 4ºC on MRS agar. For production of the fermentation inoculum the strains were cultivated in MRS broth at 35ºC over-night. The cells were harvested by centrifugation (Eppendorf MiniSpin, 10 000 g for 3 min) and washed twice with 0.85% NaCl solution. The bacterial suspension was added in a concentration of 1% (w/w) to the vegetable mix. More details on the used strains can be found in Appendix 1.

2.3.2 Ingredient preparation

Cauliflower could not be sourced directly from a producer and was purchased locally from an independent fruit and vegetable vendor. It was labelled as Swedish produced, however the exact variety and production method was undetermined. White beans were obtained directly from Swedish producer Per Modig (Kristianstad, Sweden).

The outer leaves and excess stem were removed from cauliflower heads, and remaining florets were rinsed in cold water. To prepare the raw substrate, cauliflower florets were roughly chopped and then blended in a food processor until a grainy, paste like consistency was produced with pieces no larger than 2-3mm. For the cooked substrate, cauliflower florets were chopped into approximately 2cm-sized pieces then boiled for 10 minutes.

Cooked cauliflower pieces were cooled to room temperature and then processed in a food processor until a smooth, even consistency was achieved, similar to the texture of mashed potato. White beans were soaked in cold water for 16 hours, rinsed and then added to a pan of boiling water. They were hard boiled for approximately 10 minutes, and then simmered for a further 50 minutes until soft. Cooked beans were cooled to room temperature and then processed into a smooth paste using a food processor.

For experiment 1, four portions of raw cauliflower, four portions of cooked cauliflower, and four portions consisting of a 50:50 ratio of raw and cooked cauliflower were prepared. For experiment 2, fifteen portions of raw cauliflower, fifteen portions of cooked white beans and fifteen portions consisting of a 50:50 ratio of raw cauliflower and cooked white beans were prepared. For each substrate, portions weighing 100g ±1g were measured into sterile containers with a volume of 150 ml and were combined with 2g of sea salt. Containers with only white beans also received 10ml of boiled water, cooled to room temperature, to dissolve the salt and ensure it was evenly distributed throughout the mixture. Samples were thoroughly stirred and left to rest for 10 minutes.

2.3.3 Fermentation procedure

For experiment 1, *L. plantarum* 299v was added to three containers of each substrate, and, for experiment 2, bacteria cultures from each of the four different *L. plantarum* strains were added to three containers of each substrate. For both experiments, a corresponding volume of 0.85% NaCl solution, without bacterial culture but with initial content of sterile MRS broth in the tube, was added to the remaining containers to produce control samples. Each mixture was thoroughly stirred again following the addition of bacteria or control substance. The pH of each sample was recorded immediately, and containers with bacterial culture were incubated at 30° for 120 hours (A), and 44 hours (B). The lids of the containers were placed loosely on to allow gas produced during fermentation to escape. Control samples were directly frozen at -80°C in order to prevent the degradation of the nutrient content.

2.3.4 pH measurements

To record the pH of samples, 1.5 ± 0.1g of mixture was combined with 3ml of distilled water in a test tube. Test tubes were agitated to ensure the content was well mixed before measurements were taken. In experiment 1, the pH of fermented samples was measured at 4, 23, 48 and 72-hour increments. Samples were tasted after 23 hours. After 120 hours at 30° the samples had begun to spoil, with a detectable change in colour and visible mould growth. In experiment 2, the pH of fermented samples was measured after 18 and 44 hours. Samples were tasted after 44 hours, and then transferred to a freezer at -80°C.

2.3.5 Vitamin B12 analysis

Limited funding was available to perform the B12 content analysis. Following the successful fermentation of the white-bean and cauliflower mixture, this treatment group was selected for analysis due to its unique composition and taste. Vitamin B12 analysis was performed by Eurofins, following method AOAC 952.20 - a microbiological assay procedure whereby the concentration of vitamin B12 is determined based on the observed growth of a known B12 dependent bacteria, typically *L. delbreueckii,* in supporting medium (Lawrance, 2015). The level of growth achieved by the bacteria is directly proportional to the amount of vitamin B12 in the test extract.

2.3.6 Statistical analysis of laboratory experiment

Both experiments were set up with three replicates in each treatment group and repeated once. Statistical analysis was performed using Minitab 17 for Windows. Treatment effect was tested using one-way ANOVA analysis, followed by Tukey's multiple comparison test, at significance level $P \leq 0.05$.

3. Results

3.1. Survey

3.1.1 Demographics

A total of 140 completed responses were received. The majority of respondents were identified as Swedish (81.4%), female (73.6%), and students/interns (74.3%). The average age was 30.2 years, ranging from 21 – 56 years (figure 1).

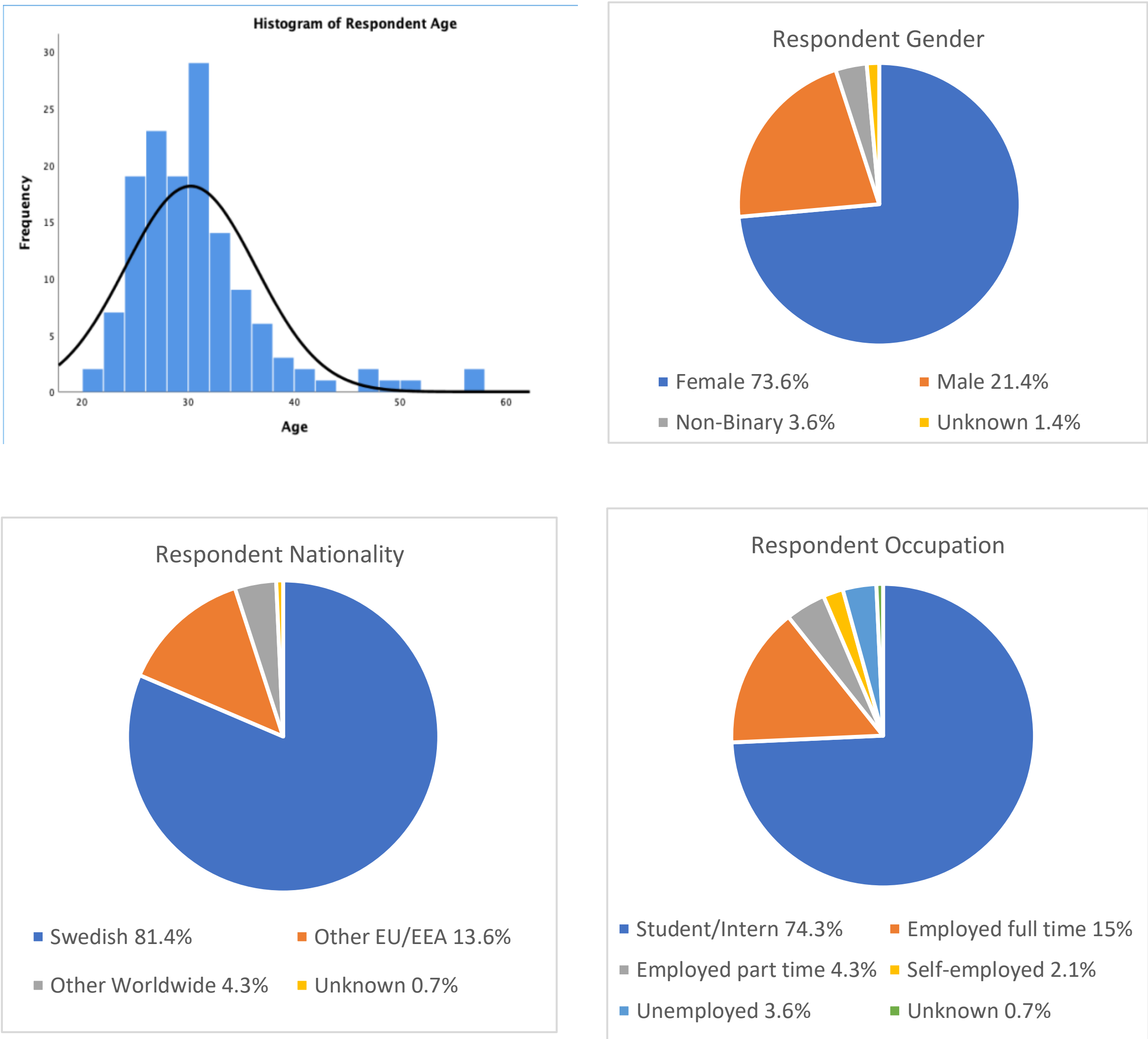

Figure 1. Visual representation of demographic data.

3.1.2 Dietary groups

When asked to self-report diet identity, 27.1% and 34.3% of respondents identified as vegan and vegetarian respectively. When respondents were categorized based on reported eating habits, 22.9% were classified as vegan, and 21.4% were classified as vegetarian. In total 55.7% were categorized as meat eaters; 39.3% as low meat eaters and 16.4% as high meat eaters.

3.1.3 Motivation

The three most frequently cited motivations for dietary choices were ethics, health and the environment (table 1). This is in line with other research that has identified these three factors as the most commonly reported motives for choosing a vegan or vegetarian diet (Janssen *et al.*, 2016). For vegans and vegetarians, these reasons accounted for >90% of all motivations given, and the only other motivations reported were social. Within meat-eating groups, the three principle motivations remained the same, however other motivations, including economic considerations, local sourcing of products and tradition, were also reported.

Table 1. Dietary motivation reported by dietary group (%).

Diet/Motivation	Ethics	Health	Environment	Economic	Local	Other
Vegan	96.9	71.9	84.4	-	-	6.3
Vegetarian	93.3	60	93.3	-	-	10
Low Meat	50.9	61.8	76.4	16.4	12.7	20
High Meat	34.8	60.9	65.2	26.1	4.3	30.4
All	67.9	63.6	80	10.7	5.7	15

Binary logistic regression indicated that diet was a significant predictor of the motivation ethics, and all other motives (economic, local and "other" combined). Vegans and vegetarians were significantly more likely to report ethics as a motivation (Chi-sq=46.6, df=8, p<0.01) and less likely to report other motivations (chi-sq=25.9, df=8, p,0.01), than the high meat group, which did not differ significantly from the low meat group. Nationality was found to be a significant predictor of the environment motivation (Chi-sq=18.0, df=8, p=0.021), with Swedish respondents being approximately 3 times more likely to quote this motive (95% CI = 1.04-8.81, p=0.042). No other variables were found to have a significant effect on dietary motivations.

3.1.4 Vitamin B12 Knowledge

80% of respondents correctly identified that people who eat a strict vegetarian diet are recommended to take B12 supplements, however only 25% identified the recommended daily intake of B12 from a multiple-choice list with four possible answers. 62% of all respondents knew at least one symptom of vitamin B12 deficiency, and 11% identified 3 or more. One-way ANOVA analysis demonstrated that dietary group had a significant effect on "B12 Knowledge" ($F_{3, 136} = 8.484$, $P<0.01$). Post hoc comparisons using the Tukey HSD test indicated that the mean score for dietary group "Vegan" (3.06) was significantly higher than all other dietary groups (Vg 1.9, LM 2, HM 1.48), which did not differ significantly from each other.

