Vitamin B_{12} and folate enrichment of kefir by *Propionibacterium freudenreichii* and *Streptococcus thermophilus* strains

by

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

DECLARATION

I, Ryan Andrew Morkel, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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Signed **Date**

ABSTRACT

In South Africa malnutrition exists due to inadequate dietary intake of micronutrients which is one of the major causes of vitamin deficiencies leading to disease. The treatment of malnutrition over the past years has been a considerable burden on the South African economy. Therefore, food fortification is one of the current strategies used to minimize malnutrition by increasing the nutritional value of staple foods.

Commercial dairy products and pharmaceutical nutritional products (food supplements) in South Africa have been developed and produced for affluent consumers. Hence the need to develop an affordable fortified dairy product for the majority of South Africans prompted this study aimed at using a “naturally” fortified kefir beverage with vitamin B_{12} and folate to increase B-vitamins levels.

Since *Propionibacterium freudenreichii* and *Streptococcus thermophilus* are known to be good producers of vitamin B_{12} and folate, respectively, and propionibacteria has the ability to grow symbiotically in the presence of lactic acid bacteria, the inclusion of these organisms with the kefir grains was an achievable objective.

In order to conduct the analysis of vitamin B_{12} and folate in the samples, sample extraction and HPLC assay techniques were developed. The extraction of vitamin B_{12} and folate were achieved by using KCN extraction buffer and the trienzymatic method, respectively. The samples were also subjected to purification and concentration using solid phase extraction for optimum results. All standards and samples were flushed with nitrogen gas and stored for a maximum of 2 weeks at –20°C to prevent B-vitamin deterioration. The HPLC assembly for the vitamin B_{12} analysis included a Luna C_{18} column and a diode array detector (DAD) for the detection and quantification. For the folate analysis it included a Zorbax SB-C_{18} and Luna C_{18} columns in tandem and the fluorescence detector (FLD) was used for the detection and quantification of THF, 5-CH_3-THF and 5-CHO-THF, while the DAD was used for PGA and pteroyltri-γ-L-glutamic acid concentration in the samples.

Propionibacterium freudenreichii J19 (PAB) and *Streptococcus thermophilus* ATCC 19258 (LAB) were selected due to their ability to produce high levels of vitamin B_{12} and folate, respectively. The vitamin B_{12} levels produced by the PAB was optimized using a rich glucose based growth media (VBM) while the *S. thermophilu*s was grown in De Man Rogosa Sharpe (MRS) medium for increased folate levels.

The inclusion of the PAB and LAB in the study was conducted using three different forms of inoculum, namely broth, freeze-dried and direct cultures. During the first and second phase of this study, the PAB and PAB-LAB inoculum concentration inoculated with the kefir grains was varied as well as the B-vitamin concentration.

During the first phase of this study, the broth culture and freeze-dried culture of PAB only was used in three inoculation treatments whereas the direct culture was inoculated once with the kefir grains. In the second phase, the broth culture and freeze-dried culture of PAB, followed by treatments with LAB cultures were used in multiple treatments (two treatments each) and the direct culture of PAB and LAB was inoculated once with the kefir grains. The multiple treatments of PAB with the kefir grains showed an increase in vitamin B_{12} in this phase of the study, while the folate had similar concentration levels in both phases of the study. The PAB-LAB treatments resulted in increased vitamin B_{12} and folate levels which were on par with a %NRV, thus achieving the objective of the study.

The DNA and PCR assays performed on the treated samples confirmed the presence of *P. freudenreichii* after fermentation. The sensory analysis of the kefir beverage in terms of sour and overall taste was preferred after fermentation for 1 d by the sensory evaluators.

In this study, the inclusion of PAB and PAB-LAB treatments with the kefir grains was shown to be successful and has the potential to improve the functionality of a food product by using food-grade bacteria to increase B-vitamin levels, promote and sustain human health. The implementation of the findings in this study may reduce the inadequate intake and deficiency of B-vitamins.

Although the PAB and PAB-LAB treated kefir grains produced ideal levels of vitamin B_{12} and folate, there were variations with regards to inoculum concentration between broth, freeze-dried and direct cultures of PAB and LAB. Therefore, further optimization techniques and methods with regards to consistently producing the same B-vitamin levels are recommended using a bioreactor for a more controlled fermentation and standardisation of the inoculum concentration.

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DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents, Samuel and Denise Morkel, for their kind words of encouragement and support.

I also dedicate this dissertation to my siblings, André and Abigail as well as my lecturers who have supported me throughout the process. I will always appreciate all they have done, especially Professor Jessy Van Wyk for helping me develop my technology skills and has generously given her time and expertise to better my research.

GLOSSARY

Freeze-drying (also known as lyophilisation, lyophilization or cryodesiccation) is a dehydration process typically used to preserve a perishable material. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase.

A cryoprotectant is a substance that is used to protect biological tissue from freezing damage (damage due to ice formation).

High Performance Liquid Chromatography (HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

Enrichment is defined as the addition of micronutrients to a food which are lost during processing.

Fortification refers to the practice of deliberately increasing the content of an essential micronutrient, i.e. vitamins and minerals (including trace elements) in a food irrespective of whether the nutrients were originally in the food before processing or not, so as to improve the nutritional quality of the food supply and to provide a public health benefit with minimal risk to health.

Folic acid (also known as vitamin B_9, vitamin B_c or folacin) and folate (the form naturally occurring in the body), as well as pteroyl-L-glutamic acid, pteroyl-L-glutamate, and pteroylmonoglutamic acid are forms of the water-soluble vitamin B_9.

Vitamin B_{12} also called cobalamin, is a water-soluble vitamin with a key role in the normal functioning of the brain and nervous system, and for the formation of blood. It is normally involved in the metabolism of every cell of the human body, especially affecting DNA synthesis. It is the largest and most structurally complicated vitamin and can be produced industrially only through bacterial fermentation-synthesis

DNA is a nucleic acid containing the genetic instructions used in the development and functioning of all known living organisms (with the exception of RNA viruses). The DNA segments carrying this genetic information are called genes. DNA is one of the three major macromolecules that are essential for all known forms of life.

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

CHAPTER 1

INTRODUCTION

1.1 Background to the research problem

Vitamins are essential for the growth, self-maintenance and functioning of human beings. They play a specific and vital function in metabolism, and their lack or excess produces specific diseases. Thirteen vitamins are recognized in human nutrition and these are classified according to their solubility. The fat-soluble vitamins are represented by vitamins A, D, E and K. The water-soluble vitamins comprise of vitamin C and members of the vitamin B group, namely thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin, pyridoxine (vitamin B_6), pantothenic acid (vitamin B_5), biotin, folate and vitamin B_{12}. With the possible exception of vitamins D and K, these vitamins must be supplied by the diet because they cannot be produced in adequate amounts by the human body (Li & Chen, 2001).

Vitamins might be lost during processing and storage of food through chemical reactions. Thus, it is extremely important to have available preparations to compensate for the possible lack of vitamins in the daily diet, which is why multivitamin pharmaceuticals are becoming widely employed. This has stimulated research on accurate and efficient analytical methods for the quality control of these complex preparations (Moreno & Salvadó, 2000).

The disguised deficiency of vitamin B_{12} by using folate is an on-going issue that is being discussed worldwide in relation to mandatory fortification of foods with folate. The metabolic pathway that is shared partially by these two B-vitamins allows that a high intake of folate can rectify anaemia caused by a vitamin B_{12} deficiency (Roddie & Davis, 2009). However, as a result, vitamin B_{12} deficiency remains unrecognized and eventually progresses to irreversible problems associated with low vitamin B_{12} status such as dementia and Alzheimer's disease, with persistent vitamin B_{12} deficiency leading to neurological damage that have been linked with psychiatric disorders (Shane, 2003; Ulrich & Potter, 2006; Refsum & Smith, 2008). The aforementioned imbalance can be prevented by administering the optimal folate-vitamin B_{12} mass ratio of 170:1 in the diet (Santos *et al*., 2008; Winkels *et al*., 2008). According to a recent World Health Organization (WHO) evaluation, folate and

vitamin B_{12} deficiencies may be a public health problem affecting millions of people (Anonymous, 2008; Hugenschmidt *et al*., 2010). Anaemia and an increased risk of birth defects in new-borns are some of the health consequences of low folate and vitamin B_{12} status. Folate can be produced both by plants and microorganisms, whereas vitamin B_{12} can only be synthesized by some bacteria and archaea (Levin *et al*., 2004).

Food-grade microorganisms, lactic acid bacteria (LAB) and propionic acid bacteria (PAB) are known to produce high levels of folate, whereas PAB can be used for the commercial production of vitamin B_{12}.

1.2 Statement of the research problem

Micronutrient malnutrition has often gone unnoticed and a consequence thereof is major health problems such as neural tube defects (NTDs) and other illnesses. The worldwide average of NTDs is 1 in 500 to 800 births which has raised concern with regards to vitamin enrichment plans, therefore, making this an important field of research (Dolk *et al*., 1991). The enrichment of food (kefir beverage) with vitamin B_{12} and folate may decrease the incidence of NTDs and other illnesses. It is cost effective due to the kefir beverage being a naturally B-vitamin enriched fermented beverage (Van Wyk *et al*., 2002).

Novel food products naturally enriched with vitamin B_{12} and folate could address insufficient vitamin intake which are common in many parts of the world, both developing and industrialised countries. Furthermore, the concept of food fortification by bacterial fermentation opens the way for development of food products directed at specific groups in society such as the elderly and adolescents. These innovative strategies could be modified by the food industry for the development of novel vitamin-enhanced functional foods with enhanced consumer appeal. Consumers globally are becoming increasingly more health-conscious and thus more discerning about their food choices. The production of fermented food products with naturally elevated levels of B-vitamins, i.e. increased nutritional value, hence eliminates any need for fortification using synthetic vitamin preparations (Burgess *et al*., 2009).

Hence there is a need to develop a process for the simultaneous production of natural B-vitamins such as folate and vitamin B_{12}, using a mixed culture of food-grade microorganisms for the production of naturally fortified food products.

1.3 Broad objectives of the study

The aims of this study were: firstly to (a) measure the vitamin B_{12}, folate and pH levels, and (b) to determine the sensorial quality of the kefir beverage, and (c) to confirm the presence of PAB in the kefir grains as a function of the form of the initial inoculum and the sampling time. Secondly, to (a) determine vitamin B_{12}, folate, pH levels, and (b) the sensorial quality of the kefir beverage co-inoculated with both LAB and PAB, and (c) to confirm the presence of PAB in the kefir grains as a function of the form of the initial inoculum and the sampling time, in order to determine which procedure results in the ideal concentration of the B-vitamins in the enriched kefir beverage and in effective preservation of PAB added in the kefir grains.

1.3.1 Specific objectives of the study

The specific objectives were the application of a mixed culture, i.e. kefir grains containing: (1) a high vitamin B_{12} producer, *P. freudenreichii* and (2) both a high vitamin B_{12} producer, *P. freudenreichii* and high folate producer, *S. thermophilus*. These vitamin-producing strains were selected for application in this study based on proven vitamin yields of folate and vitamin B_{12}. The effect of using freeze-dried *versus* broth cultures as well as the effect of sampling time (1 d and 3 d), will also be investigated. The different co-culture processes and sampling time will then be compared with the aim of identifying the process that will afford the optimum levels of folate and vitamin B_{12} in the enriched kefir, as well as the preservation of PAB in the kefir grains.

1.4 Hypotheses

All of the following hypotheses are non-statistical. It was hypothesised that the kefir grains reacted with freeze-dried cultures will produce a higher concentration of vitamin B_{12} and folate than kefir grains reacted with broth cultures (Van Wyk *et al*., 2011).

Since LAB produces folates extracellularly during milk fermentation (Lin & Young, 2000), it was hypothesised that the LAB co-inoculated with PAB used in this study will contribute a higher concentration of folates than when inoculating with PAB alone.

It was hypothesised that the sampling time at 3 d will have a higher

concentration of vitamin B_{12} and folate. However, at 1 d the beverage will have a greater sensorial acceptance.

1.5 Delineation of the research

For the production of B-vitamins, two types of microorganisms were investigated, namely *Propionibacterium freudenreichii* subsp. *shermanii* strain J19 and *Streptococcus thermophilus* ATCC 19258. Two B-vitamins were investigated, namely vitamin B_{12} and folate. The fermentation temperature that was employed will be 30°C, and two different sampling times, *viz*. 1 d and 3 d, were used.

The kefir beverage was used as a B-vitamin enriched food product in which the production of folate and vitamin B_{12} was measured.

Only High Performance Liquid Chromatography (HPLC) methods were used to determine the concentration of folate and vitamin B_{12} in the kefir grains. All statistical data analyses were performed using SPSS 19.0 for Windows®.

1.6 Significance of the study

The significance of the research is the potential impact of a vitamin enriched beverage suitable for all ages in the prevention of folate and vitamin B_{12} deficiency, hence promoting good health. Companies producing dairy products will benefit in particular because the beverage produced during this study was a fermented milk product.

1.7 Expected outcomes, results and contributions of the research

The expected outcome was to improve the functionality of a food product by using food-grade bacteria to alter the fermentation processes, which may yield a higher concentration of vitamins to promote and sustain human health. There is still significant room for growth and development of kefir beverages, and manufacturers in the dairy industry may benefit from increased marketing efforts in order to increase the consumption of kefir beverages within South African households. In order to do this, they need to ensure that they cater to the specific needs of consumers. This may be in the form of a high B-vitamin beverage with a good sensorial quality.

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CHAPTER 2

LITERATURE REVIEW

2.1 History of Kefir

Kefir dates back many centuries and originated in the Caucasus mountains in the Russian region situated between the Black Sea and the Caspian Sea (Duitschaever *et al*., 1987). The tribes and shepherds in this region first used this traditional fermented dairy product. The long life-span of the Caucasian people has been attributed to their high consumption of kefir. It was also said that the resulting kefir grains were a gift from Mohammed to Orthodox Muslims. The kefir grains or "grains of the prophet" were safely guarded as a family or tribe's wealth (Powell *et al*., 2007). Moreover, kefir grains are also considered to be “manna of milk” and it was used by the Israelites as mentioned in the Bible and Jewish Torah. Due to the reuse of these grains, the traditional method of culturing milk to produce a milk beverage has been passed on from generation to generation (Chiffolo & Hesse, 2006).

2.2 Kefir grains

The structure of the kefir grains resembles small cauliflower florets (Libudzisz & Piatkiewicz, 1990). Kefir grains are 0.3 − 3.5 cm in length, are lobed and irregularly shaped. The colour characteristics of the grains are white to yellow-white, with a firm, yet slimy texture (Garrote *et al*., 1996). The slimy texture of the grains is due to the formation of kefiran. Kefiran is an exopolysaccharide consisting of D-glucose and D-galactose produced by *Lactobacillus kefiranofaciens*, one of the vast consortium of microorganisms present in the kefir grains. The repeating hexasaccharide structures of kefiran have been determined by using mainly methylation analysis and NMR data (Yokoi *et al*., 1991; Maeda *et al*., 2004).