The variables Age, Gender, Nationality and Occupation were not found to have a significant effect on the total "B12 Knowledge", however binary logistic regression indicates that "Diet", "Gender" and "Nationality" were all significant predictors of whether a respondent correctly identified that B12 is a necessary supplement for people adhering to a strict vegetarian diet (Chi-square = 29.997, df=8, $p<0.01$). The odds ratios were reported as "Vegan" 28.367 (95% CI 2.871-280.317, $P<0.001$) and "Swedish" 7.561 (95% CI 2.367-24.157, $p<0.001$) indicating that, relative to high meat eaters and non-Swedish nationals, people who adhere to a vegan diet, and people with Swedish nationality were 28 and 7.5 times more likely to correctly identify vitamin B12 as a necessary supplement, respectively. Conversely, the odds ratio for "Male" 0.271 (0.090-0.822, $p<0.05$), indicates males are less likely than females to correctly identify this. No difference was observed between female and non-binary individuals. The model had an overall correct prediction rate of 84.7%.

3.1.5 Consumption of B12 supplements and fortified products

Binary logistic regression indicates that "Diet" is a significant predictor of the consumption of B12 supplements (Chi-sq=23.883, df=8, $p<0.01$), and B12 fortified drinks (Chi-sq=26.661, df=8, $p<0.01$). In both models the predictors Age, Gender, Occupation and Nationality were not significant. For B12 supplementation, the odds ratio for "Vegan" diet is 7.900 (95% CI 2.205 – 28.310, $p<0.01$), and for B12 fortified drinks, the odds ratio for "Vegan" diet is 9.497 (95% CI 2.523 – 35.742, $P<0.01$). These results indicate that respondents who eat a vegan diet are approximately 8 and 9 times more likely than those in the "High Meat" diet group to have consumed a B12 supplement, or drinks that have been fortified with B12 within the past two weeks, respectively. In both models, "Vegetarian" and "Low Meat" diet groups were not significantly different than "High meat". When the supplementation dependent variable was expanded to include multivitamin consumption (i.e. the respondent had taken a B12 supplement, and/or a multivitamin which is assumed to contain vitamin B12), the odds ratio increased to 55.570 (6.076-508.191, $p<0.01$), however this result has an extremely wide confidence interval, likely due to the relatively small quantity of data. None of the factors tested were found to be significant predictors of the consumption of B12 fortified foods.

Overall, 55% of respondents had taken a B12 supplement and/or multivitamin within the past two weeks. Consumption was highest among vegans (96.9%), followed by vegetarians (63.3%), and approximately even among low and high meat diet groups at 34.5% and 34.8%, respectively (figure 2A). The consumption of B12 fortified drinks and food was lower; 39% of all respondents reported consuming B12 fortified drinks, and only 24% reported consuming B12 fortified food, within the past two weeks. Consumption of B12 fortified drinks was also highest among vegans (75%), followed by vegetarians (40%). In both meat-eating groups reported consumption was relatively low at approximately 20-25% (figure 2B). There was a high degree of uncertainty about the consumption of B12 fortified foods, as between 25-50% of respondents in each group answered 'don't know' when asked if they had consumed B12 fortified products within the past two weeks. Interestingly, the highest reported consumption (40%) was in the High Meat group (figure 2C). Within both vegan and vegetarian groups only 25% of respondents reported consuming B12 fortified foods.

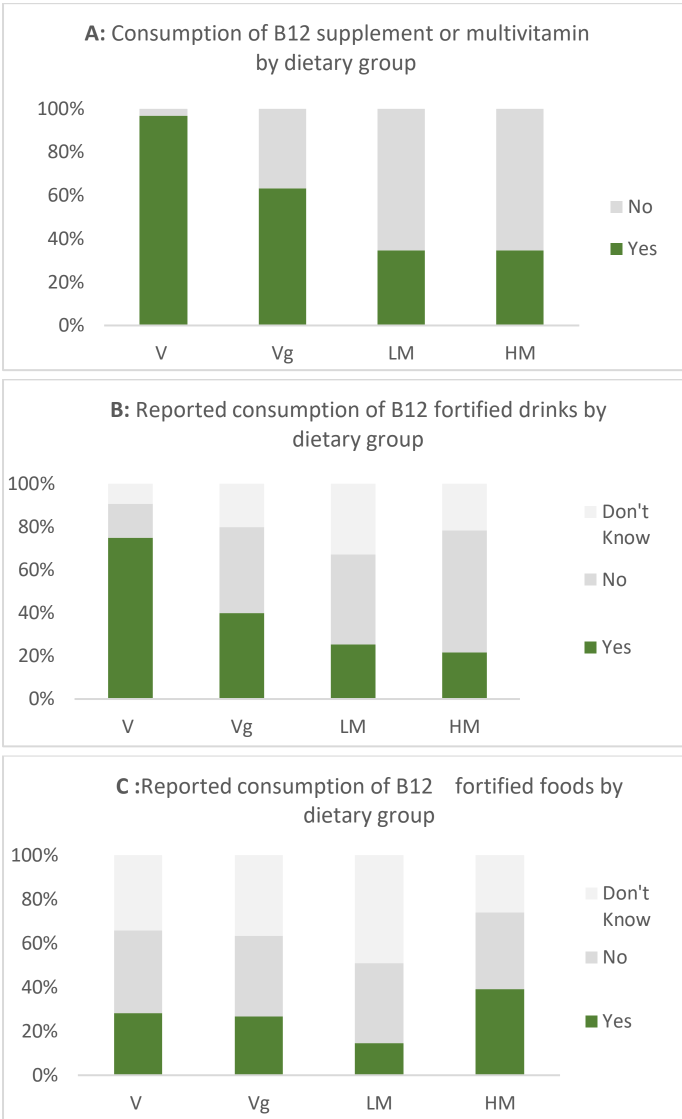

Figure 2. Percentage of respondents who consumed a B12 vitamin supplement, B12 fortified drinks, or B12 fortified foods within the past two weeks, divided by dietary group.
A –B12 supplement or multivitamin;
B – B12 fortified drinks; C – B12 fortified foods.

3.1.5 Attitudes to vitamin supplements and food fortification

Following principle component analysis of likert items, four likert scales were created; each scale combined a minimum of three likert items and had high internal consistency ($\alpha>0.7$). A fifth combination of likert items was identified by principle component analysis, however the internal consistency was low ($\alpha<0.7$), so the items were not combined for analysis.

Kruskal-Wallis analysis indicated that diet has a significant effect on the variable 'B12 knowledge perception", which measured respondents self-reported knowledge level about vitamin B12 ($H_3=15.365$, $p=0.002$). Pairwise comparisons showed that respondents in the dietary group 'Vegan' were significantly more likely to report that they are knowledgeable about B12 than all other diet groups, which did not vary significantly from each other. This is consistent with the results of the "B12 knowledge" test which showed vegans scored higher than all other dietary groups. The average score for each group was <3, indicating that respondents from all groups felt knowledgeable about B12.

Diet also had a significant effect on the variable "B12 fortification scepticism", which measured agreement with negative statements about B12 fortification of foods (e.g. "Food should not be fortified with vitamin B12"). In this instance, the vegetarian group responded, on average, more negatively to these statements than all other diet groups ($f_{3,139}=5.811$, $p=0.001$). The mean score for Vegetarian was 5.76, compared to 4.59, 4.96 and 4.66 for High Meat, Low Meat and Vegan, respectively. All of the scores are greater than 4, indicating that, on average, respondents disagree with negative statements about B12 fortification.

Diet did not have a significant effect on the remaining two likert scales, "Attitudes to B12 fortification" and "Dietary supplement value" which measured agreement with positive statements about B12 fortification of foods and the importance of dietary supplements to the respondents, respectively. Occupation had a significant effect on "Dietary supplement value" (U=2870, p,0.01). The mean score for non-students was 3.2 indicating they valued dietary supplements positively, whereas for students it was 4.6, a slightly negative response. None of the other variables tested had a significant effect on "Attitudes to B12 fortification".

Within questions relating to the B12 fortification of foods there was a relatively high regress of uncertainty, with 20-40% of respondents answering "don't know" to each of the individual items (table 2). When these responses were removed, the adjusted average scores for likert items indicated a moderately positive response to B12 food fortification. Less than 20% of respondents agreed that food should not be fortified with B12, and only 10% indicated they would avoid buying food with added B12. Approximately 25% agreed with the statement that adding B12 to foods is unnatural, however no additional questions were asked to ascertain whether there was a positive or negative association with the concept of "natural".

More than 80% of respondents agreed that fortifying foods with B12 has health benefits for them, and although only 30% indicated that they would by foods specifically for B12, almost 70% would prefer to eat B12 fortified foods if given a choice. Overall, only 17% of people reported that they depend on fortified foods to obtain their B12, however this number increased to 31% within the vegan diet group.

Table 2. Responses to likert items relating to B12 fortification of food.

[a] Percentage of agree vs. disagree adjusted to exclude neutral answers. [b] Overall neutral responses.

[c] Adjusted mean excluding neutral answers. 1-3: agree, 5-7: disagree

Item	Adjusted responses[a] (%) 1-3	Adjusted responses[a] (%) 5-7	Neutral responses[b] %	Adjusted mean[c]
Food should not be fortified with vitamin B12	17.2	82.8	33.6	5.3
It is unnatural to add vitamin B12 to foods	24.5	75.5	24.3	5.1
I would avoid buying foods with added B12	10.3	89.7	31.3	5.6
Where there is a choice, I would prefer to eat foods that are fortified with B12	67.0	33.0	28.6	3.7
I rely on fortified foods to meet my vitamin B12 requirements	17.0	83.0	20.0	5.6
Fortifying foods with B12 has health benefits	81.0	19.0	40.0	3.0
I would buy foods specifically for the added vitamin B12	30.6	69.4	22.9	4.9
I would prefer to take B12 supplements that have it added to foods	45.1	54.9	27.1	4.3

3.2 Literature Review

3.2.1 Vitamin B12 content of fermented foods

Assessment of the vitamin B12 content of traditional fermented food products is limited and for this review only four original research papers, published in English, were identified. Table 3 provides an overview of the products tested and the B12 content reported.

Three out of four papers focused on the B12 content of fermented soya bean products from across East and South East Asia. The earliest paper was published in 1977 and examined the vitamin B12 content of two traditional fermented Indonesian foods: tempeh, made from soya beans, and ontjom, made with by-products such as peanut press cake (from peanut oil) and soya pulp (from tofu) (Liem et al., 1977). Tempeh and ontjom are predominately fermented by *Rhizopus* and *Neurospora* mould, not bacteria, however microbiological assay analysis of purchased samples revealed vitamin B12 levels up to 6.3 µg/100g in tempeh, 3.1 µg/100g in peanut ontjom and 2.3 µg/100g in soya ontjom. Since mould is unable to synthesis B12, Liem et al. (1977) hypothesised that B12 content was produced by contaminant bacteria during the fermentation process. They proceeded to isolate a bacterium (species unknown) from tempeh with an elevated B12 content and demonstrated, via inoculation experiments, that B12 was present only in tempeh that had been contaminated with the bacteria during fermentation. Tempeh produced from with a pure *Rhizopus sp.* culture did not contain vitamin B12.

Traditional Korean fermented foods include three different soya bean products, doenjang, chungkookjang and ganjang (soy sauce), as well as kimchi (cabbage) and gochujang (red chili paste) (Kwak *et al.*, 2008; Kwak, *et al.*, 2010). Low levels of B12 were detected in all products, but significant variation was found between different samples of the same products (table 3). Homemade products had consistently higher B12 content than commercially produced products, with the highest concentration being found in homemade doenjang (9.82 µg/100g). In kimchi, a product that has become popular in Europe, B12 concentration ranged from 0.18 - 0.24 µg/100g. This study used microbiological assay methods to assess the B12 content, and the specific cobalamin analogues were not reported. No analysis was performed to identify the species responsible for fermentation of the tested products.

The most recent study, published in 2017, reported the B12 content of a broad range of soya bean products, determined by high-performance liquid chromatography (HPLC) analysis (Xu, *et al*. 2017). All products contained detectable levels of B12, although these levels varied significantly ($P<0.05$) among all the products sampled. Variation was observed between different products, but also between the same products sourced from different producers (table 3).

The highest concentration was 73.2µg/100g, found in Liubiju, a dry yellow soybean paste. Other products with notable B12 content included doenjang (53.1µg/100g) black bean douche (52.5µg/100g), and tempeh (50.9µg/100g). Although HPLC was used to determine the B12 concentration, the cobalamin analogue was not specified in the results. As in the previous study, no analysis was performed to identify the species responsible for fermentation of the tested products.

The fourth paper examined the nutritional and functional composition of a range of traditionally prepared fermented bamboo shoot (FBS) products, collected from the Indian states of Arunachal Pradesh and Manipur (Sonar *et al.*, 2015). Following HPLC analysis, the B12 content of FBS products was found to be significantly higher than that of unfermented bamboo shoots. The vitamin B12 content was reported to range from 6.7 – 75.6 mg/100g, however it is likely this has been mis-reported as these values are three orders of magnitude (mg instead of µg) higher than all other reported results. The cobalamin analogue was reported to be the bioactive form cyanocobalamin. No analysis was performed to identify the bacteria spp. present in the ferments, but the authors suggested the rise in B12 content was likely a result of LAB activity during fermentation.

Table 3. Summary of reported vitamin B12 content in traditional fermented plant-based food products. ~ Unknown, [a]mg/100g [b] µg/100ml

Product	Production method	Product origin	Vitamin B12 content µg/100g	B12 analysis methods	Reference
Yellow Soybean Paste	Commercial	China, Hongkong, Korea	29.4 - 73.3	HPLC	(Xu, Cai and Xu, 2017)
Sufu	Commercial	China	28.7 - 36.8		
Miso	Commercial	China	35.5 - 48.4		
Natto	Commercial	China, Japan	27.9 - 35.1		
Black bean sauce	Commercial	China	24.6 - 30.7		
Black bean douchi	Commercial	China	26.5 - 52.5		
Yellow bean douchi	Commercial	China	22.9 - 27.6		
Stinky tofu	Commercial	China	29.9		
Moldy tofu	Home-made	China	42.2		
Tempeh	Home-made	Indonesia	50.9		
Various fermented bamboo shoot products	~	North East India	6.7 - 75.6[a]	HPLC	(Sonar *et al.*, 2015)
Doenjang	Commercial	Korea	0.07 - 0.49	Microbiological assay	(Kwak *et al.*, 2008; Kwak *et al.*, 2010)
(yellow soybean paste)	Home-made	Korea	0.3 - 9.82		
Chungkookjang	Commercial	Korea	0.08 - 0.31		
(soybeans)	Home-made	Korea	0.05 - 1.4		
Gochujang	Commercial	Korea	0 - 0.14		
(red chili paste)	Home-made	Korea	0.02 - 0.43		
Kimchi (Cabbage)	~	Korea	0.18 - 0.24		
Ganjang (Soy sauce)	Home-made	Korea	0.02 - 6.76[b]		
Tempeh (soybean)	Commercial	Canada, Indonesia, USA	0.4 - 6.3	Microbiological assay	(Liem *et al.*, 1977)
Peanut ontjom	~	Indonesia	3.1		
Soybean ontjom	~	Indonesia	2.3		

3.2.2 Vitamin B12 production during fermentation by *Propionibacterium freudenreichii*

P. freudenreichii is a vitamin B12 producer with GRAS status, which is commonly used in the food industry. It is known to predominately produce a bioactive analogue of B12, and minimal pseudo-B12 forms (Deptula *et al.*, 2015).

Tempeh

As described above, in 1977 it was reported that tempeh contaminated with an unknown bacteria contained elevated levels of vitamin B12 (Liem et al., 1977), and subsequent analysis identified *Klebsiella pneumoniae* and *Citrobacter freundii* as two species capable of producing vitamin B12 during co-fermentation of tempeh (Keuth and Bisping, 1993, 1994; Wiesel, Rehm and Bisping, 1997). *P. freudenreichii* was reported not to produce vitamin B12 during tempeh fermentation and it was suggested that soya beans might be an unsuitable growth medium for this species (Keuth and Bisping, 1993). Two more recent studies have been performed to investigate the potential for co-fermentation of lupin-based tempeh by *Rhizopus spp.* and *P. freudenreichii* (Signorini *et al.*, 2018; Wolkers *et al*., 2018). Lupin is a native European grain legume species commonly grown for fodder, but which has recently been investigated as a sustainable source of plant-based protein for humans. In both studies, the co-fermentation of lupin by Rhizopus spores and *P. freudenreichii* significantly increased vitamin B12 content.

Signorini et al. (2018) co-fermented three different species of lupin, *Lupinus albus, L. angustifolius* and *L. mutabilis with Rhizopus oligosporus* and *P. freudenreichii.* Two different fermentation processes were tested: sequential, whereby lupin seeds were fermented with propionibacteria for 48 hours prior to inoculation with Rhizopus spores, and simultaneous, whereby lupin seeds were inoculated with a mix of Rhizopus spores and propionibacteria. Both methods produced significantly higher levels of vitamin B12 than fermentation with pure cultures of either *R. oligosporus or P. freudenreichii.* Sequential fermentation produced a dry weight concentration of approximately 68.4 µg/100g B12, whereas simultaneous fermentation produced up to 123.0 µg/100g (dw), a 44% increase from the sequential method.

Wolkers-Rooijackers, Endika and Smid (2018) reported similar results. Following co-fermentation of lupin seeds with *R. oryzae* and *P. freudenreichii* a significant increase of B12 was observed, up to 0.97 µg/100 g fresh weight. In both cases, 100g of tempeh would provide approximately 50% of the daily B12 RDI.

Cereals

Four papers were identified that explored the production of B12 during fermentation of cereal products by *P. freudenreichii.* This method is proposed as an alternative to chemical fortification.

During fermentation of rye malt extract and barley malt extract *P. freudenreichii* is reported to produce 1.93 µg/100g and 1.57 µg/100g respectively (Chamlagain *et al.*, 2015). A subsequent study explored the effect of adding B12 precursors, (cobalt, 5,6-dimethylbenzimidazole (DMBI), riboflavin and nicotinamide) to various cereal matrices. The presence of B12 precursors had a notable impact on the B12 content of the fermented products. The highest concentrations were observed in a malted barley flour matrix (BM); fermented BM contained 1.2 - 3.7 µg/100g, whereas BM with added cobalt, riboflavin and nicotinamide contained 71.2 µg/100g. The highest concentration, 148 µg/100g, was observed in BM with added cobalt and DMBI, however it was noted that DMBI is not permitted to be used in food production (Chamlagain *et al.*, 2018).

Xie *et al.* (2018) fermented non-sterile durum flour, whole-wheat flour and wheat bran for seven days with *P. freudenreichii* and reported average B12 levels of 3.3, 8.7 and 15.5 µg/100g (dw) respectively. However, they also observed that pathogenic endogenous microbiota could potentially grow in these conditions, leading to safety concerns and limit the use of these fermented products in food applications. A follow up study examined the effect of co-fermenting wheat bran with *P. freudenreichii* and *L. brevis* ATCC 14869, with controlled and uncontrolled pH levels, as a possible method to improve the microbiological safety (Xie *et al.*, 2019). In this experiment, fermenting wheat bran with *P. freudenreichii* and a controlled pH of 5.0, produced the highest concentration of B12: an average of 3.57 µg/100g (dw) after day one, which remained stable until day three. Samples that were co-fermented with *P. freudenreichii* and *L. brevis,* with a controlled pH of 5.0, contained an average of 3.32 µg/100g (dw) B12 after three days. Co-fermented samples with an uncontrolled pH contained a significantly lower average of 1.83 µg/100g (dw) B12 after day three, however they exhibited the strongest inhibition of pathogenic microbes.

3.2.3 Vitamin B12 production during LAB fermentation

Lactobacillus reuteri

L. reuteri was the first *Lactobacillus sp*. identified as a vitamin B12 producer (Taranto *et al.*, 2003). It was demonstrated that *L. reuteri* CRL1098, isolated from sourdough, was able to co-ferment glycerol, a B12 dependent process, in B12-free growth medium. Furthermore, cell extract from *L. reuteri* facilitated the growth of three B12 dependent microbial species in B12-free growth medium; *L. delbrueckii* subsp. *lactis* ATCC 7830, *Salmonella enterica* serovar Typhimurium (*metE cbiB*) and *Escherichia coli* (*metE*). The vitamin B12 was produced intracellularly and later examination of the chemical structure demonstrated that the main analogue produced was a form of pseudo-vitamin B12 which is not proven to be bioavailable to humans (Santos *et al.*, 2007). However, it has also been shown that modifying the growth medium can alter both the quantity produced, and the analogue. The omission of single amino acids from the growth medium, including alanine, lysine and cysteine, was demonstrated to enhance the B12 production by *L. reuteri;* in particular, the omission of cysteine combined with the addition of glycerol produced a 17-fold increase in the production of vitamin B12, although the specific analogues were not reported (Santos *et al.*, 2009). Under optimized conditions, where the compounds δ-aminolevulinic acid (ALA) and 5,6-dimethylbenzimidazole (DMB), which are required for vitamin B12 production, were added to the growth medium, *L. reuteri* has been shown to produce biologically active vitamin B12 (Mohammed *et al.*, 2014).

Two reports have been published from experiments designed to examine the effect of fermenting plant-based food products with *L. reuteri.* Bao *et al.* (2019) inoculated furu, a traditional Chinese fermented tofu product (also known as sufu) with *L. reuteri* during large scale fermentation. They reported that vitamin B12 content was increased up to 14.17 µg per 100g (ww) and was predominantly present as the bioavailable forms AdCbl and Cbl; at this level, approximately 17g of furu would be sufficient to obtain the recommended daily intake of 2.4 µg of vitamin B12. The authors reported this concentration was significantly higher than in their control group (3.6 µg/100g), and higher than concentrations previously reported by Li et al. (2004), who observed that B12 concentrations in furu were highly variable, ranging from 0.42-0.78 µg per 100g dw (study unavailable in English).

The co-fermentation of glycerol and fructose in soy-yoghurt by *L. reuteri* produced up to 18 µg/100ml of vitamin B12 (Gu *et al.*, 2015a). At these levels, consuming 14 ml of soy-yoghurt would be sufficient to obtain the recommended daily intake, however it must be noted that the authors did not include evidence to support their conclusion that the B12 produced was bioavailable, and the paper has been criticized for the misleading use of the term "vitamin analogues" to distinguish pseudo vitamin B12 (Varmanen *et al.*, 2016).

Lactobacillus plantarum

A variety of screening processes have been used to identify B12 producing strains of *L. plantarum* in vitro and various research has explored their potential to fortify plant-based food products during fermentation.

Intracellular production of vitamin B12 by *L. plantarum* was first reported by Madhu *et al.* (2010). In this study the production of B12 was optimized by controlling the fermentation conditions, and by the addition of zinc chloride ($ZnCl_2$) to the growth medium. An unspecified strain of *L. plantarum* was isolated from Kanjika, a traditional Indian sour liquid made from powdered rice, and identified by 16S rRNA sequencing. Bacteria were inoculated into a modified B12-production medium, and fermented for 48 hours under anaerobic conditions, followed by 48 hours under aerobic conditions. The highest B12 concentration, 1.31µg/100g dw, was reported when the growth medium contained 0.69% Zn Cl_2. The B12 analogue was identified as Cbl under HPLC analysis, and bioassay experiments performed with *E. coli* ATCC 11105 confirmed its bioavailability.