2.3 Microorganisms present in kefir

The symbiosis of the various LAB and yeasts present in kefir grains plays an important role in the distinctive flavour thereof. In recent years, molecular techniques, such as PCR-DGGE and DNA purification have been used to identify the microbial population in kefir grains (Garbers *et al*., 2004). The bacterial genera

present in kefir grains are homofermentative and heterofermentative *Lactobacillus* (*Lb*.), *Lactococcus* (*L*.) and *Leuconostoc* (*Leuc*.) (Simova *et al*., 2002; Santos *et al*., 2003; Yüksekdağ *et al*., 2004). The organisms that have been isolated and identified include *Lb. brevis*, *Lb. viridescens*, *Lb. gasseri*, *Lb. fermentum*, *Lb. casei* (Kandler & Kunath, 1983; Marshall *et al*., 1984; Angulo *et al*., 1993), *Lb. acidophilus* (Marshall, 1993) and *Lb. kefiranofaciens* (Fujisawa *et al*., 1992; Mukai *et al*., 1992). *Lb. kefirgranum*, *L. parakefir*, *Lb. plantarum*, *Lb. lactis* subsp. *lactis*, *Lactococcus lactis* subsp. biovar *diacetylactis*, *Lb. kefir* and *Lb. paracasei*, as well as *Leuc. mesenteroides* and *Acetobacter*, were also identified (Takizawa *et al*., 1994; Garrote *et al*., 2001; Santos *et al*., 2003). Vancanneyt *et al*. (2004) reported that *Lb. kefirgranum* should be reclassified as *Lb. kefiranofaciens* subsp. *kefirgranum* due to the similarity of the 16S rDNA sequence of these organisms. *Lb. kefiranofaciens* is a homofermentative lactic acid bacterium that differs from other homofermentative species of the genus lactobacillus in terms of its carbohydrate fermentation. Moreover, four different strains of *Lb. kefiranofaciens* have been isolated in kefir grains from Argentina (Fujisawa *et al*., 1992).

The yeasts isolated from kefir include *Saccharomyces cerevisiae* (Rohm *et al*., 1992), *Candida kefir*, the anamorphic form of *Kluyveromyces lactis* (Engel *et al*., 1986), *Torulopsis holmii* (Iwasawa *et al*., 1982), *C. holmii*, *S*. *unisporus* (Angulo *et al*., 1993), *K. marxianus* (Rohm *et al*., 1992), *T. delbrueckii*, *C. friedricchi* and *Pichia fermentans* (Rohm *et al*., 1992). Latorre-Garcia *et al*. (2007) isolated yeast colonies from various homemade and commercial kefir samples. The use of DNA purification with PCR amplification revealed that the most prevalent yeast species in these kefir samples were *K. lactis*, *Issatchenkia orientalis*, *S. unisporus*, *S. exiguus*, and *S. humaticus*, while Wang *et al*. (2008) identified *K. marxianus*, *S. turicensis* and *P. fermentans* in Taiwanese kefir grains.

2.4 Storage and cultivation of kefir grains

An important limitation in industrial kefir production is the slow kefir grain growth. Schoevers and Britz (2003) studied the factors that affect kefir grain proliferation in terms of parameters such as incubation temperature, culture medium enrichment and cultivation with or without agitation. A significant increase in biomass occurred when grains were cultured in low-fat milk with enrichment media. However, when low-fat or full-fat milk was used without enrichment, the kefir grain biomass also

increased. Gorsek and Tramsek (2007) determined the effects of process parameters on kefir grain mass increase by using an experimental design by Taguchi. They determined that the rotational frequency of the stirrer had some influence on kefir grain growth, whereas glucose concentration and culture temperature had less effect. Kuo and Lin (1999) established that incubation temperature had an effect on the mass increase of kefir grains and the length of time required to make kefir. At a low incubation temperature (15°C), the time to reach pH 4.7 was 31 – 35 h, with the total mass increase of kefir grains 12 – 16%. At a high incubation temperature (30°C), the incubation was completed in 14 – 15 h, but the total mass of the kefir grains increased to approximately 8 – 11% only. Hence, the lower temperature favoured biomass increase.

Newly purchased kefir grains become fully active after two or three propagations (Kosikowski & Mistry, 1999). The viability of the kefir grains depends on a daily transfer into fresh milk, allowing growth for approximately 20 h. During this period, the initial mass is increased by up to 25% (Hallé *et al*., 1994). However, water used to wash kefir grains may cause a reduction in its microbiological viability. Therefore, in commercial production it is recommended that grains are kept viable by means of daily transfers into a fresh batch of milk and that replacement of the grains only occur when their ability to ferment milk is impaired (Farnworth, 2005).

Kefir grains that replicate in milk at 4°C have been shown to have microbiological profiles that are different to that of fresh kefir grains (Pintado *et al*., 1996). Moreover, kefir grains have a limited shelf-life when left wet. When stored at 4°C in this condition, they lose activity within 8 to 10 d if not used (Tietze, 1996). To extend their shelf-life, kefir grains can be dried at room temperature for 36 – 48 h and stored in a cool and dry place until reused, or grains can be frozen and kept at −20°C for extended storage (Garrote *et al*., 1997).

2.5 Kefir beverage

Kefir fermentation originated when milk was kept in sheep-skin bags by the Caucasian shepherds under uncontrolled conditions in terms of temperature and the mixed culture of lactic acid bacteria present in the bag. The formation of the grains was attributed to the rocking motion of the bag when carried on the back of the shepherd (Powell *et al*., 2007). Once the grains were formed, the milk was then added to the kefir grains for fermentation to occur, followed by separation from the

grains to provide a refreshing beverage. Hence, traditionally the kefir beverage was produced by adding kefir grains that consisted of a complex consortium of microorganisms, embedded in a complex protein and polysaccharide matrix, to milk. The beverage is the result of the microbial action of lactic acid bacteria (LAB), yeast and acetic acid bacteria contained in the grains (Hallé *et al*., 1994).

The commercial kefir beverage is traditionally manufactured from cows' milk, but buffalo and goats' milk have also been used. The kefir beverage is a viscous and slightly carbonated fermented dairy beverage that contains small quantities of alcohol (Koroleva, 1988). The initial kefir grain mass ratio used as the inoculum in the manufacturing of the beverage affects the quality of the beverage in terms of the pH, viscosity and the microbial profile of the final beverage (Garrote *et al*., 1998). In certain manufacturing processes, the activated starter culture is added to homogenized and pasteurized milk containing 2 – 5% milk fat. It is reported that the optimum ratio of grain to milk ranges from 1:30 to 1:50 (Koroleva, 1991). When using a inoculum concentration of 2 – 5% kefir culture in milk, the pH drops gradually to 4.6 and the fermentation at 25°C is completed in approximately 20 – 24 h, which gives sufficient time for the formation of taste and aroma substances (Simova *et al*., 2002; Ertekin, 2008). After fermentation, the product is stored at refrigeration temperatures (Garrote *et al*., 1997).

The complex mixture of microorganisms, constituting the kefir grains, produces distinctive and unique sensorial properties in the fermented milk (Farnworth & Mainville, 2003). The taste of the natural kefir beverage has been described as a refreshing "yeasty" taste and the terms "sparkling" and "prickling" have been used to describe the mouth-feel of the kefir beverage caused by the liberation of CO_2 (Kemp, 1984). The distinctive taste of kefir is a result of several flavour compounds which are produced during fermentation (Beshkova *et al*., 2003).Compounds such as diacetyl and acetoin have received particular attention owing to their flavour contribution to kefir (Güzel-Seydim *et al*., 2000; Yüksekdağ *et al*., 2004). Another distinctive aspect of kefir is the acidic taste created by the relatively high levels of lactic and some propionic acid. While Bottazzi *et al*. (1994) reported the occurrence of acetic acid in the kefir beverage, others reported that no acetic acid was present (Güzel-Seydim *et al*., 2000). Although not universally unacceptable, Duitschaever *et al*. (1987) showed that in Canada, 40% of the people evaluating the taste of natural kefir beverages for the first time gave it a low taste

rating due to the overwhelming acidic taste. The addition of flavours or the modification of the fermentation process to achieve less acidity and a creamier taste increased the acceptability of the beverage, compared to the standard kefir beverage (Duitschaever *et al*., 1991; Muir *et al*., 1999).

As mentioned, major end products of the fermentation are lactic acid, acetaldehyde, acetoin, diacetyl, ethanol and CO_2 (Güzel-Seydim *et al*., 2000). However, the chemical and microbiological composition of the kefir beverage varies widely and depends on the origin of the grains, the fermentation method and the different types of milk used during fermentation (Kneifel & Mayer, 1991; Farnworth & Mainville, 2003). The fermentation leads to substantial changes in nutrient composition (Bottazzi *et al*., 1994). Firstly, lactic acid, the organic acid present in a relatively high concentration after fermentation, is derived from approximately 25% of the original lactose present in the starter milk (Alm, 1982; Dousset & Caillet, 1993).

Secondly, during the first hours of fermentation, the bacteria in the grains consume free amino acids found in milk. As the fermentation rate decreases and the kefir enters its ripening stage, the proteolytic activity of other microorganisms such as acetic acid bacteria and yeasts allows the formation of more peptides and free amino acids in a manner similarly found in other fermented milk products (Farnworth & Mainville, 2003). During fermentation, the amino acids valine, leucine, lysine and serine are formed *de novo*, while the quantities of alanine and aspartic acid are increased when compared to raw milk (Alm, 1982).

Thirdly, Kneifel and Mayer (1991) found that substantial amounts of pyridoxine, vitamin B_{12}, folic acid and biotin were synthesized during kefir production, depending on the source of kefir grains used. These authors also reported a reduction in thiamine and riboflavin levels. These results contrast with Alm (1982) who reported decreases in biotin, vitamin B_{12} and pyridoxine and significant increases in folic acid, as compared to non-fermented milk.

Fourthly, yeasts and heterofermentative lactic acid bacteria in the symbiotic mixture of microorganisms are responsible for the production of ethanol and CO_2 during the fermentation of the kefir beverage (Kuo & Lin, 1999). Ethanol and CO_2 levels depend on the production conditions during the fermentation of kefir. The CO_2 content in kefir is fairly low compared to other fermented beverages (Koroleva, 1982), with 0.85 − 1.05 $g.L^{-1}$ of CO_2 produced by the yeasts (Beshkova *et al*., 2002; Simova *et al*., 2002). The ethanol content may be between 0.0 – 0.1 $g.100\ mL^{-1}$

when kefir is made with a starter culture during fermentation and 0.03 – 1.8 g.100 mL^{-1} in kefir produced by kefir grains (Bottazzi *et al*., 1994).

Lastly, kefiran is a polysaccharide obtained from kefir and comprises of a water-soluble branched glucogalactan having equal amounts of D-glucose and D-galactose units. Kefiran is produced by the microorganisms contained in the kefir grains and is the major polysaccharide in the embedding matrix (Rimada & Abraham, 2006).

Other LAB also produce EPS other than kefiran. EPS produced by LAB can be subdivided into two groups: (1) homopolysaccharides and (2) heteropolysaccharides. Homopolysaccharides consist of four subgroups, namely α-D-glucans, β-D-glucans, fructans and polygalactan. In terms of the structure, homopolysaccharides are primarily composed of α-1,6-linked glucose residues with variable degrees of branching i.e. α-D-glucans (dextrans) produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuc. mesenteroides* subsp. *dextranicum* and β-D-glucans, (composed of β-1,3-linked glucose molecules with β-1,2 branches, are produced by *Pediococcus* spp. and *Streptococcus* spp.). Fructans are mainly composed of β-2,6-linked D-fructose molecules, such as levan with some β-2,1 branching, while polygalactan is composed of structurally identical units with different glycosidic linkages. Heteropolysaccharides are produced by mesophilic LAB (*Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Lactobacillus casei*, *Lb. sake*, *Lb. rhamnosus*) and thermophilic LAB (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus* and *S. thermophilus*) (De Vuyst *et al*., 2001; Ruas-Madiedo *et al*., 2002). The kefiran produced by *Lb. kefiranofaciens* adds viscosity and mouth-feel to the beverage (Rattray & O'Connell, 2011).

2.6 Antimicrobial activity of kefir

Kefir grains possess antimicrobial activity due to the wide variety of lactobacilli which are capable of producing a wide range of antimicrobial compounds, including organic acids (lactic and acetic acids), carbon dioxide, hydrogen peroxide, ethanol, diacetyl and peptides. The peptides have been shown to act as bacteriocins (Powell *et al*., 2006). Yüksekdağ *et al*. (2004) reported that 11 out of 21 strains of kefir lactococci produced hydrogen peroxide, while *C. albicans* and *S. cerevisiae*, *Leuc. mesenteroides* and *Lb. plantarum*, isolated from kefir grains, have been shown to produce antimicrobial compounds that are present in kefir.

These antimicrobial compounds are beneficial in reducing food-borne pathogens and spoilage bacteria during food production and storage (Powell *et al*., 2006). The inhibitory effect of the organisms present in kefir against *Listeria innocua* was attributed to the production of bacteriocins by the microbial population. However, the inhibitory effect on *Escherichia coli* was attributed to the combined effect of the acids and hydrogen peroxide. Garrote *et al*. (2000) also confirmed the inhibitory activity against Gram-negative and Gram-positive bacteria in cow's milk which was fermented with kefir grains. Their results confirmed that Gram-positive microorganisms were inhibited to a much larger extent than Gram-negative microorganisms. These authors also demonstrated that milk containing lactic acid or a combination of lactic acid and acetic acid, both present in the kefir beverage, had inhibitory activity against *E. coli*. In addition to the aforementioned organisms, fresh kefir grains were found to inhibit the growth of *Streptococcus aureus* and *Klebsiella pneumonia* (Brialy *et al*., 1995; Yüksekdağ *et al*., 2004).

Moreover, the bacteriostatic properties of the organic acids produced during kefir fermentation could occur even in the early stages of fermentation (Pacheco & Galindo, 2010), while fermentations of kefir and yoghurt combinations proved to be more effective at pathogen suppression than single inoculum fermentations (Gulmez & Guven, 2003). In addition, the antimicrobial effects inherent in kefir grains can be preserved during lyophilisation, especially when glycerol is added as a cryoprotectant (Brialy *et al*., 1995).

2.7 Health benefits of kefir

Kefir is a natural probiotic. Probiotics in foods that contain live bacteria are beneficial to health (Salminen *et al*., 1998). One of the criteria for probiotic bacteria is the ability to withstand the harsh conditions of the gastrointestinal tract, including extreme pH conditions present in the stomach and the action of bile salts and digestive enzymes (Lee & Salminen, 1995). It is also believed that one way in which probiotic bacteria could protect against pathogenic bacteria would be to compete with or displace pathogenic bacteria by adhering to intestinal epithelial cells (Kirjavainen *et al*., 1998; Fujiwara *et al*., 2001; Gibson & Rastall, 2003). Kefir, which is a milk based product, is able to buffer the pH in the stomach when ingested and thereby provide time for many of the bacteria to pass through to the upper small intestine (Farnworth *et al*., 2005). The study of the effect of diet on the intestinal microbial population is limited

to the analysis of faecal samples, although no detailed human study has been published in which kefir has been used. Marquina *et al*. (2002) used mice to study the effect of consuming kefir in a feeding study that lasted 7 months. The study showed that the numbers of lactic acid bacteria in the mouse small and large intestines increased. Streptococci increased by 1 Log, while sulphite-reducing clostridia decreased by 2 Logs. In addition to their texture-modifying properties, of some polysaccharides produced by LAB, they are shown to have prebiotic (Gibson & Roberfroid 1995), immunostimulatory (Hosono *et al*., 1997), antitumoral (Kitazawa *et al*., 1991) and cholesterol-lowering effects (Nakajima *et al*., 1992).