L. plantarum BHM10, isolated from human milk samples, and BCF20, isolated from human faecal samples, were identified as vitamin B12 producers by genetic screening for cbiK, a characteristic gene of anaerobic vitamin B12 synthesis. Following HPLC analysis the average intracellular level of Cblm in BHM10 and BCF20 was reported as 1.09µg/100ml and 2.39µg/100ml, respectively (Bhushan *et al.,* 2016). Further analysis of the species' techno-functional characteristics found that BHM10 produced approximately 0.4µg/100ml extracellular Cblm during the fermentation of soymilk (Bhushan *et al*., 2017). At this concentration 600ml of soymilk would be sufficient to obtain the recommended daily intake of 2.4 µg.

Extracellular production by *L. plantarum* CN-225, DW12, L295 and CY2 has been reported (Masuda *et al.*, 2012; Kantachote *et al.*, 2017; Li *et al.*, 2017). Following the isolation of 233 LAB from Japanese pickles, *L. plantarum* CN-225 was reported to produce approximately 0.2µg/100ml of extracellular vitamin B12. This study also reported *L. coryniformis* CN-229 as an extracellular B12 producer. The analogue produced was not reported for either species (Masuda *et al.*, 2012) *L. plantarum* DW12 was reported to produce 15.93 µg/ml intracellular and 14.11 µg/ml extracellular vitamin B12 during 48 hour fermentation of mature coconut water (Kantachote *et al.*, 2017). Following the same methods, *L. casei* L4 has also been reported to produce up to 11.47 µg/mL B12 after 48 hour fermentation of coconut water (Giri *et al.*, 2018). *L. plantarum* L295 and CY2 were identified as high extracellular vitamin B12 producers following solid phase extraction and HPLC analysis (Li *et al.*, 2017). L295 was reported to produce 95 ± 15 µg/l of AdCbl and MeCbl, and CY2 60 ± 9µg/l of AdCbl. The same study reports high intracellular production by 8 additional *L. plantarum* strains.

One additional report identified five strains of different *Lactobacillus spp*. as potential vitamin B12 producers following seven rounds of successful growth on B12-free medium; *L. plantarum* PB5067, *L. reuteri* PBS072, *L. fermentum* PBS073, *L. rhamnosus* PBS070 and *Bifidobacterium animalis* subsp. *lactis* PBS075 (Presti *et al.*, 2015). However, no further analysis was performed to confirm the analogue produced, or the quantity, and it has been observed that growth in B12-deprived conditions is not sufficient to confirm B12 production (Bhushan, Tomar and Mandal, 2016).

3.3 LAB Fermentation of Cauliflower and White Beans

3.3.1 Experiment 1

No biologically meaningful difference was observed in the outcome of fermentation between raw, cooked or mixed cauliflower samples. Within 24 hours the pH of every sample had decreased to the range of 3.65 - 3.88, and after 72 hours it had dropped to 3.37 - 3.60 (figure 3). The cooked samples had the lowest average pH (3.38), followed by the mixed sample

(3.47) and finally the raw sample (3.60). As this experiment is part of a larger product development project, consideration was given to the over-all number of steps required for each process. In order to minimize the time and energy required, it was decided that raw cauliflower would be used for experiment 2.

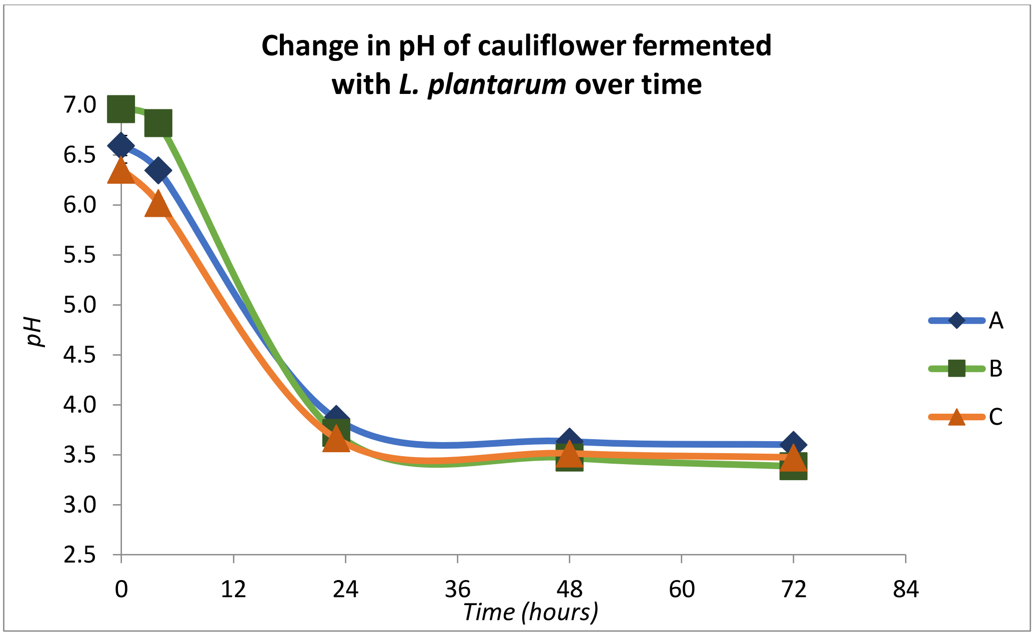

Figure 3. Average (± 1 SD) pH of fermented cauliflower recorded over 72 hours. A: raw cauliflower, B: cooked cauliflower, C: 50:50 cooked/raw mixture.

3.3.2 Experiment 2

Fermentation rate

A significant difference was observed between the fermentation rate of the white beans compared to the cauliflower and cauliflower/white bean mixtures (figure 4). All samples of cauliflower, and cauliflower mixed with beans reached a pH ≤3.91 within the first 18 hours, and ≤3.58 within 44 hours. The samples containing only white beans initially experienced a drop in pH, to the range of 4.75 - 4.9 within 18 hours, however after 44 hours the pH did not decrease any further and for many samples an increase was observed. The differences observed between the average pH for each substrate group were statistically significant (at 44 hours, F_2=2866.0, $p<0.01$).

For each of the three mediums there was no biologically meaningful difference between the fermentation achieved by the different strains of bacteria. Similarly, no significant difference in taste and/or texture could be identified among the cauliflower samples, or the mixed cauliflower/bean samples. Due to low change in pH observed in the pure white beans, these ferments were considered unsuccessful and were excluded from the taste analysis.

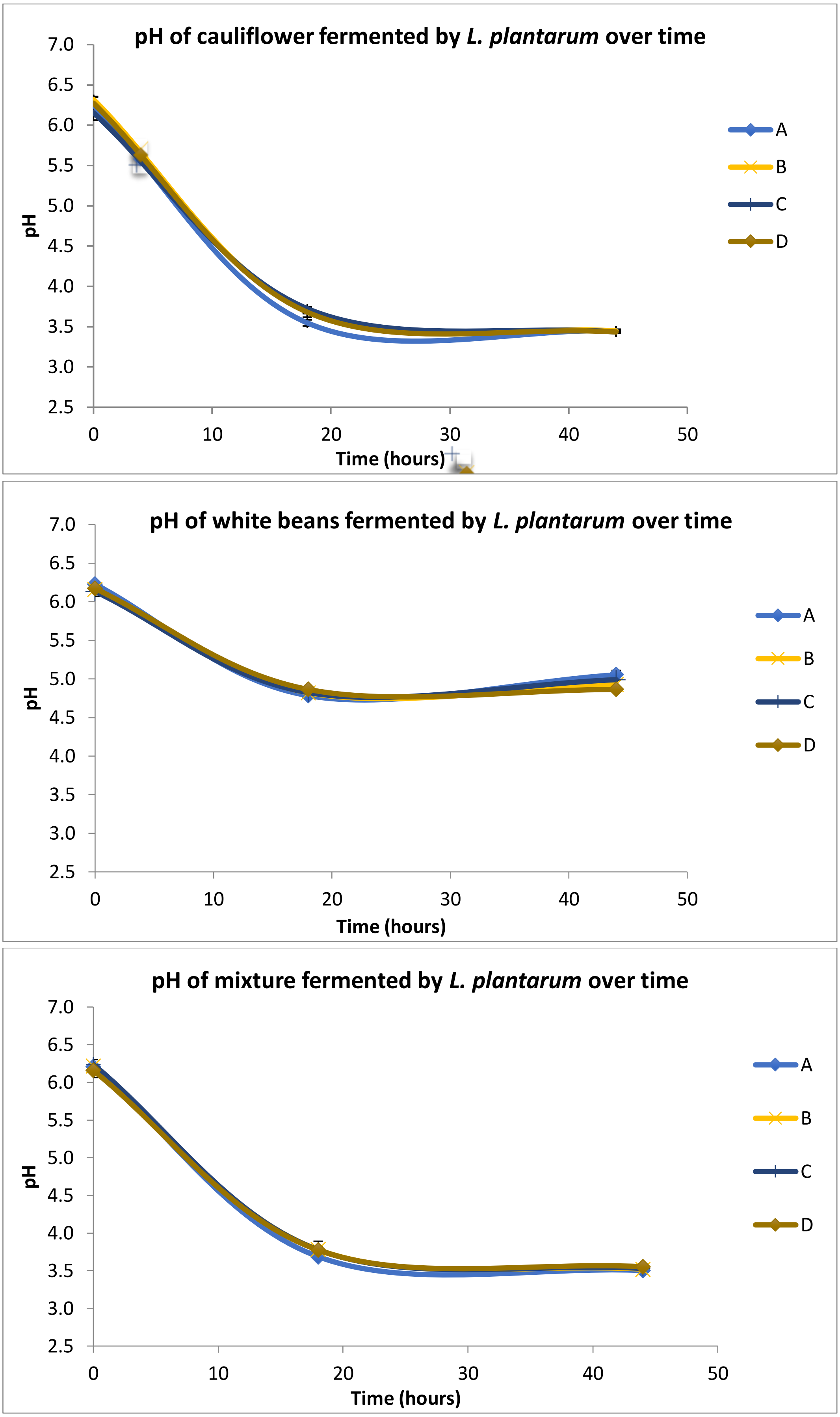

Figure 4. Change in pH of fermented cauliflower, white beans and cauliflower/white bean mixture, recorded over 44 hours (mean ±1SD). A: 299v, B: Lp900, C: 299, D: Heal19

The most interesting difference observed during the taste analysis was that between the pure cauliflower and the mixture of cauliflower and white beans. As expected, the fermented cauliflower had a fresh, clean acidity with a slight cabbage taste, not unlike mild sauerkraut. The cauliflower and bean mixture also had a fresh acidity, but with a dryer mouth feel and a deeper, umami flavour, giving an overall richer experience.

3.3.3 Vitamin B12 analysis

A small but statistically significant difference in the concentration of B12 was observed following the fermentation of 50:50 white beans and cauliflower mixture with *L. plantarum* 299 (table 4). The average concentration increased by 66% relative to the control, up to 0.048µg/100 g (ww). No statistically significant differences were observed between the other 3 treatment groups and the unfermented control. Analysis was not performed to identify the B12 analogue(s), so the bioavailability is unknown.

Table 4. Vitamin B12 content (µg/100 g fresh weight) of white bean and cauliflower samples.

Treatment	**B12 content** *Mean ± 1SD*
Control	0.029 ± 0.002a*
299v	0.033 ± 0.004ab
Lp900	0.034 ± 0.011ab
299	0.048 ± 0.013b
Heal19	0.034 ± 0.004ab

*Different letters indicated significant differences (Anova, $p<0.05$).