2.8 *Propionibacterium freudenreichii* – a vitamin B_{12} producing micro-organism

In food products, PAB are widely known for the formation of the “holes” in Swiss cheese. This is the result of CO_2 produced by PAB, accompanied by propionic acid and vitamin B_{12}. Propionibacteria are Gram-positive, non-motile, non-spore forming and pleiomorphic rods (coccoid, bifid or branched). They are anaerobic to aerotolerant and mesophilic bacteria. They belong to the class of Actinobacteria and the order of Actinomycetales (Gautier *et al*., 1992). The genus *Propionibacterium* is divided into “cutaneous” and “dairy” propionibacteria. Based on the DNA homology and cellular similarities (Johnson & Cummins, 1979), dairy PAB are classified into six species (Vorobjeva, 1999), namely *Propionibacterium freudenreichii, P. jensenii, P. theonii, P. acidipropionici, P. coccoides,* and *P. cyclohexanicum.* Cutaneous PAB are also classified into six species: *P. acnes, P. avidium, P. granulosum, P. lymphophilum, P. propionicum,* and *Propioniferaxinnocua.* In contrast to cutaneous PAB, dairy organisms do not produce indole and cannot liquefy gelatin. Therefore, the dairy and cutaneous PAB differ based on their typical natural habitats (Vorobjeva, 1999). The sequenced type strain is *Propionibacterium freudenreichii* subsp. *shermanii* and the strain has a circular chromosome of about 2.5 megabase (Mb) (Gautier *et al*., 1992). This strain belongs to the dairy PAB, grows most rapidly at 30°C under anaerobic conditions and form cream colonies on yeast-extract lactate agar medium in 5 – 6 days.

The dairy propionibacteria, particularly *Propionibacterium freudenreichii* subsp. *shermanii*, also produce characteristic EPS (Montere-Alhonen, 1995). However, less research into the synthesis of exopolysaccharides produced by

propionibacteria (PAB) have been conducted as have been EPS produced from lactic acid bacteria (Laws *et al*., 2001; Ruas-Madiedo & de los Reyes-Gavilan, 2005). The most frequently identified monosaccharides in polysaccharides formed by PAB species are glucose, galactose, mannose and small amounts of glucosamine, galactosamine, fucose, and rhamnose (Crow, 1988; Rancine *et al*., 1991; Gorret *et al*., 2003). With regards to *P. freudenreichii* subsp. *shermanii* (Nordmark *et al*., 2005; Dobruchowska *et al*., 2008), the presence of D-glucose, D-mannose and D-glucuronic acid have been confirmed as the primary structure of the EPS produced.

In the production of vitamin B_{12}, many fermentative processes routinely focus on bacterial growth to achieve high cell densities (Rickert *et al*., 1998). For example, fermentation with cross-flow filtration, fermentation coupled with activated charcoal adsorption, extractive fermentation and electrodialysis have been reported as methods to achieve this (Piao *et al*., 2004b). The nutrient composition of the culture medium, such as the amino acid or mineral composition including cobalt ions, affects the production of vitamin B_{12}. The following two experimental findings led to major improvements in the production of vitamin B_{12}: (i) addition of the precursor dimethylbenzimidazole (DMBI), and (ii) periodic fluctuation of anaerobic and aerobic conditions, with anaerobic incubation in the last phase of fermentation (Ye *et al*., 1996; Ye *et al*., 1999).

2.9 Vitamin B_{12}

Vitamin B_{12} is a compound that is of the cobalt corrinoid family, particularly the cobalamin group (Martens *et al*., 2002). The final products of vitamin B_{12} biosynthesis that occur in nature are 5′-deoxyadenosylcobalamin (coenzyme B_{12}) and methylcobalamin (MeCbl), whereas vitamin B_{12} manufactured industrially is by definition cyanocobalamin (CN-Cbl) (Arkbåge, 2003). The vitamin B_{12} molecule is comprised of three parts: a central corrin ring which contains the central cobalt ion; a lower (alpha) ligand, the 5,6 dimethylbenzimidazole (DMBI); and an upper (beta) ligand, the deoxyadenosyl group or a methyl group (Fig. 2.1) (Chandra & Brown, 2007).

Dairy products contain primarily methylcobalamin (Me-Cbl) and hydroxocobalamin (OH-Cbl) (Kumar *et al*., 2010). Hydroxocobalamin, which is a heat unstable form of the vitamin can be lost during extraction procedures (Jägerstad & Arkbåge, 2003). Therefore, the inclusion of potassium cyanide (KCN) in the

extraction buffer before autoclaving converts the OH-Cbl and various coenzymes into the more heat stable cyanocobalamin (CN-Cbl) while liberating all protein-bound cobalamin. Hence, the extraction buffer used for vitamin B_{12} determination by HPLC analysis usually contains KCN (Van Wyk & Britz, 2010). This synthetic derivative of vitamin B_{12} and hydroxocobalamin are available for medical use, food fortification and multi-nutrient supplements (Arkbåge, 2003). Other types of cobalamins include aquacobalamin (H_2OCbl), nitritocobalamin (NO_2Cbl) and sulphitocobalamin (SO_3Cbl) (Hannibal *et al*., 2004)

2.10 Biosynthesis of vitamin B_{12}

As mentioned, PAB produce vitamin B_{12}. The vitamin is retained intracellularly while propionic acid and acetic acid are excreted extracellularly. All tetrapyrroles are biosynthesised from the same precursor, 5-aminolaevulinic acid (ALA) (Storbeck *et al*., 2010). In most bacteria ALA is made from glutamate and the pathway is known as the C5 pathway. In animals, yeasts and photosynthetic bacteria, ALA is made from glycine and succinyl CoA (Bykhovsky *et al*., 1997), with the reaction known as the Shemin pathway. The rest of the biosynthetic pathway of haem is common to all organisms (Vogt & Renz, 1988). During the biosynthesis of the corrin ring, the ALA molecules are transformed into porphobilinogen (PBG) (Fig. 2.2) (Vorobjeva, 1999). The formation of uroporphyrinogen III is affected by the condensation of the four PGB molecules by means of PBG deaminase (hydroxymethylbilane synthase) (Leeper, 1985). At precorrin-2, the aerobic and anaerobic pathways are quite distinct (Murooka *et al*., 2005). In the aerobic pathway, the insertion of cobalt occurs at the precorrin-2 (Fig. 3) (Scott & Roessner, 2000; Van Wyk, 2002), while in the anaerobic pathway the precorrin-2 is chelated with cobalt to produce cobalt-precorrin-2 (Quesada-Chanto *et al*., 1998). In the aerobic pathway, the methylation of the precorrin-2 at C-20 by methyltransferase produces precorrin-3 (Velišek & Cejpek, 2007). The early insertion of cobalt during the anaerobic pathway requires enzymes with distinct substrate characteristics in comparison to the metal-free intermediates of the aerobic pathway. A further distinction between the anaerobic and aerobic pathway is the manner in which the (corrin) ring structure is promoted. Under aerobic conditions, the C-20 atom of precorrin-3 is oxidized by molecular oxygen and sustained by a iron- sulphur protein (CobG) releasing the C-20 as acetate (Scott *et al*., 1996). Under anaerobic conditions, the ring contraction may be mediated by

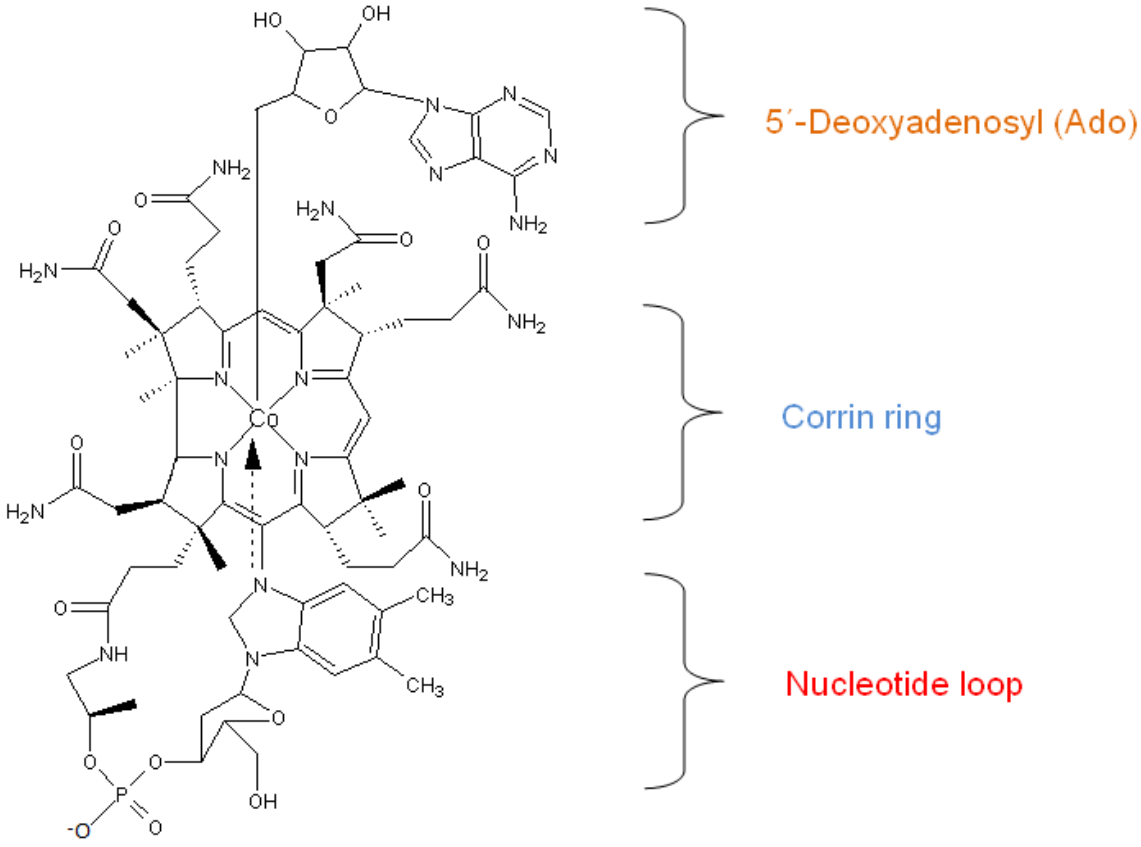


Figure 2.1 The vitamin B_{12} molecule comprising three parts (Chandra & Brown, 2007).

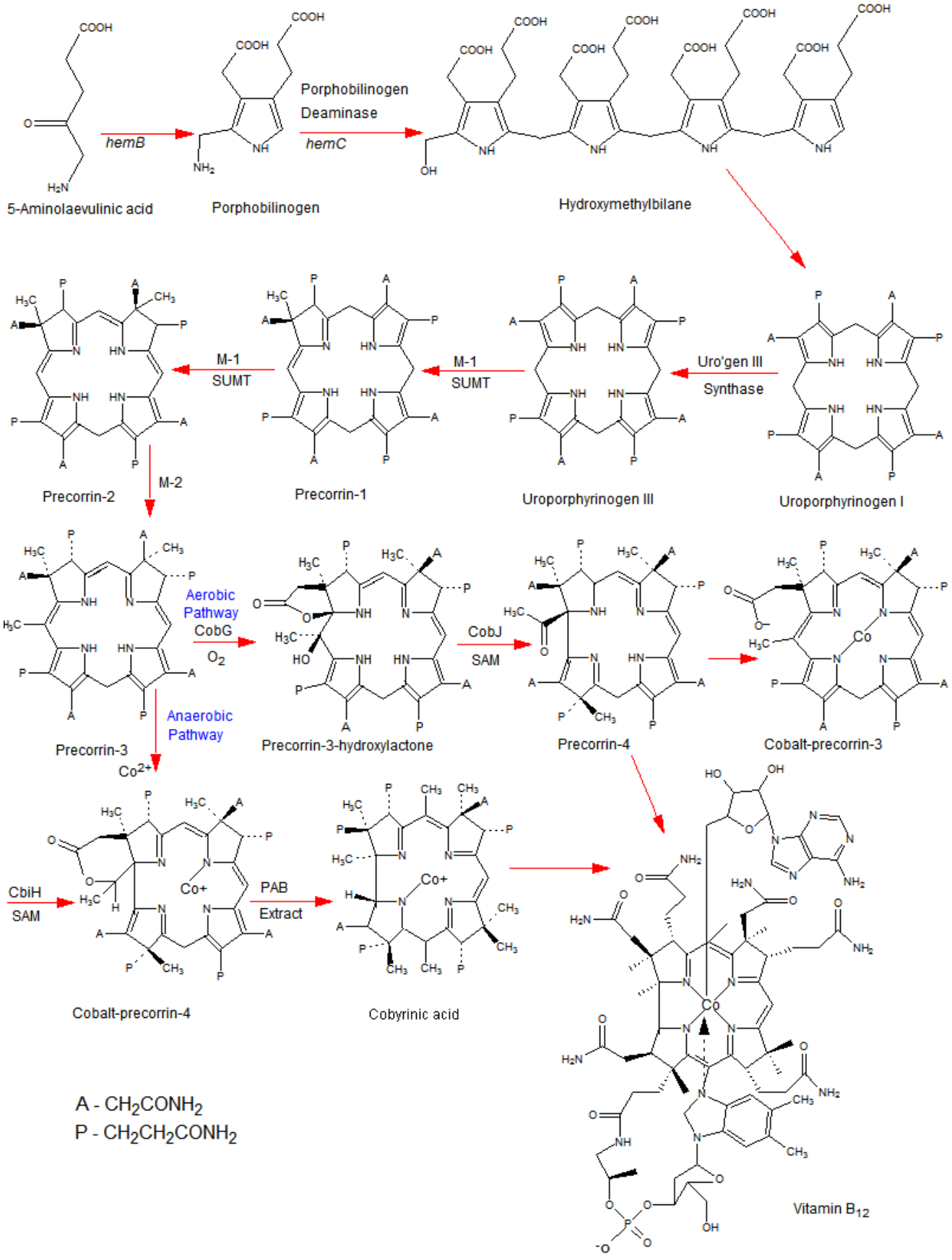


Figure 2.2 The anaerobic and aerobic pathways of vitamin B_{12} biosynthesis (Scott & Roessner, 2000; Van Wyk, 2002)

means of the complex cobalt ion which assists in oxidation, resulting in C-20 being released as acetaldehyde (Fig. 2.2) (Survase *et al*., 2006). While the vitamin B_{12} biosynthetic pathways diverge at precorrin-2, they re-join at the step of cobalt-precorrin-4 (Murooka *et al*., 2005). The formation of the cobalt-precorrin-4 occurs when the enzyme CbiH catalyse methylation at C-17 of cobalt-precorrin-3 allowing a ring contraction to form at cobalt-precorrin-4 (Scott & Roessner, 2000). The cobalt-precorrin-4 is then converted to cobyrinic acid by a cell-free lysate of *P. shermanii* leading to the formation of the vitamin B_{12} (Fig. 2.2) (Santander *et al*., 1997; Scott & Roessner, 2000; Van Wyk, 2002).

2.11 Industrial production of vitamin B_{12}

The industrial production of vitamin B_{12} requires more than 70 steps and the chemical methods to produce vitamin B_{12} are technically demanding and expensive (Martens *et al*., 2002). Therefore, natural vitamin B_{12} has been produced on an industrial scale using the batch process of microbial fermentation (Yongsmith, 1982). Microorganisms, including those of the genera *Bacillus*, *Methanobacterium*, *Propionibacterium* and *Pseudomonas*, have been used to produce vitamin B_{12} industrially. Since some species of *Propionibacterium* have been generally recognized as safe (GRAS) in the United States by the Food and Drug Administration (FDA) and have been awarded European QPS (qualified presumption of safety), it is safe to use these microorganisms as it does not produce endotoxins or exotoxins (Salminen *et al*., 1998; Anon, 2008a; Van Wyk & Britz, 2010). During the production of vitamin B_{12}, the fermentative processes emphasise on bacterial growth to achieve high cell densities (Rickert *et al*., 1998). One of the methods used to achieve a high production of vitamin B_{12} by means of cell densities is to alter the nutrient composition of the culture medium with amino acids or the mineral composition with cobalt ions. The addition of the precursor dimethylbenzimidazole (DMBI) and aerobic incubation in the latter phase of fermentation have led to major improvements in the production of vitamin B_{12} (Roth *et al*., 1996). The primary problem for vitamin B_{12} synthesis using propionibacteria is that the end products such as propionic acid inhibit the cell growth (Hsu & Yang, 1991). Therefore, it is critical to remove the end-product *in situ* to improve the cell growth, which results in the improved production of vitamin B_{12}. A number of fermentation processes have been developed to remove propionic acid and acetic acid (Jin & Yang, 2008). Other

methods to enhance vitamin B_{12} production, such as the use of gene manipulation, have been successful (Survase *et al*., 2006). A common method to enhance vitamin B_{12} production is by using random mutagenesis to generate mutant strains producing increased levels of vitamin B_{12} (Pouwels *et al*., 1999; Martens *et al*., 2002; Piao *et al*., 2004b). Propionibacteria which are cobalt-resistant strains have also been reported to show enhanced vitamin B_{12} production (Seidametova *et al*., 2004). Moreover, increased vitamin B_{12} production in *Propionibacterium freudenreichii* by applying different metabolic engineering strategies has been achieved (Piao *et al*., 2004b). A high yield of vitamin B_{12} has been achieved by treating the microorganism with mutagenic agents such as UV light or chemical reagents (Martens *et al*., 2002).

Moreover, an increase in the precursors or intermediary metabolites of vitamin B_{12} in the cells is expected to result in the overproduction of vitamin B_{12} when using genetic recombinant DNA technology. An example of this is the *P. freudenreichii* strain which was genetically modified with a plasmid having the genes *hemA* from *Rhodobacter sphaeroides* and homologues of *hemB* and *cobA*. These genes caused a 2.2-fold overproduction of vitamin B_{12} (Piao *et al*., 2004a). It was found that multigene expression systems can be used for the improvement of vitamin B_{12} production levels in propionibacteria (Murooka *et al*., 2005). Moreover, advances in molecular biology and biochemistry related to the biosynthesis of vitamin B_{12} have led to the isolation of several enzymes responsible for the synthesis of vitamin B_{12} (Piao *et al*., 2004).

2.12 Bioavailability and deficiency of vitamin B_{12} in humans

Vitamin B_{12} nutrition has two unique features in terms of human nutrition: firstly is the vitamin B_{12} restriction to foods of animal origin; and secondly, the unique human autoimmune disease pernicious anaemia (PA) (Stabler & Allen, 2004). Since plants do not produce or contain vitamin B_{12}, the usual dietary sources of vitamin B_{12} are meat and certain fermented dairy products (Table 2.1) (Arkbåge, 2003; Mozzi *et al*., 2010; Clemens *et al*., 2011). Vitamin B_{12} binds to proteins present in food which is released by gastric enzymes and stomach acid. Once released, it binds to the gastric intrinsic factor (GIF) which is a glycoprotein that is synthesised by gastric cells in the stomach, which is absorbed in the ileum of the small intestine (Allen, 1996). Two proteins, known as haptocorrin and gastric IF, can bind vitamin B_{12} along all levels of

Table 2.1 Presence of vitamin B_{12} in fermented milk products

Product	**Vitamin B_{12} ($\mu g.L^{-1}$)**	**Reference**
Whole cows' milk	0.36	Clemens *et al*., 2011
Blue cheese	1.0 – 2.1	Arkbåge, 2003.
Buttermilk	1.7 – 2.0	Mozzi *et al*., 2010
Kefir	0.8 – 1.0	Mozzi *et al*., 2010

the digestive tract (Guent & Grasbeckl, 1990). Pernicious anaemia is the most common effect of vitamin B_{12} deficiency. It is an autoimmune disease which causes the body's immune system (which normally protects against infection) to hinder the body's healthy cells (Stabler & Allen, 2004).

Vitamin B_{12} deficiencies have been studied in people with PA who have a normal nutritional status. Over time, they tend to develop macrocytic anaemia with characteristic abnormalities of the cell tissue in bone marrow. Due to the resemblance of megaloblastic anaemia caused by folate deficiency, this may lead to difficulty in recognizing vitamin B_{12} deficient megaloblastic anaemia (Allen, 1996). Therefore, the correlation between megaloblastic anaemia and the central nervous system disease and the severity thereof prevents the recognition of vitamin B_{12} deficiency whereby megaloblastic anaemia is used as the indicator of deficient status (Healton *et al*., 1991; Savage *et al*., 1994). Moreover, patients suffering from PA show symptoms of spinal cord, cranial or peripheral nerve, or even cerebral demyelination, while vitamin B_{12} deficiencies in infants and young children have been shown to cause movement disorders. Infants have shown symptoms, which include irritability, abnormal reflexes, and feeding difficulties and, due to delayed diagnosis of the deficiency, the failure of brain growth may cause permanent developmental disabilities (Monagle *et al*., 1997; Graham, 2004). The treatment of milder vitamin B_{12} deficiency may also be important in minimising hyper-homocysteinemia which may be a risk factor for arteriosclerosis. Studies of methylmalonic acid and homocysteine have been compared to vitamin B_{12} levels in patients with PA and it had been proven that these metabolites are elevated prior to development of clinical abnormalities (Lindenbraum *et al*., 1990).

The contribution of vitamin B_{12} deficiency to nutritional anaemia worldwide may have been underestimated due to the frequently coexisting deficiencies of iron, folate and other vitamins resulting from diets with low animal protein (Allen, 1996). Over the past three decades, the diagnosis of PA in African populations has increased. In 1973, South African researchers described patients who had megaloblastic anaemia, but due to the coexistence of malabsorption syndromes, the authors were unable to determine whether it was a true case of PA (Hift *et al*., 1973a; 1973b). However, the comparison of PA in African-American patients from Washington, D.C. and Johannesburg, South Africa, drew the attention to young African women who had a high occurrence of anti-intrinsic factor antibodies and

severe neurologic symptoms (Solanki *et al*., 1981). PA in 80% of the patients also caused the severity of vitamin B12 deficient megaloblastic anaemia from Gambia (Abdalla *et al*., 1986). Another report from Nigeria in 1992 described that eleven patients had PA (Akinyanju & Okany, 1992). PA was the most common cause of megaloblastic anaemia in most hospitals situated in Harare, Zimbabwe (Savage *et al*., 1994). The extreme severity of megaloblastic anaemia and neurologic problems suggests that these cases are only the beginning (Savage *et al*., 1994). These findings emphasise the importance of preventing vitamin B_{12} deficiency.

2.13 Methods of quantifying vitamin B_{12}

The determination of total vitamin B_{12} can be performed by using various methods such as microbiological assays, including AOAC Official Methods 952.30 and 986.23 (Anon, 2006), radioisotope dilution methods (Osterdahl & Johansson, 1988), and HPLC methods (Heudi *et al*., 2006; Van Wyk & Britz, 2010). The microbiological assay is most widely used for the determination of total vitamin B_{12} in foods. However, radioassay kits for clinical samples are not useful for analysis of food samples. Radioisotope dilution methods lack the intrinsic factor (protein) used for the assay which can bind other cobalamins (Indyk *et al*., 2002), while the HPLC method lacks the sensitivity to measure vitamin B_{12} in non-fortified food products.

Extraction procedures are used to liberate cobalamins from protein and, therefore, for converting the naturally occurring cobalamins into a stable form, such as cyanocobalamin or sulphitocobalamin (Ball, 2006; Van Wyk & Britz, 2010). To protect cobalamins in samples, the samples may be flushed with nitrogen gas eliminating the presence of oxygen due to the sensitivity of some forms of vitamin B_{12} to oxygen as described by Heudi *et al*. (2006). If the sample extracts are exposed to light before analysis, adenosylcobalamin and methylcobalamin are completely converted into hydroxocobalamin, so that vitamin B_{12} activity cannot be measured accurately (Muhammad *et al*., 1993). The use of a cyanide-containing buffer obviates this step (Van Wyk & Britz, 2010). The vitamin B_{12} concentrations in foods are expressed in milligrams or micrograms of vitamin B_{12} per 100 g (Table 2.1).

2.14 Folate

Folate is a generic term for a water-soluble vitamin and includes naturally occurring food folates and folic acid found in dietary supplements and those used in food fortification. According to the International Union of Nutritional Sciences (IUNS) Committee on Nomenclature, "folate" is the generic term used to classify all compounds that have similar chemical and nutritional properties to that of pteroyl-L-glutamic acid (folic acid) (Blakley, 1988). The structure of folate may vary due to the reduction state of the pteridine moiety (Vahteristo *et al*., 1996). The pteridine ring present in the folic acid molecular structure exists in an oxidised form, whereas in naturally occurring folates the reduction of the pteridine ring occurs to form dihydrofolate or tetrahydrofolate by the addition of two or four hydrogen atoms (Witthöft *et al*., 1999).

Dietary folate often exists as a one carbon substituent form of pteroylglutamate and may have up to fourteen glutamyl residues linked to the para-aminobenzoic moiety by means of peptide linkages (Fig. 2.3) (Garrat *et al*., 2005; Le Blanc *et al*., 2007; De Brouwer *et al*., 2008). There are five various types of one carbon substituents known to link at the positions N 5- and/or N 10- on the pteroyl group, namely CH_3-(5-methyltetrahydrofolate), HCO-(5-or 10-formyltetrahydrofolate), CHNH-(5-formiminotetrahydrofolate), -CH_2-(5,10 methylenetetrahydrofolate), or CH=(5,10-methenyltetrahydrofolate). Tetrahydrofolate (THF) is one of the reduced folate derivatives that exists without substituent's (Arkbåge, 2003).

All folates may undergo oxidative degradation which is enhanced by oxygen, light and heat. The degradation of folate may result in the complete disintegration of the molecule into inactive forms (Eitenmiller & Landen, 1999). The stability of folate differs amongst various forms of reduced folate with THF the least stable. Moreover, the stability of folate is also pH dependent (Gregory, 1996). One of the major end products of folate undergoing oxidative cleaving is para-aminobenzoylglutamate which occurs at pH 4, 7 and 10 (Arkbåge, 2003). The highest stability of folate is achieved at pH 4 – 8 at 37°C, except tetrahydrofolate and dihydrofolate, which are degraded at low pH (De Brouwer *et al*., 2007). The increased state of oxidative stability are usually present at the substituent positions N 5-and/or N 10- of the reduced folates. Therefore, the vulnerability of folate to undergo major loss during food processing depends on the food matrix and composition as it is less stable than folic acid. In order to prevent folate degradation, antioxidants such as ascorbic acid

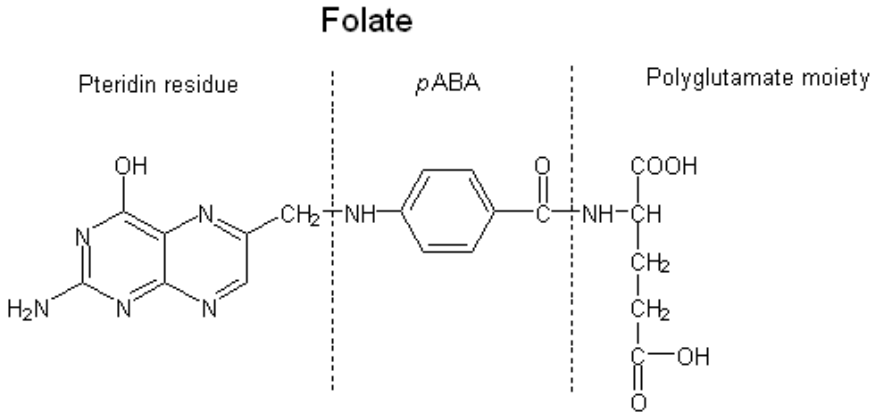
and 2-mercaptoethanol are usually used as reducing agents which protect and stabilise folates during analyses (Quinlivan *et al*., 2006).

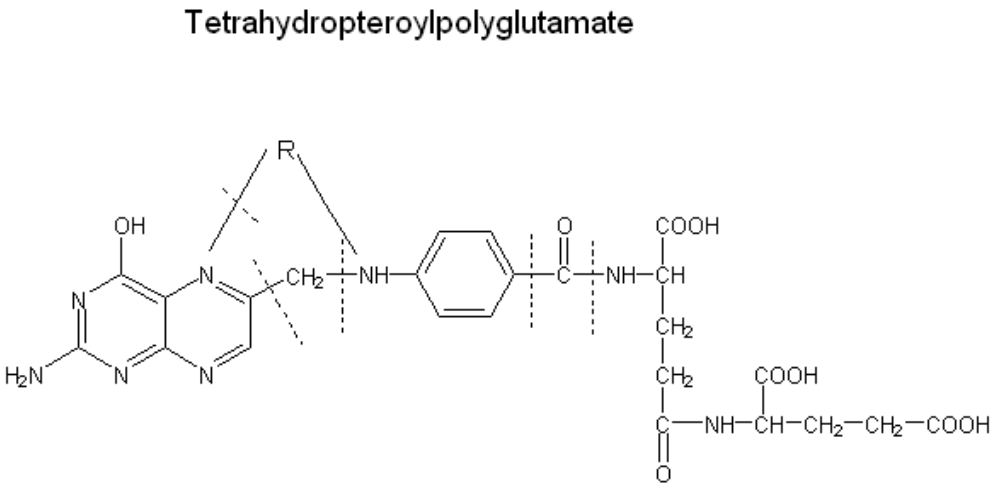
2.15 Folate producing microorganisms

Numerous studies concerning the synthesis of folate-producing lactic acid bacteria have been reported (LeBlanc *et al*., 2007). Industrial starter bacteria *Lactococcus lactis* and *Leuconostoc* species have the ability to synthesize folate (Lin & Young 2000; Hugenholtz & Smid, 2002). Although yoghurt starter cultures such as *L. lactis* have the ability to produce folate, this important vitamin may also be synthesised by other LAB species such as *Lb. acidophilus, Leuconostoc lactis, Bifidobacterium longum* and some strains of propionibacteria which also produce large amounts of folate (Lin & Young, 2000; Hugenholtz & Smid, 2002; Crittenden *et al*., 2003; Sybesma *et al*., 2003b). Propionibacteria may have similar characteristics as *Streptococcus thermophilus* with regards to folate production, but *S. thermophilus* has been shown to produce more folate (Smid *et al*., 2001; Mousavi *et al*., 2013).

However, *S. thermophilus* may also consume folates during the fermentation process (Hugenholtz & Smid, 2002; Holasova *et al*., 2004). Therefore, the major limitation when utilising these organisms for bio-fortification of folate, is the possibility that the folate present in the fermented milk product may be utilised by co-cultures when used as a starter (Iyer & Tomar, 2009).

However, among these food-grade bacteria and dairy starters, *S. thermophilus* has been regarded as a good folate producer. This organism produces folate extracellularly in the milk products during fermentation (Iyer & Tomar, 2009), unlike *Lb. lactis* and *Leuconostoc* which accumulate folate intracellularly, and therefore, the folate is not excreted into the milk. *Streptococcus salivarius* subsp. *thermophilus* (common name *Streptococcus thermophilus*) is a Gram-positive bacterium and homofermentative facultative anaerobe which does not form endospores (Prescott *et al*., 1996). It is classified as a lactic acid bacterium (LAB) and is generally regarded as safe (Courtin & Françoise, 2003). This organism is generally used in the production of yoghurt alongside *Lactobacillus bulgaricus* (Kiliç *et al*., 2006). The folate produced by *S. thermophilus* may be used by *Lb. bulgaricus* for purine synthesis, but *S. thermophilus* has the ability to produce folate at a higher quantity in comparison to other LAB (Crittenden *et al*., 2003; Papapstoyiannidis *et al*., 2006). It was shown to elevate folate levels in skim milk, whereas lactobacilli have been found





Substituent (R)	Position
$-CH_3$ (Methyl)	5’(5-MeTHF)
-CHO (Formyl)	5’ or 10’(5-CHOTHF)
=CH- (Methenyl)	5’ and 10’(5, 10-CH-THF)

Figure 2.3 The structure of folic acid and natural food folate (Le Blanc *et al*., 2007; De Brouwer *et al*., 2008).

to deplete the available folate in the skim milk. Sybesma *et al*. (2003b) reported that the highest folate level produced by *S. thermophilus* was as high as 0.214 µg.L^{1}, while Holasova *et al*. (2004) reported a 6-fold increase in 5-MeTHF content produced by *S. thermophilus*. Tomar *et al*. (2009) confirmed that the culture strain NCDC177produced 35 µg.mL^{-1} of folate and was regarded the best folate producer among the *S. thermophilus* cultures.