4. Discussion

4.1 B12 knowledge, behaviour and attitudes

The present research indicates that the surveyed population (predominately Swedish female students) is aware of and has basic knowledge relating to B12. The majority of respondents (80%) were able to identify B12 as a necessary supplement for people adhering to a strict vegetarian diet (though only 25% could identify the RDI) and approximately two thirds (63%) could identify at least one symptom of B12 deficiency. The survey was conducted online and unsupervised, so it is possible the respondents opted to look up answers to questions assessing B12 knowledge, influencing the results. However, given the average time to complete the survey was under 9 minutes, less than the anticipated 10-15 minutes, and that 75% of respondents did not identify the correct RDI for B12, it is considered unlikely that this occurred to a significant degree. Vegan diet was the only factor found to have a significant effect on “B12 knowledge”; respondents in this diet group had a significantly higher average score than all other groups, which did not differ significantly, indicating they are more knowledgeable. This result is encouraging as it indicates the most vulnerable subgroup of the surveyed population is aware of their B12 needs, and the potential consequences of becoming deficient. Women were more likely than men, but not non-binary people, to correctly identify B12 as a necessary supplement; an interesting result given that preventing micronutrient deficiency is particularly important for women of child-bearing age, and the average age within the survey population was approximately 30 years old.

The results also indicate that the consumption of dietary supplements containing B12 and B12 fortified drinks is widespread within the vegan, and to a lesser extent vegetarian, subgroup of the surveyed population, a positive indicator that people within these groups are taking steps to reduce their risk of B12 deficiency. Consumption of supplements containing B12 was reported by 97% of vegans and 63% of vegetarians, compared to 34% of meat eaters. Consumption of B12 fortified drinks was lower in both groups, at 75% and 40%, respectively, though still notably higher than among meat eaters (21%). Consumption of B12 fortified food was low among all respondents; only one vegan respondent reported consuming no B12 supplements or fortified drinks, indicating that they depend on B12 fortified foods to meet their RDI. The research methods employed depend on self-reported consumption within the past two weeks. This method has been used to assess dietary supplement consumption elsewhere in the literature (Molster *et al.*, 2009) however, it must be recognised that these results function as a proxy measure, and do not provide evidence that respondents are consuming sufficient quantities to meet their nutrient needs. More in depth analysis of behaviour patterns using methodology such as the theory of planned behaviour (Conner *et al.*, 2003) coupled with clinical analysis of blood serum levels would provide more robust evidence that the population is protected against B12 deficiency.

Limited conclusions can be drawn from the present results about attitudes towards dietary supplementation and the consumption of B12 fortified foods. No demographic factors were found to influence attitudes, and there was a high degree of uncertainty for many of the questions asked, with up to 40% of respondents answering “don’t know” in some cases. From the data presented there is some indication that, among those who answered positively or negatively, B12 fortified foods are viewed positively. A clear majority disagreed with statements indicating that foods should not be fortified with B12 and agreed that fortifying foods with B12 has health benefits, but it should also be observed that the majority also disagreed that they would buy foods specifically for B12. This indicates that although consumers aren’t likely to reject foods containing B12, they currently do not look to fortified foods as a regular source. Previous research has identified that Swedes are significant consumers of “functional foods”, products that are marketed as having enhanced nutritive value and health benefits, such as probiotic products, juice containing added vitamins or minerals, and bread with added omega 3 fatty acids (Özen *et al.*, 2014). It is reported that the typical Swedish consumer of functional foods is health-conscious, highly educated, health motivated and accepting of health claims associated with functional foods (Landström *et al.*, 2007). It is therefore considered that a B12 enriched fermented product could likely be marketed to the Swedish population with some success, however, as is seen with current dietary supplement marketing and consumption patterns, the potential for such a product to protect against B12 deficiency in the wider population may be limited.

4.2 Dietary motivations

The most frequently reported motives were environmental, which were stated by 80% of all respondents. Furthermore, nationality was a significant predictor of environmental motives with Swedish respondents citing them more often than non-Swedish nationals. This gives a positive indication that there is a widespread awareness that dietary choices can impact environmental issues, and that campaigns designed to influence dietary choices in order to improve environmental outcomes could be well received by the Swedish population. Demonstrating low environmental impact of products may also be beneficial to their marketing. Unsurprisingly, dietary group was also identified as a significant predictor of motivation, with vegans and vegetarians being significantly more like to state ethics related motives than meat eaters. Conversely, they were less likely to report “other” motives than meat eaters; most notably local sourcing of food and economic reasons were reported by 12.7-16.4% of low meat eaters, and 4.3-26.1% of high meat eaters, respectively, but were not stated by any vegans or vegetarians. Health was the other most commonly reported motivation, cited by 63% of all respondents. It did not differ significantly between dietary groups or demographic factors and was not associated with differences in attitudes towards B12 supplementation or product fortification.

The main limitations to this survey are the small sample size, and the uneven demographic representation. Binary regression analysis highlighted some significant differences between different factors, however in most cases the confidence interval was very wide, likely reflecting the small quantity of data included in the analysis. The formulation of questions used to assess attitudes could also be refined to reduce the number of neutral (don't know) answers.

4.3 B12 production through fermentation of plant-based foods

Research into the B12 content of traditional fermented plant-based products is limited to a small number of studies. There is some evidence that B12 may be present at nutritionally meaningful concentrations in some products, although the quantity of B12 is highly variable, even within different variations of the same product (Xu *et al.,* 2017). The microorganisms responsible for B12 production were not experimentally determined in any of the studies that reported B12 content of traditional fermented products but, where it was discussed, LAB spp, as well as Rhizopus, Mucoraceae and Aspergillus fungi were recognized as the microorganisms responsible for fermentation (Liem *et al.* 1977; Xu *et al.* 2017). Fungal species do not produce B12 (Martens *et al.*, 2002) and therefore the B12 content of fungal fermented products must arise via contamination, as was reported by Liem *et al.* (1977), or during lactic acid fermentation which is sometimes employed as a final processing step. The high levels of B12 reported in some home-made products relative to their commercial counterparts may reflect diversity among the bacteria present, contamination with other bacteria spp, or differences in the fermentation process compared to commercial processes. For example, Kwak *et al.* (2010) recognized that the traditional method of producing home-made Doenjang takes approximately 10 months, whereas commercial Doenjang is prepared in as little as 3-4 months under tightly regulated conditions whereby microorganism diversity is controlled by inoculation. The commercialization of fermentation processes typically relies on known starter cultures to reduce the risk of contamination and ensure food safety; it is interesting to considered that in some cases this may have a deleterious effect on the final product quality in terms of reduced nutrient content, potentially impacting the health outcomes of populations that have previously depended on traditional fermented products to meet their micronutrient needs. The present research only considers B12, however it is reasonable to suspect other vitamins could be affected in a similar way. Careful isolation and selection of vitamin producing bacteria that can be safely utilized for fermentation or co-fermentation with fungal spp. could be beneficial to ensure the nutritive quality of products remains consistent under commercial production methods.

These results are corroborated by data from studies that have demonstrated the production of B12 during fermentation of plant-based media under experimental conditions. To date, multiple strains of four different Lactobacillus spp. (*L. plantarum, L. reuteri, L. coryniformis* and *L casei)* have been reported to produce B12 during fermentation. *P. freudenreichii* has also been successfully used to produce B12 during fermentation of plant-based foods, and during co-fermentation with *Rhizopus spp and L. brevis.* A further three strains of LAB (two Lactobacillus and one Bifidobacterium) have been identified as possible producers following growth on B12 free media, however this method alone is not regarded as sufficient to prove B12 production and further analysis would be needed to confirm their production potential (Bhushan *et al,,* 2016). A variety of experimental and analytical methods have been employed to examine B12 production during fermentation, and although nutritionally significant quantities are reported in several cases (Bhushan et al., 2017; Kantachote *et al.*, 2017), evidence that it is present as a bioavailable analogue is often lacking. Furthermore, several studies report intracellular concentrations which would require cell lysis to occur, either during processing at an early stage of digestion, for the B12 content to be nutritionally meaningful. None-the-less, extracellular bioavailable analogues are also reported (Masuda *et al.*, 2012; Kantachote *et al.*, 2017; Li *et al.*, 2017) and it is reasonable to conclude that lactic acid fermentation, under controlled conditions, could be developed as a tool to produce plant-based products that are naturally rich in B12.

In the present work, a small but statistically significant increase was observed in the concentration of B12 within samples of mixed white-beans and cauliflower fermented with *L. plantarum* 299, compared to unfermented control samples. No method was employed to induce cell lysis and it is considered that the increased concentration reflects extracellular B12 production. However, the average concentration observed (0.048µg/100g (ww)) is low relative to the RDI of 2.4µg. Furthermore, the results were obtained by microbiological assay and there is no data to demonstrate which B12 analogue was present. The fermented white-bean-cauliflower matrix therefore cannot be considered a significant source of B12 for humans. The four *L. plantarum* strains examined had not previously been reported to produce B12. The experimental design could be improved by the inclusion of LAB strains, such as *L. plantarum* L295 and CY2, that have already been identified as extracellular B12 producers or by integrating a screening processes to identify likely B12 producers, such as microbiological assay or genetic analysis, into the experimental design. Assessing the B12 content of sauerkraut samples could also be employed as a strategy to identify suitable LAB bacteria strains for production during the fermentation of vegetables.

4.4 Developing a novel fermented food

Lactic acid fermentation of vegetables is traditional to many countries around the world; in northern European cabbage is the most commonly utilized crop, used for the production of sauerkraut. Cauliflower is another member of the family *Brassicaceae* that is consumed throughout Europe. It is not widely used in fermented products, which gave rise to questions about its suitability for fermentation in its raw form, however the present work indicates that it can be successfully fermented both as a raw ingredient and after a short period of boiling.

Legumes are well known for their high oligosaccharide content which is associated with poor digestibility (Messina, 2014). It is recognised that fermentation may ease digestive problems by reducing oligosaccharide content, however, with the exception of soya, grain-legumes are not widely used for the production of fermented foods. When soya is used, it is predominately fermented by fungi, although LAB are sometimes used in the final processing stage, e.g. furu is made via lactic acid fermentation of tofu (Bao *et al.*, 2019). Results presented from experiment 2 indicate that common white beans are not a suitable medium for fermentation by *L. plantarum* as the pH did not decrease to less than 4, which is required to ensure a stable product (Montet *et al.*, 2014). If this holds true for other LAB and bean varieties, the inability of LAB to ferment grain-legumes, combined with climatic differences, may explain the absence of fermented legume foods in traditional European diets – the cooler, dryer climate is less suitable for spontaneous fermentation by fungi which presumably was essential to the development of fermented soya products in Asia.

The fermentation of a mixed matrix including beans and vegetables is a novel approach to producing a fermented legume product that has not been reported elsewhere. In the present work, the combination of white-beans and cauliflower proved to be a successful matrix for fermentation with *L. plantarum;* a stable pH of approximately 3.5 was produced within 48 hours. The carbohydrate content was not assessed so there is, as yet, no evidence that this method can be employed to reduce oligosaccharide content and improve digestibility, however two hypotheses are suggested. The first is that *L. plantarum* can metabolize carbohydrates from white beans in the presence of an alternative energy source. In this case, during fermentation of the cauliflower-bean matrix, available monosaccharides may enable the fermenting bacteria to create and sustain a stable environment for sufficient time to degrade the more complex oligosaccharides present in the beans. This could lead to the development of a product with improved digestibility, increasing consumer acceptance of white beans. The second is that *L. plantarum* cannot metabolize carbohydrates from white beans. In this case, beans within a fermented cauliflower-bean matrix will likely be preserved by the high lactic acid content, but remain relatively unchanged within the mixture, as the bacteria ferment only the vegetable derived carbohydrates. In this instance digestibility would presumably not be improved, but other factors such as enhanced taste and improved shelf-life may also be appealing to consumers. Further research is required to establish the oligosaccharide content of the fermented mixture, and its digestibility.