2.16 Folate concentration in fermented and non-fermented foods

Folate is an essential B-vitamin in the human diet as it involves many metabolic, pyrimidine and amino acid biosynthetic pathways (Iyer & Tomar, 2009). Vegetables play an important role in a well-balanced diet providing sufficient amounts of vitamins, including folate (Johansson *et al*., 2007). Vegetables and pulses are rich sources of folate with concentrations of up to 600 µg.100g^{-1} in some beans and chick peas and leafy vegetables with typical concentrations of 200 µg.100g^{-1} (Witthöft *et al*., 1999). In meat and meat products folates are present in fairly low to moderate concentrations, whereas milk has a typical concentration of 5 µg.100g^{-1}, irrespective of its source. Milk products such as cheese have been shown to have a higher concentration of folate (40 µg.100g^{-1}) (Mozzi *et al*., 2010) as a result of fermentation and the starter cultures which produce folate extracellularly (Wigertz, 1997).

2.16.1 Fermented dairy products

As mentioned, milk is not rich in dietary folate. However, microbial fermentations produce a variety of dairy products with significantly increased folate concentrations in the final product (Table 2.2). In most countries around the world, yoghurt constitutes a greater portion of the *per capita* consumption of fermented milk products (Kneifel *et al*., 1989). In contrast, kefir is a newly discovered milk product in certain countries, although its popularity has been well established in countries such as Hungary, Poland and the former Soviet Union (Ötles & Cagini, 2003). It is also well known in some Scandinavian countries as well as Germany, Greece, Austria, Brazil and Israel (Hallé *et al*., 1994). In addition to kefir's attractive sensory aspects as a fermented milk product, it is also rich in folate causing consumers to become aware of the nutritional and physiological properties of kefir (LeBlanc *et al*., 2007).

2.16.2 Non-dairy foods

The use of microorganisms has the capability to increase the folate content in a variety of non-dairy foods. The fermentation of rye sourdough is often accompanied by an increase in folate content (Kariluoto *et al*., 2006). Proper strain selection, i.e. exchanging folate consuming LAB with folate producing ones could significantly increase folate content in these breads. It has also been reported that it is possible to select lactic acid-producing starter cultures that produce significant amounts of 5-MeTHF (almost doubling its concentration) during fermentation of vegetables (Jägerstrada *et al*., 2004).

2.17 Biosynthesis of folate

All cells require reduced folate cofactors for the biosynthesis of cellular components. Tetrahydrofolate serves as a donor of one-carbon units in a variety of biosynthetic processes, namely the formation of methionine, purines and thiamine (Bermingham & Derrick, 2002). As mentioned, folate is required and produced by plants and microorganisms, while animals do not synthesize folate and it is, therefore, required exogenously. Moreover, bacteria and plants can synthesize the vitamin *de novo* by means of essentially the same biosynthetic pathway (Fig. 2.4) (Bermingham & Derrick, 2002). The common precursor of the folate molecule is 6-hydroxymethyl-7, 8- dihydropterin pyrophosphate (DHPPP). The DHPPP then binds to the para-aminobenzoic acid (*p*ABA) (Dosselaere & Vanderleyden, 2001) and this step plays an important role in the *de novo* biosynthesis of folate.

The biosynthetic pathway of DHPPP commences by means of the transformation of guanosine triphosphate (GTP). The first step is catalysed by GTP cyclohydrolase I (EC 3.5.4.16) which involves the conversion of GTP *via* the Amadori rearrangement, forming a pterin ring structure. Dephosphorylation of the pterin molecule occurs as well as aldolase and pyro-phosphokinase reactions, which produce the activated pyro-phosphorylated DHPPP (Rossi *et al*., 2011). The pyruvate undergo the Shikimate pathway, which allows the biosynthesis of aromatic amino acids and *p*ABA (Dosselaere & Vanderleyden, 2001) *via* intermediates. The first intermediate is shikimate, which is converted to chorismate. Aminodeoxychorismate synthase (EC 2.6.1.85) then converts chorismate into 4- amino-4-deoxyhydrochorismate. Cleaving

Table 2.2 Folate concentrations in dairy products

Product	Folate(μg.L^{-1})	Reference
Milk	30 – 40	Mozzi *et al*., 2010
Yoghurt	110	Sybesma *et al*., 2003a
Kefir	40 – 50	LeBlanc *et al*., 2007

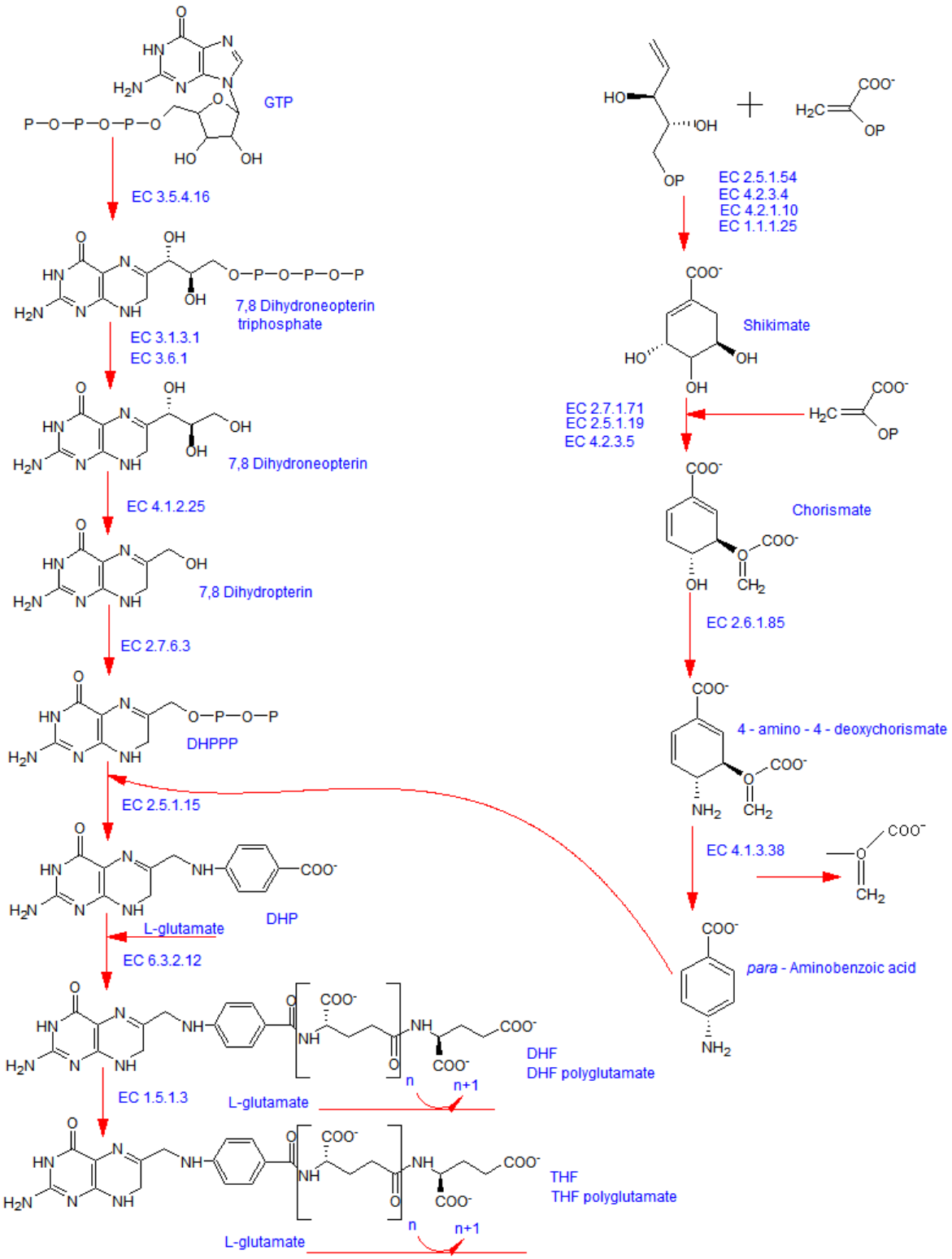


Figure 2.4 The biosynthesis of tetrahydrofolic acid (Rossi *et al*., 2011).

of pyruvate by 4-amino-4 deoxyhydrochorismate lyase (EC 4.1.3.38) leads to the formation of *p*ABA (Haussmann *et al*., 1998). The continuation of the folate biosynthesis proceeds with the formation of a C-N bond joining DHPPP to *p*ABA. This condensation reaction is then catalysed by dihydropteroate synthase (EC 2.5.1.15) to yield 7, 8 dihydropteroate (DHP). DHP is glutamylated by dihydrofolate synthase (EC 6.3.2.12) forming dihydrofolate (DHF). DHF is then reduced by DHF reductase (EC 1.5.1.3) to the biologically active cofactor tetrahydrofolate (THF) which is exposed to an addition of multiple glutamate moieties by folylpolyglutamate synthase (EC 6.3.2.17) to produce THF-polyglutamate (Fig. 2.4) (Rossi *et al*., 2011). Polyglutamylation may occur before the reduction step, being catalysed by DHF synthase or by a bi-functional enzyme which is responsible for both dihydrofolate synthase and folylpolyglutamate synthase activities in many bacteria (De Crécy-Lagard *et al*., 2007).

2.18 The necessity of fortification of food products with folate

Folate is a vitamin that cannot be synthesized by humans and, therefore, it is required exogenously. Folate is present in foods such as legumes (beans, nuts and peas), leafy greens (spinach), citrus, some fruits, vegetables (broccoli, cauliflower), liver, and fermented dairy products (Eitenmiller & Landen, 1999). The recommended daily intake (RDI) of folate for adults is 400 µg per day, while 600 µg is recommended for pregnant women and 500 µg for lactating women (LeBlanc *et al*., 2007). Pregnant women are especially susceptible to become folate deficient because there is a considerable increase in folate requirement during pregnancy (Pietzrik & Thorand, 1997). Folate is essential for a normal human metabolism. However, deficiencies of folate are prevalent (Konings *et al*., 2001; O'Brien *et al*., 2001). One of the methods used to compensate nutritional deficiencies in food is by fortification. While many countries have adopted a national folic acid fortification programme due to the potential adverse effects of folic acid deficiency, such as the delayed diagnosis of vitamin B_{12} deficiency by high intake levels of folic acid, other countries have not (Bailey *et al*., 2003). The excessive intake of folic acid has the potential to mask vitamin B_{12} deficiency leading to early haematological manifestations, such as pernicious anaemia (Sweeney *et al*., 2007). However, the fortification of food products with natural folates such as tetrahydrofolates produced by microorganisms do not cause "masking" of pernicious anaemia that occurs at high

concentrations of folic acid and should thus be considered as a viable alternative to folic acid fortification programmes (Scott, 1999). Therefore, the fortification of fermented milk and milk products with B-vitamins has been deemed a suitable means to prevent vitamin deficiencies (Papastoyiannidis *et al*., 2006).

Dairy products and fermented milk products are considered as a potential matrix for folate fortification due to the presence of folate binding proteins in milk used to improve folate stability and bioavailability of both 5-methyltetrahydrofolate (Aryana, 2003; Verwei *et al*., 2003).

2.19 Folate deficiencies

Folate, a water-soluble B vitamin, acts as either a donor or receiver of one-carbon moieties in numerous enzyme-catalysed reactions (Wagner, 1995). The importance of folate in the diet is the observation that a deficiency of this vitamin may increase the risk of certain types of cancer and many other diseases, including megaloblastic anaemia, neural tube defects (NTDs) and heart disease (Ames, 1999; Green & Miller, 1999). Although the mechanisms by which folate deficiency may cause these diseases remain to be elucidated, evidence accumulated by means of epidemiologic, metabolic and laboratory investigations over the years show substantial data to support the importance of folic acid in the prevention of NTDs, vascular disease, certain cancers and neurologic diseases such as Alzheimer's disease (Choi & Mason, 2000).

Neural tube defects are the most distressing genetic defect, manifesting as a type of malformation of the embryonic brain and/or spinal cord. The prevalence of this defect at birth differs in various countries and is related to ethnic and socioeconomic factors. The ratio of the defect ranges from 1:2500 births in Finland, 1:300 in Mexico, and 1:80 births in South-Wales, whereas the worldwide average is 1:500 (Dolk *et al*., 1991). Although NTDs are among the most common congenital malformations, little is understood about the underlying developmental mechanisms in humans. Neural tube formation is a multifactorial process, determined by both extrinsic and intrinsic factors, with both genetic and environmental factors, including maternal nutritional status, having been proposed to affect the risk for NTD (Copp *et al*., 1990). It is now known that folate intake is an important environmental factor in the aetiology of NTDs (Posey *et al*., 1996). Periconceptional folate administration

reduces the occurrence as well as the recurrent risk of NTDs (Czeizel & Dudas, 1992).

2.20 Methods of folate determination

There are various types of methods used when determining folates in foods, depending on the food matrix. These methods include the microbiological assay (Hyun & Tamura, 2005) using the AOAC Official Method 2004.05 and for total folate determination in cereal foods, HPLC or GC with mass spectrometry (MS) have been used (Rychlik & Freisleben, 2002).

Although the microbiological assay method is widely used for total folate determination, HPLC methods can measure various forms of folates. Studies have shown that the trienzyme treatment of food homogenates as an extraction technique with α-amylase, protease and folate conjugase may yield an increased concentration of folate in assays (Tamura *et al*., 1997). The use of enzymes such as α-amylase and protease permits a complete extraction of folate bound to carbohydrates and proteins in the food matrix, thus becoming widely used. During analysis, labile folates are treated with antioxidants, both ascorbic acid and mercaptoethanol to prevent oxidative stress.

Folate determination using a microbiological assay with *Lb. rhamnosus* after extraction with folate conjugase has a greater response to the γ-glutamyl folate polymers compared to the other assay organisms (Ball, 2006). AOAC Official Method 2004.05 is the microbiological method that uses *Lb. rhamnosus* after extracting samples by the trienzyme procedure. This method is used to determine the turbidity semi-automatically by using 96-well microtiter plates (Anon, 2006).

Recently developed HPLC systems used for the separation of folates either by ion-pair or reversed-phase chromatography with UV, fluorometric or electrochemical detection are commonly used for the analysis of food folates in conjunction with the trienzyme treatment (Van Wyk & Britz, 2012). Some studies have shown increased levels of folate when using the trienzyme extraction method, while others failed to demonstrate an appreciable increase. The use of the α-amylase and protease is to liberate folate from the food matrix. Since HPLC systems have the capability to detect only monoglutamates, human or rat plasma conjugase and not chicken pancreas conjugase is utilized to deconjugate polyglutamyl folates to monoglutamyl folates (Johansson, 2005). The removal of interfering impurities in

the sample extracts require a purification step with solid-phase extraction using silica-based strong anion-exchange cartridges. Due to its sensitivity and selectivity, fluorescence detection is most commonly used, particularly for reduced folate forms (Patring, 2007).

2.21 Fortification in Africa and Southern Africa

A dairy product such as milk is a complete food as it consists of all the important ingredients necessary for the human diet. Milk contains proteins (mainly casein), fat, salt, lactose as well as vitamins A, C, D, certain vitamins B and other minerals (Shah, 2000).

Commercial dairy products in South Africa have traditionally been developed and produced mostly for affluent consumers. The price and the technology of these products make them unsuited to the majority of South Africa's population with a low income and specific living conditions, i.e. lack of refrigeration facilities. Moreover, in rural communities refrigeration facilities are seen to be problematic and the storage temperature of the milk may differ between 10°C and 35°C. The majority of the milk is then sold for household use while the rest of the milk is used to produce traditional fermented milk beverages such as Maas (Van Wyk *et al*., 2002). Kefir is an example of such a fermented milk beverage that contains vitamins, minerals and essential amino acids. The benefits of consuming kefir as part of a diet are numerous. The consumption thereof assists the body with its nutritional intake leading to healthier bodily maintenance and also contains easily digestible proteins (Hosono *et al*., 1990).

The treatment of malnutrition over the past number of years has been a considerable burden on the South African economy, especially in terms of the health budget. The prevention of malnutrition and vitamin deficiencies was initially established to prevent and save unnecessary costs as part of nation development. Globally, the economic losses due to malnutrition were calculated by the World Bank to be between 6 – 12% of the gross national product (GNP) of the developing countries (Anon, 1993).

Protein energy malnutrition (PEM) in South Africa occurs mostly in children in underprivileged communities (Hansen, 1989). Poverty, food insecurity and poor nutrition education has allowed a persistent lack of knowledge surrounding the malnutrition problems associated with children and adults (Ndaba, 1984). In order to

determine and sustain a suitable solution to the problem, food fortification and enrichment programs along with nutrition education, living and work conditions should be seen as a national priority (Stampfer, 1993).

South Africa's society has undergone a major transition, which manifested concerns relating to disease and nutrition problems. The National Food Consumption Survey indicated that low-income households in South Africa had a fairly low dietary intake of many vitamins and minerals (Labadarios *et al*., 1999). This is linked to the population sector not being proficient in the self-production of food causing compromised food availability impacting food security (Maxwell & Smith, 1992; Mater & Gordon, 1996; Pelletier *et al*., 2001).

In South Africa, the vitamin A and B-vitamin status of population groups are poorly defined. However, the deficiency of B-vitamins is a health concern issue in the country, due to low levels of vitamin B_6, folic acid, thiamine, niacin and riboflavin intake (Bourne *et al*., 1995). In South Africa, the afore-mentioned are linked to several diet-related issues that are of concern with regards to public health (Maunder *et al*., 2001). For example studies have indicated that micronutrients such as vitamin E have the ability to reduce coronary heart disease and that folic acid prevents neural tube defects amongst pregnancies. Furthermore, in order to resolve these problems, the implementations of Food-Based Dietary Guidelines (FBDG) is established (Vorster *et al*., 2001). Therefore, the fortification and enrichment of the kefir beverage with vitamin B_{12} and folate promotes an affordable food commodity, which is vital for good health.

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CHAPTER 3

VITAMIN B_{12} AND FOLATE IN KEFIR BEVERAGE AFTER CO-INOCULATION WITH A *PROPIONIBACTERIUM FREUDENREICHII* STRAIN

3.1 Abstract

Two types of regimes regarding the inclusion of *Propionibacterium freudenreichii* in kefir grains were performed. These regimes were used (1) to measure the vitamin B_{12}, folate and pH levels in the kefir beverage; (2) to determine the sensorial quality of the kefir beverage; and (3) to confirm the presence of PAB in the kefir grains and kefir beverage after treatment or co-inoculation with PAB. Multiple treatments of the PAB broth culture and freeze-dried culture were included to ensure the presence of the PAB in the kefir grains and beverage from the start of the fermentation, as well as the concomitant elevated vitamin B_{12} and folate levels. These multiple treatments resulted in the inclusion of PAB in the kefir grains. The increase in vitamin B_{12} and folate levels in the PAB-treated samples at 1 d and 3 d was measured and the PCR assays confirmed the presence of PAB in all kefir grains and beverages, except the non-treated grains and beverage (control sample).

However, with regards to the vitamin B_{12} and folate production, both multiple treatments and concentration of PAB, the highest vitamin B_{12} and folate concentration measured at 1 d was 11.80 ± 2.18 µg.100 mL^{-1} (freeze-dried culture) and 458.45 ± 34.91 µg.100 mL^{-1} (broth culture), respectively. During this study, the broth culture produced the highest vitamin B_{12} and folate concentration measured at 3 d, which were 29.81 ± 3.90 µg.100 mL^{-1} and 655.87 ± 15.2 µg.100 mL^{-1}, respectively.

The preferred beverage, according to the sensory analysis with regards to sour taste and overall taste, was the broth culture sample at 1 d and 3 d. However, since the typical kefir fermentation is 24 h, the treatment results at 1 d was favoured. Hence, the results also confirmed that this type of co-inoculation may have the potential to enrich the kefir with B-vitamins resulting from a natural biosynthetic process.

3.2 Introduction

In South Africa malnutrition exists due to inadequate dietary intake of micronutrients, which is one of the major causes of vitamin deficiencies leading to disease. At present, under- and over-nutrition is evident among South Africa's population (Mvo, 1999; Faber & Wenhold, 2007).

In this context, the status of micronutrients, including B-vitamins, is a health concern, due to low intake levels of pyridoxine (vitamin $B_{6)}$, folic acid (vitamin B_9), cyanocobalamin (vitamin B_{12}), thiamine (vitamin B_1), niacin (vitamin B_3) and riboflavin (vitamin B_2) by adults and children (Bourne *et al*., 1995). Vitamin B_{12} and folate are vitamins that have co-dependent roles in nucleic acid synthesis. The deficiencies thereof can lead to serious health problems such as pernicious anaemia and megaloblastic anaemia (Klee, 2000). Moreover, persistent vitamin B_{12} deficiencies may result in irreversible nerve degeneration (Kirke *et al*., 1997). Although some studies show an improvement in nutritional status, the prevalence of some deficiencies increased over the past decade. The aforementioned studies that describe the malnutrition status in South Africa are primarily directed towards infants, young children and pregnant women in rural and urban poor areas (Anon., 1994).

Furthermore, since South Africa's major transition in 1994, an increase in urban areas occurred causing high population density and unemployment in these areas (Van Wyk *et al*., 2002). Although malnutrition continues to affect the lives of millions of children in South Africa it also has both short-term and long-term effects impacting on educability, productivity and mortality, especially in low-income urban households and rural areas. Moreover, the National Food Consumption survey indicated that the intake of a number of important micronutrients was less than the recommended level. It also showed that a great majority of children in South Africa, consumed a diet of poor nutrient density, where the nutrient intake of those living in rural areas was considerably lower than children living in urban areas. This is due to the rural population sector not being proficient in the production of food and increases in food prices can therefore have a significant impact on the ability of low-income households to afford food, which seriously threatens the food security of such households (Maxwell & Smith, 1992; Mater & Gordon, 1996; Pelletier *et al*., 2001; Anon., 2007). There are various reasons causing food prices to be higher in certain rural areas, namely (1) increasingly higher fuel prices which play a role as food items are transported over a greater distance to rural settlements than to urban

settlements and (2) fewer large retailers, with mostly small businesses operating in rural areas where the transaction cost of making food items available to consumers is high. The latter may also be due to insufficient competition (Anon., 2007).

Since there is a high prevalence of under-nutrition and micronutrient deficiencies among rural and urban poor households, the integrated nutrition strategies for South Africa were formulated and approved by the Department of Health (Anon., 1994). This was later developed into the Integrated Nutrition Program (INP) for South Africa (Anon., 1998). The INP focuses on eight strategies dealing with nutrition deficiencies and diseases (Labadarios *et al*., 2005). One of which is micronutrient malnutrition control or micronutrient intervention which, is used to address micronutrient deficiencies in the population. Micronutrient malnutrition control is divided into supplementation, food fortification, promotion of dietary diversification, and related public health measures (Labadarios *et al*., 2005).

Food fortification is one of the strategies currently used to increase the nutritional quality of foods, namely staple foods. Financial sustainability plays an important role with regards to the cost of staple foods, the nutritional status and food availability in rural and urban households (Cade *et al*., 1999).

Commercial dairy products and pharmaceutical nutritional products (food supplements) in South Africa have traditionally been developed and produced for affluent consumers. The cost and the technology of these products make them unsuited to the majority of South Africans that have a low income with specific and unfavourable living conditions, particularly the lack of refrigeration facilities. This lead to milk either being sold for household use or being used to produce traditional fermented milk beverages such as Maas (Van Wyk *et al*., 2002), with the main purpose of fermentation achieving their preservation (Caplice & Fitzgerald, 1999).

In fermented milk beverages, the preservative activity of LAB by lowering the pH between 3 – 4 by means of acid production inhibits the growth of pathogenic and spoilage microorganisms in foods (Ananou *et al*., 2007). This allows fermented food products to have a prolonged shelf-life. Therefore, the lactic acid produced by LAB in fermented foods serves as the main preservative. In South Africa and Zimbabwe, Amasi is a traditional fermented milk beverage and was shown to inhibit the growth of pathogens, such as *Salmonella enteridis* and *Escherichia coli*, which are common contaminants in raw milk (Mufandaedza *et al*., 2006). Hence, fermented food products have an important socio-economic role due to preserved status, but they

may also serve to increase essential amino acids, vitamins and protein bioavailability (Chelule *et al*., 2010).

Kefir is an example of a fermented milk beverage that contains LAB as well as vitamins, minerals, essential amino acids, produced at room temperature by means of fermentation. Kefir and kefir grains are complex systems of symbiotic microorganisms with probiotic characteristics which have been found not only to be nutritionally beneficial, but was proven to inhibit a number of food-borne pathogens and spoilage microorganisms (Păucean & Socaciu, 2008). Moreover, although kefir does not produce high levels of vitamin B_{12}, it may be a source of B-vitamins such as folate, pantothenic acid and vitamin B_{12} (Farnworth & Mainville, 2003; Powell, 2006; Van Wyk *et al*., 2011). Certain microorganisms are known to be beneficial to human health such as *Lactobacillus, Bifidobacterium*, *Streptococcus* and *Propionibacterium*. Propionibacteria provides certain probiotic characteristics, the most important characteristics being the production of vitamin B_{12} and minute quantities of folate in milk products i.e. cheese (Montere-Alhonen, 1995).

During cheese ripening, propionic acid and vitamin B_{12} production are possible due to the ability of the propionibacteria to metabolize lactate as a carbon substrate. Furthermore, stimulatory effects of LAB on propionic acid bacteria have been observed (Marcoux *et al*., 1992; Piveteau, 1999).

Hence, the combination of PAB added to kefir may offer the possibility of a B-vitamin enriched beverage containing a good source of vitamin B_{12} and folate with a nutrient reference value (NRV) of 2.4 $\mu g.d^{-1}$ and 400 $\mu g.d^{-1}$, respectively (Anon., 2003). Therefore, this novel food product naturally enriched with vitamin B_{12} and folate may address vitamin deficiency prevalent both in developing and industrialised countries as well as rural and poor urban areas. These innovative strategies by the food industry for the development of novel vitamin-enhanced functional foods with enhanced consumer appeal may be directed at specific groups such as the elderly and adolescents requiring a sustainable vitamin intake, while minimizing malnutrition caused by B-vitamin deficiency. The production of fermented food products with naturally elevated levels of B-vitamins, i.e. increased nutritional value, would thus minimizing any need for fortification using synthetic vitamin preparations (Burgess *et al*., 2009).

The aim of this study was to measure the vitamin B_{12}, folate and pH levels, to determine the sensorial quality of the kefir beverage and to confirm the presence of

PAB in the kefir grains, as a function of the form of the initial inoculum, the state of the preservation of the grains and the fermentation time in order to determine which procedure results in the optimum yield of the B-vitamins in the enriched kefir beverage, the effective preservation of PAB in the kefir grains and a beverage with consumer acceptability.

3.3 Materials and methods

3.3.1 Chemical reagents and other chemicals

Unless otherwise specified, all the chemicals used in this study were of Analar grade and chemical reagents were prepared according to standard analytical procedures. The methanol (Merck) and acetonitrile (Merck) were of HPLC grade. Milli-Q water (18.2 $M\Omega.cm^{-1}$), purified using the Milli-Q water purification system (Millipore, Microsep, South Africa), was used for making dilutions and solutions. Vitamin B_{12} and the four folate vitamers were used as external standards during the HPLC assays as described in 3.3.2.1 and 3.3.3.1. All prepared reagents were stored under conditions that will prevent deterioration or contamination.

3.3.1.1 Growth media

3.3.1.1.1 Trypticase soy broth/agar (TSB or TSA)

To 1000 mL Milli-Q water the following were added ($g.L^{-1}$): yeast extract (Biolab, Merck) 10.0; trypticase soy (Merck) 10.0; sodium lactate syrup (16.6 mL of a 60% solution) (United Scientific) 20.0; and Tween 80 (Sigma Aldrich) 1.0. The pH was adjusted to 7.0 using concentrated NaOH (Sigma Aldrich). Inoculation and growth was 7 d at 32°C. The preparation of solid media required the addition of 1.5 g bacteriological agar (Merck).

3.3.1.1.2 Vitamin B_{12} medium (VBM)

The vitamin B_{12} medium was prepared as described by Van Wyk and Britz (2010). To 1000 mL of Milli-Q water the following were added ($g.L^{-1}$): polypeptone (BBL) 5.0; casamino acids (Difco) 11.0; yeast extract (Difco) 2.5; $NaH_2PO_4{\cdot}2H_2O$ (Merck) 2.25; $MgCl_2{\cdot}6H_2O$ (Merck) 40.0; K_3PO_4 (Merck) 1.76; $CoCl_2{\cdot}6H_2O$ (Merck) 0.018; $FeSO_4{\cdot}7H_2O$ (Merck) 0.01; Ca-pantothenate (Fluka) 0.004; (+) biotin (Fluka) 0.0003;

Tween 80 (Saarchem) 1.0; glucose (BDH chemicals) 40.0 and 5,6-dimethyl-benzimidazole (DMBI) (Merck) 0.07.

After preparation, all media (i.e. TSA, TSB and VBM) were autoclaved for 15 min at 121°C. To ensure that sterilization was achieved, the media were kept at room temperature for one week and inspected for absence of growth before use.

3.3.1.2 Microbial cultures

Freeze-dried cultures of *Propionibacterium freudenreichii* subsp*. shermanii* strain J19 was obtained from the Department of Food Science and Technology, Cape Peninsula University of Technology. The kefir grains were obtained from the Department of Food Technology, Cape Peninsula University of Technology.

3.3.1.3 Reconstitution of freeze-dried cultures

The reactivation of the freeze-dried PAB occurred in the vial containing the original culture by adding 1 mL of sterile Milli-Q water using a sterile precision pipette, thus rehydrating the contents in the vial.

3.3.1.4 Freeze-drying of cultures

The reconstituted freeze-dried culture was inoculated into VBM at a concentration of 200 µL.100 mL^{-1}. This was followed by incubation for 7 d at 32°C. Broth cultures were then transferred to sterile 250 mL centrifuge tubes and centrifuged for 10 min at 10 000 X *g* at 4°C and the supernatant discarded. This was followed by the addition of 10 mL sterile Ringer's solution, resuspension of the pellet by shaking the capped tube and centrifugation. The Ringer's solution was discarded and a sterile milk/lactose mixture was used as the medium for freeze-drying. Two millilitre aliquots of the cell suspension were dispensed into sterile freeze-dryer vials. The milk/lactose mixture contained 12% (m/v) endospore free skim milk powder (Merck) and 5% (m/v) lactose monohydrate (Merck) (Joubert & Britz, 1987). Two millilitre aliquots of the cell suspension were dispensed into sterile freeze-dryer vials, followed by freeze-drying.

3.3.1.5 Sampling and cell enumeration

Kefir samples were collected and analysed once a day at day 1 and 3 for the following analyses: the measurement of the extracellular folate derivatives by HPLC; measurement of vitamin B_{12} by HPLC; pH measurement; and DNA/PCR analysis.