Formal taste-testing was not performed on the fermented samples and further research focussing on taste, texture and overall consumer acceptance would be beneficial to understand if the fermented cauliflower-bean matrix presented here has potential to be developed into a novel consumer product. In this instance, B12 production was not sufficient to be nutritionally meaningful, but there are several other micronutrients that can be produced during fermentation that could also add nutritional value to the product and should be considered. For example, the concentration of folate and riboflavin, two other essential B-vitamins, was also analysed and is reported elsewhere (Thompson *et al.*, 2020 - Appendix 1). Results from the survey component of this study indicate that most consumers (within the surveyed demographic) have at least a basic knowledge and awareness of vitamin B12. However, no attempt was made to measure understanding of B12 production and whether consumers would recognize a difference between B12 present as a fortification and B12 produced in situ during fermentation. In the event that a product was developed containing B12 produced during fermentation, attitudes towards different B12 production methods and sources could influence consumer interest in the product and willingness-to-pay. Further consumer research, such as conjoint analysis, could be employed to assess the value of fortified and *in situ* produced B12 to the consumer, however this would also require supporting evidence that consumers understand the difference.

The survey also indicates that consideration is already being given to the environmental impact of food choices, and that some value is placed on sourcing food locally. The development of products based on locally sourced ingredients and with a demonstrated low environmental impact may be appealing to a wide range of consumers within the surveyed Swedish demographic and could potentially be used to facilitate the consumption of more plant-based foods that support the development of sustainable agricultural systems in Sweden. Increasing the consumption of legume-based products via the production of novel fermented foods could create a more stable market, encouraging and enabling more farmers to integrate these ecologically valuable plants into their cropping systems, reducing their need for nitrogen fertilizers, enhancing overall biodiversity and contributing to improved soil health.

5. Conclusion

The development of ecologically sustainable food systems will require radical changes to current product and consumption patterns, but it must not come at the expense of human health goals. The growing acceptance and popularity of vegan and vegetarian diets is an encouraging trend that indicates many people are prepared to make changes in their own behaviour in order to influence positive societal outcomes. Those advocating the adoption of vegan diets must recognise there are associated nutritional challenges, including the increased risk of B12 deficiency, and that current attempts to overcome existing micronutrient deficiencies often fall short, even in wealthy populations with ample access to food and dietary supplements. It cannot be assumed that education alone will be sufficient to ensure a vegan population would meet its B12 requirements, and steps must be taken to ensure that a variety of affordable B12 sources are made available. The development of novel fermented foods is identified here as a strategy to provide a plant-based source of B12 while simultaneously encouraging the diversification of cropping systems to include more legumes. The present experimental work did not produce a sufficient quantity of B12 to provide a viable source, however other work in the field indicates that it is likely possible to achieve this outcome by working with known B12 producing bacterial strains under controlled conditions. In the surveyed population knowledge of B12 is high among recognised risk groups, and environmental concern is a strong driving force for dietary choices. These factors could be employed in the marketing strategy for introducing a novel B12 enriched fermented legume product.

6. Critical Reflections

The overall aim of this project was ambitious, and the results of both the experimental procedure and population survey are limited. The design of survey items used to measure attitudes towards supplementation and B12 fortification could be greatly improved; insufficient questions were included, although the question design was drawn from similar research, there was limited agreement between items and few valuable likert scales could be produced. The results were also constrained by the small sample size and homogeneity among respondents. A more random population sampling method and wider distribution would great improve the quality of this research. Alternative methods, such as conjoint analysis, may have been a more suitable to examine the value of B12 containing fermented products, and consumer’s willingness to buy.

The fermentation experiment was well executed; however, the results were heavily restricted by the expense of outsourcing B12 analysis. If further research is conducted it would be beneficial to invest in the development of inhouse B12 analysis capabilities, or to work in collaboration with a lab that already has these capabilities. Genetic screening of LAB prior to testing would likely have increased the likelihood of identifying B12 producing strains, as has been reported elsewhere in the literature.

7. References

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Fakta från Biosystem och teknologi

Utveckling av nya växtbaserade livsmedel - effekt av mjölksyrafermentering

Helen O. Thompson, Gun Hagström och Malin Hultberg

Mjölksyrafermentering är en traditionell metod att påverka ett livsmedels kemi och förlänga hållbarheten. Detta arbete fokuserar på effekten av fermentering av ett växtbaserat livsmedel med avseende på proteinkvalitet och B-vitaminer.

Bakgrund

Studier kring framtida hållbara livsmedelssystem pekar på ökad konsumtion av grönsaker, inklusive baljväxter (Karlsson et al., 2017). I denna kontext är vitamin B_{12} av intresse eftersom den främst tillförs genom animaliska livsmedel. Vitamin B_{12} produceras av vissa grupper av mikroorganismer och under senare år har man isolerat stammar av mjölksyrabakterier som producerar detta vitamin. Parallellt har mjölksyrafermentering länge spelat en viktig roll i människans kost och används exempelvis för produktion av surkål och surdegsbröd. Vid mjölksyrafermentering omvandlas tillgängliga kolhydrater till organiska syror, huvudsakligen mjölksyra och ättiksyra, beroende på vilken bakterieart som domineras. Vid spontan mjölksyrafermentering av grönsaker är *Lactobacillus plantarum* den dominerande bakteriearten (Di Cagno et al., 2013) och en sänkning av pH till runt 4.0 har rapporterats att ge en stabil produkt (Montet et al., 2014). En fördel med att använda välkända mjölksyrabakterier, som *L. plantarum* för fermentering, är att de ingår i listan "Qualified Presumption of Safety (QPS)", och är godkända för användning i livsmedels- och foderkedjan inom EU.

Syftet med mjölksyrafermentering är i allmänhet att förlänga hållbarhet genom att hindra tillväxt av andra mikroorganismer som kan fördärva livsmedlet. Det är emellertid en komplex process som påverkar livsmedlets kemi. Fermenteringsprocessen styras av livsmedlets sammansättning, vilka stammar av mjölksyrabakterier som dominerar och abiotiska faktorer som temperatur. Bortsett från den direkta effekten på livsmedlet är också vissa stammar av mjölksyrabakterier associerade med probiotiska egenskaper. De stammar av *L. plantarum* som använts i denna studie har tidigare visats ha olika hälsoeffekter exempelvis förbättrade symtom hos personer med IBS (Ducrotté et al., 2012) och ökad järnabsorption (Hoppe et al., 2017).

I denna studie fermenterades blomkål, vita bönor samt en blandning av blomkål och vita bönor med hjälp av fyra olika stammar av *L. plantarum* som tillhandahölls av företaget Probi AB (https://probi.com/). Förändringen av pH följdes över tid och effekten av fermenteringen på viktiga näringsparametrar som total protein, aminosyrasammansättning och riboflavin, folat och vitamin B_{12}-innehåll studerades.

Metod

Mixad rå blomkål, kokta och mixade vita bönor samt en blandning av blomkål och vita bönor (1:1 vikt/vikt) användes i försöken. Bakteriesuspensioner (*L. plantarum*), producerad enligt gängse metodik, tillsattes i en koncentration av 1% (vikt/vikt). Försöken genomfördes på **SLU Open Food Lab*,** Alnarp, sattes upp med tre replikat i varje behandling och upprepades en gång. Behandlingarna inkuberades vid 30° C under 44 timmar och efter detta avsmakades behandlingarna och blandningen av blomkål och vita bönor valdes för fördjupad analys. Den totala mängden protein i proverna bestämdes med Dumas-metod. Bestämning av riboflavin utfördes enligt europeisk standard EN14152. Bestämning av den totala mängden folat utfördes enligt europeisk standard EN1413. Extraktion av vitamin B_{12} utfördes enligt metod AOAC 952.20. Bestämning av riboflavin och den totala mängden folat utfördes vid Livsmedelsverket, Uppsala, Sverige. Vitamin B_{12} och aminosyrasammansättning bestämdes av Eurofins Food & Agro Testing Sweden AB, Linköping, Sverige.

Fig. 1 Fermentering av olika slag påverkar produktens organoleptiska egenskaper och är en mycket gammal metod för livsmedelskonservering.

Resultat och diskussion

Både blomkål och vita bönor hade liknande initialt pH på cirka 6.2. Efter 18 timmars fermentering var pH emellertid signifikant lägre i de behandlingar som innehöll blomkål, 3.7 respektive 3.8, jämfört med behandlingen som endast innehöll vita bönor, där pH var 4.8. När försöket avslutades hade pH sjunkit ytterligare i de behandlingar som innehöll blomkål, till 3.4 respektive 3.5, medan pH stigit till 5.0 i behandlingen med vita bönor. Sammanfattningsvis tillförde blomkålen kolhydrater som var tillgängliga för L. plantarum och gav en effektiv fermentering där pH sjönk under 4.0.

Fördelen med att inkludera vita bönor i produkten var tydlig, då den totala mängden protein i livsmedlet var högt och varierade mellan 21.1-23.2% av torrvikten. Efter fermentering sågs en liten ökning av aminosyrorna alanin, glycin, histidin, isoleucin,

leucin och valin jämfört med den ofermenterade kontrollen. Den totala mängden protein påverkades inte av fermenteringen.

Riboflavin är viktigt för funktionen av flera enzymer och de viktiga källorna är mjölk och mejeriprodukter, samt spannmål och kött. Det är ett vattenlösligt vitamin som inte lagras i kroppen med ett rekommenderat dagligt intag av 1,6 mg för vuxna (EFSA, 2017). Koncentrationen av riboflavin ökade signifikant jämfört med kontrollen i alla behandlingar fermenterade med L. plantarum. Ökningen var mellan 76-113% av kontrollens värde och det högsta värde som uppmättes var 91.6±0.6 µg/100 g färskvikt. Halterna av riboflavin i den slutliga fermenterade produkten var generellt låga jämfört med det rekommenderade dagliga intaget.

För folat observerades ett liknande mönster som för riboflavin, med en signifikant ökning i alla fermenterade prover jämfört med kontrollen. Ökningen varierade mellan 32-60% av kontrollen och det högsta värdet som uppmättes var 58.8±2.0 µg/100 g färskvikt. Liksom riboflavin syntetiseras folater av både växter och mikroorganismer, och viktiga källor är bladgrönt, mejeri- och fullkornsprodukter. Folater har en nyckelroll i cellmetabolismen men biotillgängligheten för naturliga folater varierar och de bryts lätt ned. Folatbrist kan förekomma och i vissa länder sker en berikning med folsyra i utvalda livsmedel. Med tanke på ett genomsnittliga rekommenderade intaget av 250 µg folatekvivalenter per dag för en vuxen (EFSA, 2014), är den fermenterade produkten som producerats i denna studie intressant.

Produktion av vitamin B_{12} är begränsad till några få arter av bakterier och arkéer, och att ett tillräckligt intag av detta vitamin är ett problem med växtbaserade dieter. I denna studie var ökningen av vitamin B_{12} i de fermenterade behandlingarna mindre uttalad än vad som sågs för riboflavin och folat. En signifikant ökning av vitamin B_{12} sågs endast efter fermentering med en av stammarna av *L. plantarum*. För denna behandling sågs en ökning med 66% jämfört med kontrollen, till 0.05 ± 0,01 µg/100 g färskvikt. Ett intag av 4 ug vitamin B_{12} per dag rekommenderas enligt EFSA (2015) och den fermenterade produkten kan bara ge en mindre del av det totala behovet trots den signifikanta ökningen.