Serial dilutions of reconstituted PAB inoculum was carried out in sterile Ringer's solution according to the method of Malik (1994). The dilutions were made from 10^{-1} to 10^{-7} and plated using the relevant growth medium for viable cell enumeration. PAB was enumerated on TSA agar. The plates were incubated anaerobically at 30°C for 5 d for PAB. The cell counts were performed in duplicate and mean values was expressed. This was performed to establish the inoculation concentration in colony forming units (cfu) per mL. Aseptic protocol was strictly adhered to throughout.

3.3.1.6 Reactivation of kefir grains

Ultra-high temperature (UHT) full cream milk (Clover, South Africa) purchased from a local supermarket was used during the study for the reactivation and fermentation of the kefir grains. To achieve satisfactory aseptic conditions, the milk bottle, including the cap and the protective seal, was sprayed with 70% ethanol and then left to dry in the laminar flow cabinet. The kefir grains were removed from storage at −20°C and allowed to defrost in the laminar flow cabinet at room temperature. The kefir grains were handled aseptically throughout. A sterile spatula was used to apportion and transfer 10 X 10 g of the kefir grains into 10 sterile 250 mL media bottles. This constitutes the total grain mass to be used, including the control samples required for the first treatment. The protective seal on the milk bottle was removed aseptically and 100 mL milk was added to the grains in each media bottle. After tightening the bottle cap, the bottle was agitated using the Orbital shaker (Gerhardt RO 20, Bonn, Germany) at a speed level of 7 and a temperature of 30°C for 12 h. Once the agitation step was complete, a sterile metal strainer was used to separate the activated granules from the fermented beverage. After straining for 5 min, the kefir grains (with some beverage retained) were transferred into clean sterile media bottles. The subsequent schedule of treatments resulted in four distinctly different inocula of kefir grains reacted with PAB. The schedule of treatments was based on the combination with PAB broth culture and combination with PAB freeze-dried culture (Fig. 3.1).

3.3.1.7 Reaction of the kefir grains with PAB culture only

The treatment entailed the inoculation of the activated kefir grains with the PAB broth

and freeze-dried cultures, respectively (Fig. 3.1). The broth cultures were centrifuged for 10 min at 10 000 X *g* at 4°C, the supernatant was discarded and the pellet of the PAB broth culture was used for co-inoculation with the activated kefir grains in the 250 mL media bottle, whereas the 1 mL reconstituted freeze-dried PAB culture was co-inoculated directly with the activated kefir grains. This procedure was applied to 2 X 10 g grain samples for each inoculum type, i.e., two replicates per treatment were prepared. The resultant preparations were allowed to react for 4 d at 30°C, with gentle agitation on the Orbital shaker at a speed set at level 3. A 100 mL aliquot of full cream UHT milk was then added and agitated for a further 4 d at 30°C before being strained with a sterile strainer. The latter was repeated thrice. The concentration of PAB inoculum volume was 200 µL per 100 mL broth, the broth culture was 5 X 10^6 cfu.mL^{-1}, while the freeze-dried culture contained 2 X 10^5 cfu.mL^{-1} and reconstituted PAB culture contained 1 X 10^5 cfu.mL^{-1}. The resulting kefir grains and remaining fermented beverage sampled at 1d and 3d was assayed by DNA extraction, followed by PCR and gel electrophoresis to confirm the presence of PAB, while vitamin B_{12} and folate was analysed using the HPLC, and sensory evaluation was done using a trained panel of sensory evaluators.

3.3.2 Vitamin B_{12} analysis

3.3.2.1 Analytical standards

The vitamin B_{12} (cyanocobalamin) (Sigma Aldrich, South Africa) was prepared and used as an external standard for vitamin B_{12} determination.

3.3.2.2 Preparation of the calibration standards

The standard used for the calibration was cyanocobalamin. Two milligrams of the cyanocobalamin was weighed and added to 100 mL of KCN- buffer solution in a 100 mL amber volumetric flask. The KCN-buffer contained 544.32 g Na-acetate trihydrate (Univar, Saarchem) and 20.0 g KCN in 1 litre Milli-Q water. The pH was adjusted to 4.5 using glacial acetic acid. All standards were flushed with nitrogen and stored at –20°C for a maximum of 2 weeks.

3.3.2.3 Sample extraction for vitamin B_{12} determination

KCN-acetate buffer was used for the sample extraction. The ratio of the buffer solution to the sample was 10:4 (v.v^{-1}). All the sample tubes were wrapped in

aluminium foil to protect vitamin B_{12} from deterioration by light. The samples were homogenised and autoclaved at 121°C for 25 min, followed by rapid cooling and centrifugation (Avanti J-E, Beckman) at 15000 X *g* for 10 min at 4°C. Once the centrifugation step was complete, the supernatant was filtered through a 0.45 µm syringe filter (Ministart, National Separations) into amber HPLC sample vials with screw caps with septa (Chemetrix, South Africa). Samples that were not immediately analysed by HPLC were stored immediately at 4°C in a dark environment for a maximum of 4 d. These storage conditions ensured complete retention of vitamin B_{12} activity (Van Wyk, 2002; Hugenschmidt *et al*., 2011).

3.3.2.4 Sample purification and concentration

Due to the low concentration of vitamin B_{12} reported in literature for some dairy products, sample purification and concentration were performed to improve the sensitivity of the analysis. Solid Phase Extraction (SPE) columns were used for the purification and concentration of the vitamin B_{12} samples. A preconditioned SPE column (Chromabond SB/ 3 mL/ 500 mg, Macherey-Nagel, Düren, Germany) was used to purify the sample. The purification step involved the application of 9 – 12 mL sample extract and the eluate (E1) was collected, in a clean sample vial. After eluting the purified vitamin B_{12}, the SPE column contains mostly impurities, but also retains some vitamin B_{12}. Hence, one column volume (c.v) of 400 $mL.L^{-1}$ methanol (Merck) was used to elute the remaining vitamin B_{12}. This eluate (E2) was collected separately from the first. The sample was then concentrated using a preconditioned SPE column (Chromabond C_{18} EC/ 6 mL/ 1000 mg column, Macherey-Nagel). This was achieved by applying eluate (E1), to the column. The vitamin B_{12} in the column was then eluted using the second eluate (E2 or eluate collected separately), followed by 1 mL 900 $mL.L^{-1}$ methanol. The methanol present with the sample then underwent flash evaporation using a rotary evaporator at 40°C under vacuum to concentrate the sample, which was diluted with Milli-Q water to 3 mL in a volumetric flask. The sample was filtered into amber vials prior to HPLC analysis.

The HPLC separations of the standards and samples were performed using an Agilent 1100 HPLC system. The HPLC system consisted of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment set at 30°C, an autosampler with an injection volume of 20 µL and a G1315C Diode Array Detector (DAD) set at 360.4 nm with a reference wavelength of 360.2 nm was

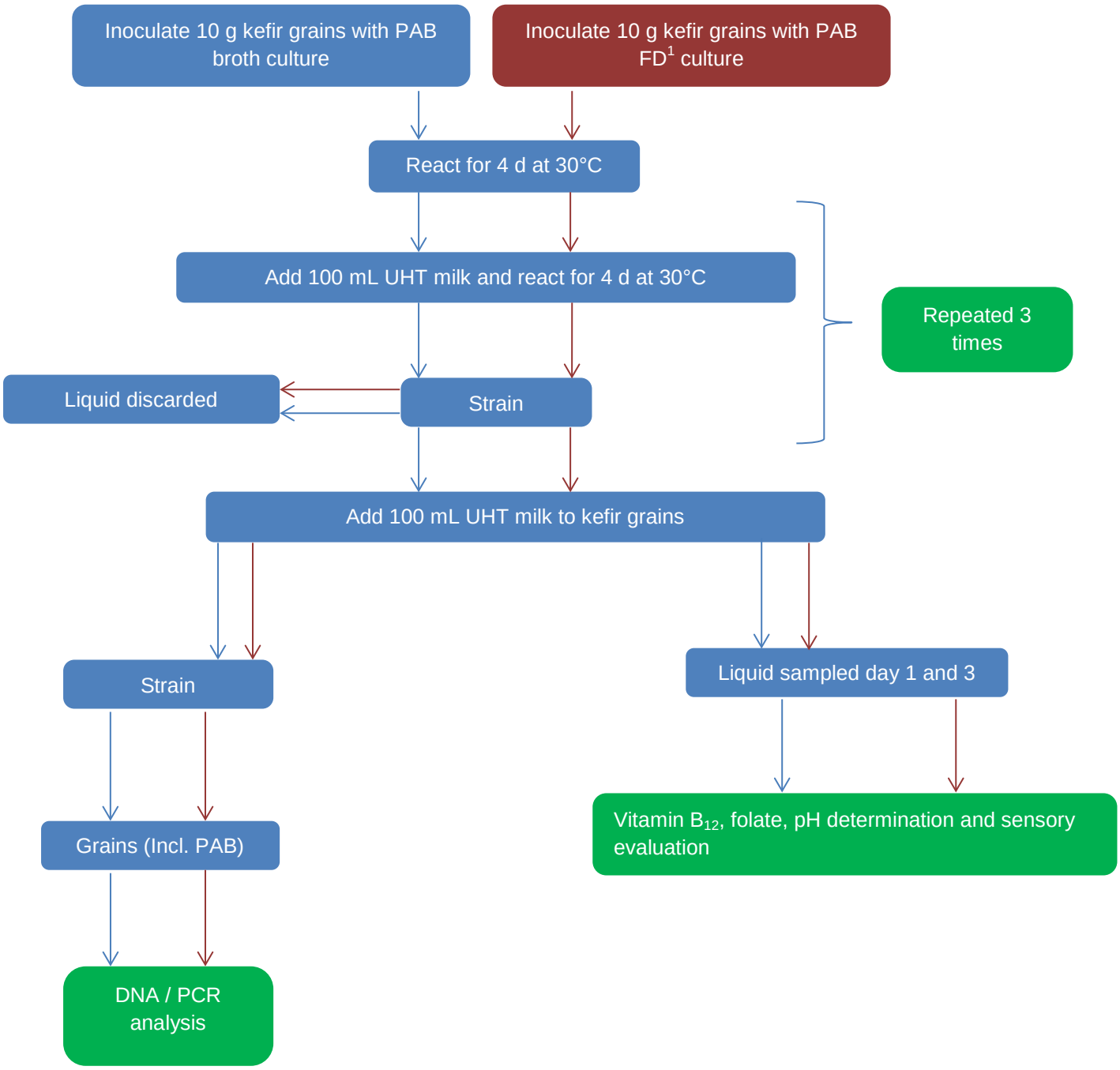


Figure 3.1 Diagram of the regimes followed to achieve inclusion of PAB in the form of freeze-dried and broth cultures into kefir. [1]FD – freeze-dried culture.

used during the analysis. The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) integrated peak areas and was also used to record and store data.

3.3.2.5 HPLC assay

The analytical column used was a reversed phase 3 µm C_{18} column (Luna 250 mm X 4.6 mm; Phenomenex Inc., Torrance, CA, USA) with a reversed-phase C_{18} guard cartridge (ODS 4.0 mm X 4.0 mm; 5 µm; Phenomenex Inc). The guard cartridge was replaced after every 150 injections. The mobile phase was a 95:5 to 50:50 (v/v) acetonitrile-water linear gradient over 35 min with a flow rate of 0.5 $mL.min^{-1}$. The cyanocobalamin was determined under these conditions.

The utilisation of the calibration function of the Agilent Chemstation software to construe the calibration curve and quantification of the vitamin B_{12} levels of samples were performed by the external standard method. In terms of quantification, a standard solution, diluted from 20 $\mu g.mL^{-1}$ to 0.01 $\mu g.mL^{-1}$ and analysed in duplicate at the start of each working day and the software programmed to use the resultant peak area to calculate the actual concentration of vitamin B_{12} in the kefir sample extracts. The column was restored for further use between injections by means of a post-run flushing with 100% acetonitrile (5 min), followed by 100% Milli-Q water (5 min), and then recycled to initial conditions (5 min).

3.3.2.6 Linearity, LOD and LOQ

A linearity curve was construed using vitamin B_{12} standard solutions at 0.01, 0.03, 0.10, 0.25, 0.50, 1.0, 1.50, 2.0, 2.50, 5.0, 10.0 and 20.0 $\mu g.mL^{-1}$. Twelve samples per concentration (n = 12) were analysed and the multiple correlation coefficient (R^2) and regression coefficient (R) were used to determine whether the peak area plotted would be linear over the concentration range. The linearity curve was assessed to find the suitable linear region that can be used to quantify the vitamin B_{12} concentration in the kefir beverage sample. The LOD and LOQ were calculated based on signal to noise ratios of 3 and 10 (S/N = 3 and 10), respectively. The standard deviation (SD) of the response and the slope (S) of the calibration curve were used to approximate the LOD according to the formula: LOD = 3 (SD/S). The LOQ was determined using the response SD and the slope of the calibration curve according to the formula: LOQ = 10 (SD/S). The standard deviation of the response

can be determined based on the standard deviation of y-intercepts of regression line. The values of SD and slope was obtained from the LINEST function, when creating the calibration curve in SigmaPlot® (Systat Software, San Jose, USA). The SD of y-intercept was the standard used for LOD and LOQ calculation.

3.3.2.7 Precision

Repeatability precision was done by analysing one sample numerous times. This includes intra-day and inter-day assays. Seven replicates of the vitamin B_{12} standard was analysed in two sessions on one day and the intermediate precision was determined by analysing two sets of 14 replicates of the vitamin B_{12} standard on two consecutive days. The mean ± standard deviation (SD) and the relative standard deviation (RSDr) were calculated. The Horwitz ratio of acceptance was used to determine repeatable precision. This was determined by the HORRATr value which has an acceptable range, namely 0.3 to 1.3 for a single laboratory validation (Anon., 2002).

3.3.2.8 Accuracy

The recovery analysis was performed by using the standard addition method. The sample or placebo (i.e. a sample that initially contained no cyanocobalamin) was divided into three aliquots each containing 4 mL. Ten mL of standard spike solutions containing 20, 50 and 80 $\mu g.mL^{-1}$ cyanocobalamin respectively, was added separately to each sample aliquot. A control sample was also prepared (to determine native cyanocobalamin). After the normal extraction procedure, cyanocobalamin were determined in two replicate samples at each level of addition , the percentage recovery, the relative standard deviation (RSD), mean and standard deviation (SD) were calculated (Table 3.3). According to the AOAC guidelines a 10% concentration of the standard added should have a range between 95 – 102% for an acceptable recovery limit (Anon., 2002).

3.3.3 Folate analysis

3.3.3.1 Analytical standards

Four folate vitamers were used as external standards during the HPLC analysis, namely tetrahydrofolic acid (THF) and Folic acid Neat (PGA) (Merck, Darmstadt, Germany), 5-methyl-tetrahydrofolic acid (5-CH_3-THF disodium salt) and 5-formyl

tetrahydrofolic acid (5-CHO-THF calcium salt) (Sigma Aldrich, South Africa). Pteroyltri-L-γ-glutamic acid ($PteGlu_3$) was obtained from Dr. Schirck's Laboratories (Jona, Switzerland). Standard solutions were prepared as described by Van Wyk (2002) and Arkbåge (2003).

3.3.3.2 Stock solutions

The stock solutions of the four folate vitamers were prepared by dissolving 10 mg into 100 mL 0.1 M phosphate buffer (pH 7.0) containing 1.0% ($m.v^{-1}$) sodium ascorbate. In the case of THF, the phosphate buffer contained 0.1% ($v.v^{-1}$) 2-mercaptoethanol (MCE) in addition to prevent the oxidation of the labile THF. The stock solutions were flushed with nitrogen to prevent degradation and stored at –20°C for a maximum of 2 weeks.