Fig. 2 Kolonier av mjölksyrabakterien Laktobacillus plantarum.

***SLU Food Lab** – är ett välutrustat och livsmedelsgodkänt utvecklingslabb som används inom LTV-fakulteten för olika verksamheter inom främst undervisning, examensarbeten på SLU:s utbildningar för trädgårdsingenjörer, hortonomer, agronomer samt även i forskningsprojekt.

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ORIGINAL PAPER

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Fermentation of Cauliflower and White Beans with *Lactobacillus plantarum* – Impact on Levels of Riboflavin, Folate, Vitamin B_{12}, and Amino Acid Composition

H. O. Thompson[1] · G. Önning[2,3] · K. Holmgren[2] · H. S. Strandler[4] · M. Hultberg[1]

Abstract
As diets change in response to ethical, environmental, and health concerns surrounding meat consumption, fermentation has potential to improve the taste and nutritional qualities of plant-based foods. In this study, cauliflower, white beans, and a 50:50 cauliflower-white bean mixture were fermented using different strains of *Lactobacillus plantarum*. In all treatments containing cauliflower, the pH was reduced to <4 after 18 h, while treatments containing only white beans had an average pH of 4.8 after 18 h. Following fermentation, the riboflavin, folate, and vitamin B_{12} content of the cauliflower-white bean mixture was measured, and compared against that of an unfermented control. The riboflavin and folate content of the mixture increased significantly after fermentation. Relative to control samples, riboflavin increased by 76–113%, to 91.6 ± 0.6 μg/100 g fresh weight, and folate increased by 32–60%, to 58.8 ± 2.0 μg/100 g fresh weight. For one bacterial strain, *L. plantarum* 299, a significant 66% increase in vitamin B_{12} was observed, although the final amount (0.048 ± 0.013 μg/100 g fresh weight) was only a small fraction of recommended daily intake. Measurements of amino acid composition in the mixture revealed small increases in alanine, glycine, histidine, isoleucine, leucine, and valine in the fermented sample compared to the unfermented control.

Keywords B-vitamins · *Brassica oleracea* · Lactic acid bacteria · Nutritional quality · *Phaseolus vulgaris*

Introduction

Recent research has highlighted good potential of a change in diet in helping to resolve global challenges such as climate change, biodiversity loss, and food insecurity [1]. Studies exploring future sustainable food systems in the Nordic countries suggest decreasing consumption of meat by 80–90% and increasing consumption of vegetables. Legumes, with their high protein content, are of special importance in this concern due to their benefits for agricultural cropping systems via biological nitrogen-fixation [2]. Thus, there is a need for developing new plant-based products including legumes.

Fermentation has been used since ancient times for food preservation, while also having an impact on organoleptic characteristics. Several traditional Asian fermented bean products have now become popular in the West, including tofu, tempeh, and miso. Additional driving forces in developing fermented vegetable products are the growing interest in locally produced food, and consumer interest in products with less chemical additives.

During fermentation with lactic acid bacteria (LAB), available carbohydrates are converted to organic acids, mainly lactic acid and acetic acid, depending on the species used. For vegetables, a decrease in pH to around 4 has been reported to ensure a stable product [3]. The dominant species in spontaneous lactic acid fermentation of vegetables is *Lactobacillus plantarum* [4]. A benefit of using well-known lactic acid bacteria, such as *L. plantarum,* for fermentation is that they are included in the Qualified Presumption of Safety (QPS) list, which authorizes their use in the food and feed chain within the European Union.

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The aim in lactic acid fermentation is generally to preserve the food by excluding growth of spoilage microorganisms. However, lactic acid fermentation is a strain-dependent and complex process with a broad impact on the nutritional value of the food [5]. An increase in the content of important nutrients, including the B-vitamins, after fermentation of plant-based products has been reported [6]. Apart from the direct effect on the food due to the bacterial metabolism, certain strains of LAB are also associated with probiotic properties. The *L. plantarum* strains investigated in the present study have been shown to have different health effects in humans, for example improved symptoms in people with irritable bowel syndrome [7], protection against lumbar spine bone loss in postmenopausal women [8], and increased iron absorption from foods [9].

The growth and capability for efficient fermentation of LAB are affected by several factors, such as composition of the substrate, strain-specific variations, and the fermentation procedure. In the present study fermentation of vegetables, cauliflower, white beans, and a mixture (50:50) of cauliflower and white beans, was studied. Four strains of *L. plantarum* were used and the effects of fermentation on levels of important nutritional parameters such as amino acid composition and riboflavin, folate, and vitamin B_{12} content were studied. Three of the investigated strains are available commercially as food supplements and as chilled plant-based food products and have been used to ferment cereals, berries and fruit [10–12]. The ability of the included strains to ferment vegetables and produce riboflavin, folate and vitamin B_{12} have not been investigated before.

Material and Methods

Bacterial Cultures

Four different strains of *L. plantarum* (strain 299v, strain Lp900, strain 299, strain Heal19) were provided by the company Probi AB, Sweden (https://probi.com/) and are described in Table 1. For production of the *inoculum* used for fermentation, the strains were cultivated as static culture in MRS broth (BDH Chemicals, UK) at 35 °C for 16 h. After this, the cells were harvested by centrifugation (Eppendorf MiniSpin, 10,000 g for 3 min) and washed twice with 0.85% NaCl solution. The control treatment was prepared with sterile MRS broth and a similar washing procedure. The bacterial suspensions, diluted in 0.85% NaCl and with an OD_{620} of 0.8 (corresponding to 7–8 log CFU/ml), and a control suspension (0.85% NaCl only) were added in a concentration of 1% (*w*/w) to the vegetable mixtures.

Experimental Set-up

Raw cauliflower (*Brassica oleracea var. botrytis*) mixed in food processor, cooked and mixed white beans (*Phaseolus vulgaris* L.), and a mixture of consisting of a 50:50 ratio (*w*/w) of raw cauliflower and cooked white beans (cauliflower-white bean) were weighed out into plastic containers. Each portion weighed 100 g ± 1 g and was combined with 2 g of sea salt.

Suspension of *L. plantarum* was added to each container. The mixture was thoroughly stirred again following addition of bacteria or control suspension. The pH of each sample was recorded and the control samples were directly frozen at −80 °C. Containers with bacterial culture were incubated at 30 °C for 44 h. The pH of fermented samples was measured after 18 and 44 h.

After 44 h, the treatments were tasted and the cauliflower-white bean mixture was chosen for further analysis. Samples for determination of riboflavin (vitamin B_2), folate, vitamin B_{12}, total protein, and amino acid composition in this treatment were prepared and frozen at −80 °C. All samples were analyzed for total protein and vitamin content, while analyses of amino acid composition were performed on the control samples of cauliflower-white bean mixture and the samples fermented with *L. plantarum* 299.

Analysis

Determination of Riboflavin, Total Amount of Folate and Vitamin B_{12}

Determination of riboflavin was performed according to European Standard EN14152, as described by Jakobsen [13]. Determination of the total amount of folate was performed

Table 1 Strains of *Lactobacillus plantarum* used in this study

Strain	Origin	DSM number[1]
L. plantarum strain 299v	Human gastrointestinal (GI) tract	9843
L. plantarum strain Lp900	Ogi, red sorghum, Nigeria	–
L. plantarum strain 299	Human GI tract	6595
L. plantarum strain Heal19	Human GI tract	15,313

The strains deposited at DSM are available commercially as food supplements and as chilled plant-based food products

[1] German Collection of Microorganisms and Cell Cultures

according to European Standard EN1413, as described by DeVries *et al*. [14], except for use of protease in the extraction procedure. Extraction of vitamin B_{12} was performed according to method AOAC 952.20, as described by Ball [15].

Total Protein and Amino Acid Composition

Total amount of protein in the samples was determined by the Dumas method [16] and applying a conversion factor of 6.25 for total nitrogen. Concentrations of the amino acids were determined according to the method of Llames and Fontaine [17].

Statistics

The experiments were set up with three replicates in each treatment and repeated once. The data obtained were analyzed statistically using Minitab 17 for Windows. One-way Anova followed by Tukey's multiple comparison test was employed to test for effects of treatments and the significance level was set to $P \leq 0.05$.

Results and Discussion

The different strains of *L. plantarum* behaved similarly with regard to the effect of pH on the different treatments. No significant difference was observed between the strains within each reading (18 and 44 h). Data from all strains were therefore pooled for each time point for analysis of the effect of fermentation on pH (Fig. 1). Cauliflower and white bean had a similar initial pH of approximately 6.20. However, after 18 h of fermentation with *L. plantarum* strains, the pH was significantly lower in the cauliflower and the cauliflower-white bean mixture treatments (3.66 ± 0.05 and 3.75 ± 0.04, respectively, mean ± SD) than in the treatment with white bean only (4.82 ± 0.02). After 44 h of fermentation, a slight but significant increase in pH was observed in the white bean treatment, to 4.96 ± 0.01. In the treatments with cauliflower and cauliflower/white bean mixture the opposite pattern was observed, with a slight but significant decrease in pH to 3.44 ± 0.1 and 3.52 ± 0.07, respectively.

Thus, fermentation was more efficient in the treatments including cauliflower adding benefits such as increased shelf life due to the low pH. Cauliflower is reported to contain approximately 4.2% carbohydrates, with a high concentration of monosaccharides [18]. Legumes, on the other hand, are well-known for containing high amounts of complex oligosaccharides, a component of dietary fiber that is less available for microbial degradation [19]. Thus, the easily available carbohydrates provided by cauliflower most probably sustained microbial growth, followed by a decrease in pH due to production of organic acids, to a greater extent than in the white bean treatment.

The mixture of cauliflower and white bean was chosen for further studies on the content of total protein, riboflavin, folate, and vitamin B_{12}. The taste was dominated by a sour and salty flavour, similar to traditional fermented cabbage (sauerkraut), but with a deeper, underlying umami taste that brought a mild cheese-like quality. The taste was unusual, but not unpleasant, though more comprehensive taste analysis and

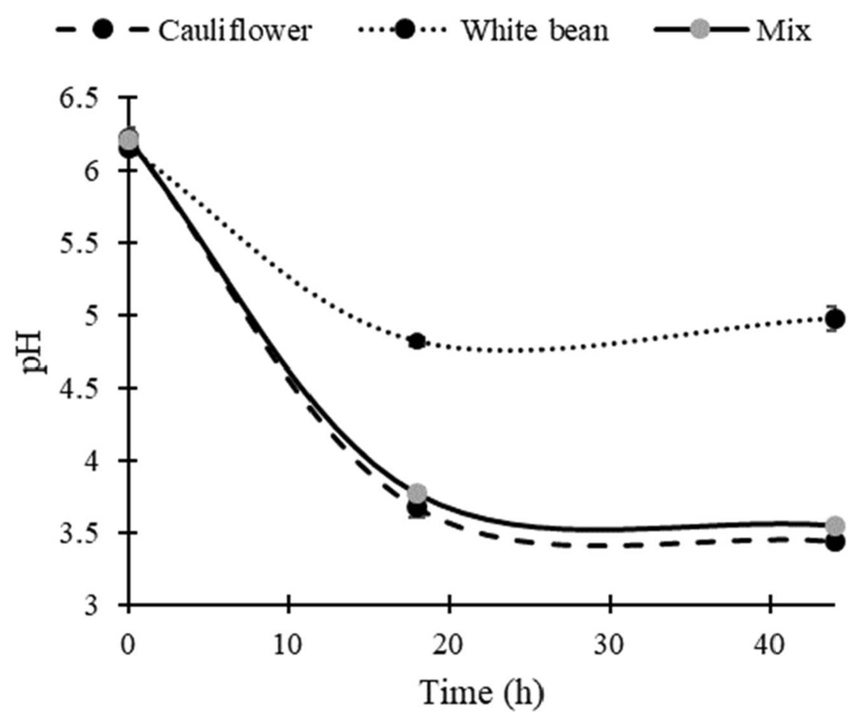

Fig. 1 Changes in pH during fermentation with *Lactobacillus plantarum* of homogenized cauliflower, white bean, and a mix (50:50) of cauliflower and white bean

consumer research would be required to determine the marketability of the product.

From a nutritional perspective the benefit of including white beans was clearly apparent, as the total amount of protein ranged from 21.1 to 23.2% of dry weight in the different bacterial treatments. Inconsistent results have been reported regarding the effect of fermentation on total protein amount [20]. Based on work with cereals, it has been suggested that the total amount of protein is generally not changed during lactic acid fermentation, although an increase can be observed in certain cases. If an increase is observed, it can often be related to a decrease in carbon ratio in the total mass due to bacterial metabolization of carbohydrates [21]. In the present study, no significant differences were observed in organic carbon content or in total nitrogen in relation to the control or between any of the treatments. Based on these results, no effect on the total protein amount was observed.

Riboflavin is important for the function of several enzymes involved in energy metabolism. It is naturally present in several different foods, including plants, and main sources of riboflavin intake are milk and dairy products, followed by cereals and meat. It is a water-soluble vitamin that is not stored in the body, and the daily dietary reference value has been set to 1.6 mg for adults [22]. Despite its presence in a wide variety of foods, riboflavin deficiency may occur. In this study, fermentation with *L. plantarum* increased the concentration of riboflavin significantly compared to the control in all treatments (Table 2). Significant differences in riboflavin concentration related to the different strains were observed. The highest value was observed after fermentation with *L. plantarum* Lp900, which gave an increase of 113% compared to the initial value, to 91.6 ± 0.6 μg/100 g fresh weight. The smallest increase, 76% of the initial value, was observed in the treatment with *L. plantarum* 299v. A similar increase in riboflavin content has been observed by Capozzi *et al*. [23] on fermenting wheat with *L. plantarum* for production of bread and pasta. However, in their study the strains used were selected for over-production of riboflavin, while such selection was not applied in the present study. Our results suggest that fermentation with *L. plantarum* can be used to increase the concentration of riboflavin in plant-based foods. However, it should be pointed out that the levels of riboflavin in the final fermented product were still generally low, at the level of μg/100 g product, compared to the recommended daily intake of 1.6 mg.

For folate, a similar pattern as for riboflavin was observed, with a significant increase in all fermented samples and variations between strains. Like riboflavin, folate is synthesized by both plants and microorganisms, with main dietary sources being leafy green vegetables, dairy products, and cereal products. This vitamin, including several related compounds play a key role in ensuring essential functions of cell metabolism, such as DNA synthesis. However, the bioavailability of natural food folates varies and these compounds are easily degraded. Thus, folate deficiency is a general concern, and a strategy based on fortification of selected foods has been adopted in some countries. In this study, the highest concentration of folate was observed after fermentation with *L. plantarum* 299v, which showed an increase of 60% compared to the initial value, to a total concentration of 58.8 ± 2.0 μg/100 g fresh weight. The smallest increase, 32% of the initial value, was observed in the treatment with *L. plantarum* 299. Considering the average recommended intake of 250 μg dietary folate equivalents/day [24], the fermented vegetable mixture is of interest. The ability of microorganisms to produce folate is strain-specific, and a decrease in folate concentration in fermented products due to microbial consumption has been reported [25]. It should be pointed out that a significant increase in folate concentration was observed for all four strains of *L. plantarum* included in the present study, and that the genes for folate biosynthesis have been identified in this species [26]. Thus, fermentation of vegetables with *L. plantarum* might be considered as a general measure to increase folate concentration.

Vitamin B_{12} has a function as an important co-factor in several enzymes in procaryotes, protists, and animals, while B_{12}-dependent enzymes have not been found in plants and fungi. Production of vitamin B_{12} has been shown to be limited to a few species of bacteria and archaea [27], and ensuring intake of adequate levels of this vitamin is a high concern with plant-based diets. In recent years, two strains of *L. plantarum* that produce vitamin B_{12} have been isolated [28]. In the present study, the increase in vitamin B_{12} in the fermentation treatments was less pronounced than that seen for riboflavin and folate. A significant increase in B_{12} content was observed after fermentation with *L. plantarum* 299 only (Table 2). An

Table 2 Concentration (μg/100 g fresh weight) of riboflavin, folate, and vitamin B_{12} before (control) and after lactic acid fermentation of a mixture of cauliflower and white beans at 30 °C for 44 h using four different strains of *Lactobacillus plantarum*

Treatment	Riboflavin	Folate	Vitamin B_{12}
Control	42.83 ± 1.20a*	36.84 ± 0.81a	0.029 ± 0.002a
L. plantarum strain 299v	75.64 ± 0.82b	58.82 ± 1.98c	0.033 ± 0.004ab
L. plantarum strain Lp900	91.60 ± 0.56c	55.88 ± 0.98c	0.034 ± 0.011ab
L. plantarum strain 299	76.36 ± 9.21b	48.74 ± 3.98b	0.048 ± 0.013b
L. plantarum strain Heal19	85.07 ± 2.14bc	53.55 ± 1.28bc	0.034 ± 0.004ab

Values shown are mean ± standard deviation

*Different letters within columns indicate significant differences ($p \leq 0.05$; Anova followed by Tukey's test) increase of 66% (to 0.048 ± 0.013 μg/100 g fresh weight) compared to the initial value (0.029 ± 0.002) was observed in this treatment. Considering that intake of 4 μg vitamin B_{12} *per* day has been set as adequate by EFSA [29], it is clear that the fermented products evaluated in the present study could only provide a very small fraction of the total requirement, despite the significant increase. For the two vitamin B_{12}-producing strains of *L. plantarum* previously isolated, it has been demonstrated that increased production of vitamin B_{12} can be achieved by addition of a B_{12} precursor such as 5-aminolevulinate [28]. Thus, a future approach to increase the concentration of vitamin B_{12} in fermented vegetables could be to ensure high concentrations of precursors before fermentation. Also, as the presence of human inactive analogues, such as pseudovitamin B_{12}, have been reported in LAB high-producing strains should be subjected to detailed chemical analysis including not only microbiological assay but also liquid chromatographic methods [30].

It should be pointed out that no cell lysing treatment was performed in the present study, apart from storage in the freezer (−80 °C), and that strains of *L. plantarum* have been demonstrated to have high stability when frozen [31]. Additionally, no difference in moisture content in any of the treatments compared to the control was observed after lyophilization (data not shown). Thus, the increased levels of vitamins observed in the present study did not represent an intracellular pool, and were not due to an increase in dry matter.

No distinguishable difference in taste could be detected in the mixture fermented with the different bacterial strains and, based on the significant increase in vitamin B_{12} level, the vegetable mixture fermented with strain *L. plantarum* 299 was chosen for analysis of amino acid composition. The results showed small increases in alanine, glycine, histidine, isoleucine, leucine, and valine in the fermented sample compared to the control (Table 3). Of these amino acids, histidine, isoleucine, leucine, and valine are essential in the human diet. Thus, fermentation with *L. plantarum* 299 can be considered to have slightly improved the protein quality of the vegetable mixture. In contrast, a recent study reported a decrease in protein quality after fermentation of pea proteins with *L. plantarum* [32]. In that study, high consumption of the sulfur-containing amino acids was observed and thus the authors recommend selection of species other than *L. plantarum* for fermentation. This discrepancy in results, despite working with the same species and a similar vegetable, reflects the strain-specific metabolism of *L. plantarum*, which has been suggested to be due to their diverse ecological niches [28]. It also highlights the need for working with several strains of the same species in order to draw sound conclusions on characteristics of the species.

Table 3 Amino acid (aa) composition (g/100 g protein, dry weight basis) of a mixture of cauliflower and white bean before (control) and after fermentation with *Lactobacillus plantarum* strain 299

Amino acid	Control	Fermented sample
Alanine	1.11 ± 0.01a*	1.13 ± 0.01b
Arginine	1.42 ± 0.04a	1.42 ± 0.05a
Aspartic acid	2.92 ± 0.04a	2.97 ± 0.01a
Cysteine	0.23 ± 0.01a	0.24 ± 0.01a
Glutamic acid	3.60 ± 0.04a	3.63 ± 0.03a
Glycine	0.97 ± 0.01a	1.03 ± 0.01b
Histidine	0.67 ± 0.01a	0.70 ± 0.01b
Isoleucine	1.09 ± 0.00a	1.13 ± 0.01b
Leucine	2.02 ± 0.01a	2.08 ± 0.02b
Lysine	1.80 ± 0.01a	1.84 ± 0.05a
Methionine	0.25 ± 0.01a	0.23 ± 0.03a
Phenylalanine	1.40 ± 0.04a	1.46 ± 0.03a
Proline	0.93 ± 0.07a	1.00 ± 0.03a
Serine	1.52 ± 0.02a	1.48 ± 0.02a
Threonine	1.09 ± 0.01a	1.10 ± 0.01a
Tyrosine	0.85 ± 0.02a	0.87 ± 0.01a
Valine	1.32 ± 0.02a	1.36 ± 0.01b
aa	23.21 ± 0.14a	23.68 ± 0.05b

*Values within rows followed by different letters are significantly different ($p < 0.05$)

Conclusions

Lactic acid fermentation is of importance for food preservation, while also having impact on taste and nutritional composition. In the present study three different vegetable substrates (cauliflower, white bean, and cauliflower-white bean mixture) were fermented using four different strains of *L. plantarum*. All strains had a similar impact on pH of the different substrates, and fermentation was more efficient in the treatments including cauliflower. Due to the efficient fermentation, with a final pH below 4, the pleasant taste and inclusion of legumes the impact of fermentation on riboflavin, folate, and vitamin B_{12} concentrations and on protein quality was studied in the cauliflower-white bean mixture. All strains of *L. plantarum* significantly increased the content of both folate and riboflavin compared to an unfermented control. Fermentation also had an impact on the content of vitamin B_{12}, with fermentation with one of the bacterial strains (*L. plantarum* 299) resulting in a significant increase in vitamin B_{12} content. In the treatment involving fermentation of a cauliflower-white bean mixture with *L. plantarum* 299, amino acid composition was analyzed. The results revealed small increases in the concentrations of alanine, glycine, histidine, isoleucine, leucine, and valine in the fermented sample compared to the unfermented control.

Thus a slight improvement in nutritional quality was obtained after fermentation, although it should be pointed out that the quantity of different vitamins produced during fermentation, particularly of riboflavin and vitamin B_{12}, was low relative to the recommended intake.

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Compliance with Ethical Standards

Conflict of Interest G Önning and K Holmgren work for Probi AB, which owns the bacterial strains investigated in the study. The other authors declare that they have no conflict of interest.

Ethical Approval This work did not involve any studies with human participants or animals performed by any of the authors.

Informed Consent All authors have the authority to publish this material and have agreed to submit it to *Plant Foods for Human Nutrition*.

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