3.3.3.3 Preparation of the calibration standards

The standards used for the calibration were THF, folic acid neat (PGA), 5-CHO-THF and 5-CH_3-THF. The calibration standards were obtained by pipetting aliquots of each stock solution into a 100 mL volumetric flask and diluting to the mark with 0.01 M sodium acetate trihydrate (Merck, Germany), 1.0% ($m.v^{-1}$) Na-ascorbate at a pH of 4.9. All standards were flushed with nitrogen and stored at –20°C for a maximum of 2 weeks.

3.3.3.4 Extraction of folate in the sample

The tri-enzyme method for the extraction of folate at pH 7 was done as described by Van Wyk & Britz (2012). The extraction protocol includes the liberation of the protein-bound folate by means of a heat treatment. Ten mL of the extraction buffer (0.15 M K_2HPO_4 containing 0.052 M ascorbic acid/ascorbate and 1% 2-mercaptoethanol) was added to 2 mL of the liquid samples. Once the extraction buffer was added, the mixture was homogenised for 15 s using a Polytron® homogeniser (Kinematica, Switzerland) at a speed level of three. The homogenisation was carried out under a continuous flow of nitrogen (Air Liquide, South Africa). This was used as a preventative measure to exclude oxygen. Twelve millilitres of the homogenate was transferred into a 50 mL centrifuge tube, flushed again with nitrogen gas and heated in a microwave oven at 75% power (610 kW) for 1 min. The centrifuge tubes were gently agitated in a waterbath at 100°C for 10 min. After heating the sample, it was

cooled to room temperature and centrifuged at 15 000 X *g* at 2°C for 20 min. Once centrifugation was completed the supernatant was decanted into a clean centrifuge tube and 40 mg of α-amylase (*Aspergillus oryzae*; Sigma Aldrich, South Africa) was added and the centrifuge tube vortexed (Vortex-Genie2^{TM}) to allow rapid dispersion of the enzyme. The sample was flushed with nitrogen gas, sealed and incubated at 37°C for 4 h. This was followed by an adjustment to pH 7 using glacial acetic acid (Merck) and the addition of 40 mg protease. The protease enzyme (*Streptomyces griseus*; Sigma Aldrich, South Africa) was dispersed using the vortex mixer and the sample was then incubated at 37°C for 4 h. The protease was inactivated by heating the sample in a waterbath at 100°C for 5 min. After heating, the sample extract was cooled to room temperature and 40 mg folate conjugase (human serum) (WP Blood Transfusion Service, South Africa) was added. The mixture was flushed again with nitrogen gas, sealed and placed into a shaking water bath at 37°C for 4 h. Once the sample was removed from the water bath and cooled rapidly, it was centrifuged at 20 000 X *g* at 2°C for 20 min. After centrifugation, 5 mL of the supernatant was dispensed by pipette into a 25 mL volumetric flask with the addition of 15 µL 2-mercaptoethanol and diluted to the mark with Milli-Q water.

The efficacy of deconjugation was performed by analysing a 5 mL aliquot of a sample type containing 110 nmol of $PteGlu_3$ prior to the addition of 40 mg folate conjugase. A control kefir sample containing 110 nmol of $PteGlu_3$ after deactivation by the enzyme was also analysed to determine whether complete deconjugation of PGA to $PteGlu_3$ was achieved.

3.3.3.5 Purification and concentration

The samples were purified as described by Van Wyk & Britz (2012), using a quaternary amine, strong anion-exchange (SAX) SPE column (Strata SAX 55 µm, 70A; 3 mL/ 500 mg, Phenomenex, Torrance, California). The activation of the columns required two c.v. of methanol (HPLC grade, Merck) followed by two of Milli-Q water. The solvents and samples were aspirated through the column using a 12-port SPE vacuum manifold (Lida, Rochester, New York). The conditioning of the SPE columns was done by using 10 mL conditioning buffer. The solvents and the conditioning buffer was aspirated at 1 $mL.min^{-1}$ followed by the application of 10 mL sample extract at 0.5 – 0.6 $mL.min^{-1}$. The impurities were removed by washing the column with a 3 mL aliquot of the conditioning buffer at 0.4 $mL.min^{-1}$, followed by

elution using 2 mL elution buffer at 0.3 – 0.4 mL.min^{-1}. Eluted samples were filtered using a 0.45 µm nylon membrane filter (Ministart, National Separations) into amber HPLC vials.

3.3.3.6 HPLC assay

Analysis of the purified samples was performed as described by Van Wyk (2002) and Van Wyk & Britz (2012). The analytical columns were a reversed-phase 3.5 µm Zorbax SB-C_{18} column (150 mm X 4.6 mm) (Agilent Technologies, Waldbron, Germany) followed by a reversed-phase 3 µm C_{18} column (Luna 250 mm X 4.6 mm; Phenomenex Inc., Torrance, CA, USA) with a C_{18} guard cartridge (ODS 4.0 mm X 4.0 mm; 5 µm; Phenomenex Inc). The Agilent 1100 HPLC system consisted of the components described earlier (section 3.3.2.4), with the addition of a G1321A fluorescence detector. The fluorescence detector was used (excitation at 290 nm and emission at 356 nm) for the detection and quantification of THF, 5-CH_3-THF and 5-CHO-THF, and the DAD was used (the DAD was set at 290.4 nm with a reference signal at 360.10 nm) for PGA and pteroyltri-γ-L-glutamic acid. Gradient elution was performed using acetonitrile and 30 mM potassium phosphate buffer, pH 2.2 at a flow rate of 0.450 mL.min^{-1}. The gradient program was used for analyte separation. The gradient started at 5% v.v^{-1} acetonitrile, which was maintained isocratically for the first 9 min and then increased to 13% within 18 min. Thereafter, the acetonitrile concentration was raised to 35% within 9 min and maintained for 2 min. The peaks were identified by their retention times, and their identities were confirmed by using a calibrant mixture sample, consisting of standard solutions of each of the four vitamers. The four folate vitamers were quantified based on the external standard method with peak areas plotted against concentrations. The calibration function of the Agilent Chemstation software was used to construe the calibration curve as describe earlier in section 3.3.2.4.

3.3.3.7 Linearity, LOD and LOQ

As described in section 3.3.2.5 a linearity curve was obtained. The folate calibrant standard solutions used contained 0.001; 0.002; 0.005; 0.01; 0.05; 0.10; 0.5; 1.0 and 5.0 µg.mL^{-1}. Nine samples per concentration (n = 9) were analysed and the multiple correlation coefficient (R^2) and regression coefficient (R) were calculated and interpreted as before. The Limits of Detection (LOD) and the Limits of Quantification

(LOQ) limits were estimated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve, as described in section 3.3.2.5.

3.3.3.8 Precision

The precision was done by intra-day and inter-day assays. Seven replicates of the calibrant mixture of folate standards was analysed in two sessions on one day and the intermediate precision was determined by analysing two sets of 14 replicates of the calibrant mixture of folate standards on two consecutive days. The mean ± standard deviation (SD) and the relative standard deviation (RSDr) were calculated. The Horwitz ratio of acceptance was used to determine repeatable precision. This was determined by the HORRATr value which has an acceptable range, namely 0.3 to 1.3 for a single laboratory validation (Anon., 2002).

3.3.3.9 Accuracy

Recovery studies were performed by adding known concentrations of standards to kefir beverage samples before the normal extraction, deconjugation and purification procedure, followed by analysis. To determine the percentage recovery, the amounts of the different folate vitamers were measured and compared to the amounts added. The addition of three different levels of the calibrant mixtures were used, namely 50, 80 and 100 $\mu g.mL^{-1}$. Folates were determined in two replicate samples at each level of addition, the percentage recovery, the relative standard deviation (RSD), mean and standard deviation (SD) were calculated. According to the AOAC guidelines, a 10% concentration of the standard added should have a range between 95 – 102% for an acceptable recovery limit (Anon., 2002).

3.4 Sensory Evaluation

The kefir beverage was evaluated by a descriptive panel of five judges which were recruited among a pool of undergraduates and staff from the Department of Food Science and Technology at the Cape Peninsula University of Technology. These judges were selected based on availability and product interest. The characteristics of sour taste and overall flavour, using a unstructured linear scale (100 mm) on standard evaluation forms, were used to evaluate the beverage. The kefir beverage was stored at 4°C for not more than six hours before being evaluated.

3.4.1 Panel training and sensory evaluation

During the panel training session, the panellists were served eight samples, all of these were selected and diluted with UHT milk to have a consistency similar to the kefir beverage. These standard samples were buttermilk; Bulgarian yoghurt 10% $v.v^{-1}$ in UHT milk; Bulgarian yoghurt 50% $v.v^{-1}$ in UHT milk; 2% $m.v^{-1}$ citric acid added to Bulgarian yoghurt 50% $v.v^{-1}$ in UHT milk; 10% $m.v^{-1}$ citric acid added to Bulgarian yoghurt 50% $v.v^{-1}$ in UHT milk, Amasi, 1 d and 3 d kefir beverage (Table 3.1). In addition to consistency, these standard samples also represented the key sensory attributes of the kefir beverage as well as the typical intensity extremes. These standards were used to orientate the panel concerning the precise meaning of the sensory attributes or descriptors, as well as typical extremes concerning their intensity. This allowed the panel to familiarize themselves with the product and the analysis protocol. As mentioned, the two descriptors were sour taste and overall taste.

To prepare the samples for the sensory evaluation, they were poured into 30 mL cups, sealed with a lid and placed in the refrigerator at 4°C for 1 h. Immediately prior to the panel evaluation, samples were removed from refrigeration and lightly shaken until the samples were homogenous. During the sensory evaluation session, eight kefir samples were presented to the panellists. The sample cups were labelled with a 3-digit code, while the random sensory order was dictated by a balanced block design, generated using a custom – designed excel programme. The panellists were asked to remove the lid and evaluate the sour taste and overall flavour prior to rating them using the 100 mm unstructured linear intensity scale. To reduce any carry-over effect, panellists were instructed to cleanse their palates by chewing a small piece of unsalted and unflavoured crackers, followed by rinsing with water between samples. Moreover, the panel rated the intensity of each of the three descriptors on the line scale, anchored on both sides with two word descriptors – “Absent” and “Intense” which was used to describe the sour taste and “Unpleasant” and Desirable” which was used to describe the overall taste (Table 3.2). The panel evaluated the individual attributes by making a vertical slash on the linear scale. The attribute intensity values previously established and the rating marks on the scale were converted into numerical values. The statistical analysis for the sensorial study was done to establish whether the differences in individual attribute ratings between samples were significant ($p < 0.05$) using of variance (ANOVA).

Table 3.1 Sensory attributes and reference standards used for the descriptive analysis

Attribute	Intensity	Reference standard
Sour taste	Slightly sour	2% $m.v^{-1}$ citric acid and 50% $m.v^{-1}$ diluted plain Bulgarian with milk mixture.
	Very sour	10% $m.v^{-1}$ citric acid and 50% $m.v^{-1}$ diluted plain Bulgarian with milk mixture
Overall taste		Amasi 50% $m.v^{-1}$ diluted plain Bulgarian with milk mixture. Buttermilk 1 d Kefir beverage 3 d Kefir beverage

Table 3.2 Sensory attributes and anchor points used for the descriptive analysis

Attributes	Anchor points
Sour taste	Absent - intense
Overall taste	Unpleasant – desirable

3.5 DNA extraction of PAB in kefir samples

The DNA isolation of the *Propionibacterium freudenreichii* strain J19 strain present from the kefir grains and beverage were performed performed according to both manufacturers' guidelines, namely Promega and Kapa Biosystems, respectively. The DNA extraction of the PAB present in the kefir grains and beverage were performed using the Wizard Genomic DNA purification kit (Promega). The kefir grain and beverage samples were removed from refrigeration and allowed to defrost overnight, 1 mL of each sample type (kefir grains and beverage) was homogenised individually, using phosphate buffered saline solution in a 1.5 mL micro-centrifuge tube. The samples were centrifuged at 16 000 X *g* for 2 min to separate the suspended cells from the supernatant. The supernatant was then removed. Subsequent resuspension of the cells of each sample followed using 480 µL of 50 mM EDTA and the addition of 120 µL of 10 $mg.mL^{-1}$ lysozyme (Sigma Aldrich, South Africa). The samples were then incubated at 37°C for 60 min. The samples were centrifuged again at 16 000 X *g* for 2 min and the supernatant was decanted. After centrifugation the cells were resuspended by gently pipetting 600 µL of nuclei lysis solution followed by incubation at 80°C for 5 min to allow the cells to lyse. After cooling the samples to 25°C, 3 µL of RNase solution was added to the cell lysate and mixed by inverting the tube 2 – 5 times. These samples were then incubated at 37°C for 30 min and cooled to 25°C. The proteins were extracted by the addition of 200 µL protein precipitation solution to the RNase treated cell lysate and vortexed to allow rapid dispersion of the solutions before placing the samples on ice for 5 min. After rapid cooling on ice for 5 min the samples were centrifuged at 16 000 X *g* for 3 min. The supernatant was then transferred to a sterile 1.5 mL microcentrifuge containing 600 µL isopropanol (25°C) and gently mixed by inversion until the DNA formed a visible mass of thread like strands this was followed by centrifugation at 16 000 X *g* for 2 min. The supernatant was decanted and 600 µL of 70% ethanol was added to the tube and gently inverted several times to wash the DNA pellet. After washing the DNA pellet the sample was centrifuged at 16 000 X *g* for 2 min and the ethanol was gently aspirated. The residual liquid in the sample tube was decanted on sterile absorbent paper and the cell pellet was allowed to air dry in the laminar flow cabinet for 15 min. After air drying the sample the DNA sample was rehydrated using DNA rehydration solution and incubated at 65°C for 1 h with periodically mixing of the solution by gently tapping the sample tube.

3.6 PCR amplification and master mix

The specific primers used in this study was the Prop 1 forward primer 5'-GATACGGGTGACTTGAGG-3' and Prop 2 reverse primer 5'-GCGTTGCTGATCTG GATTAC-3' (Whitehead Scientific, South Africa). The master mix of the 25 µL PCR reaction contained 1 X KAPA HiFi™ buffer (with $MgCl_2$), 0.3 mM dNTPs, 0.3 mM forward primer, 0.3 mM reverse primer, 125 ng BSA (Bovine Serum Albumin), 20 – 4 ng DNA template and 0.5 U of KAPA HiFi™ DNA polymerase (Kapa Biosystems, South Africa). Five microliters of the isolated DNA was added to each PCR mixture. The PCR reaction was performed in the Mastercycle gradient (Eppendorf, Germany). The PCR amplification conditions were as follows: initial denaturing was performed at 95°C for 5 min followed by denaturing at 98°C for 20 s; primer annealing at 50°C for 15 s and chain elongation at 72°C for 1 min. These three steps were repeated for 35 cycles. Final chain elongation was performed at 72°C for 5 min and the PCR reactions were cooled to 4°C. The resultant separation of the DNA fragments were isolated using the PCR assay and visualized under UV light on 1% agarose gel stained with ethidium bromide.

3.7 Agarose gel preparation

The 1% agarose gel was prepared by adding 0.5 g of agarose powder to 50 mL 1 X TAE. Once the mixture was prepared the solution was heated in the microwave to dissolve the agarose, after which 10 µL of ethidium bromide was added. Two dams were placed into the slots on each side of the gel plate and the melted agarose was poured into the gel plate in the electrophoresis box. Once completed, the comb was placed into the agarose gel and the gel was cooled to room temperature.

3.8 Electrophoresis procedure

When the gel had solidified, the comb was carefully removed and 250 mL of 1 X TAE (electrophoresis buffer) was poured over the gel. Once the buffer was poured over the gel 10 µL of the sample consisting of the loading dye was carefully pipetted into the sample wells. The sample was then electrophoresed at 100 Volts for 45 min until the sample had migrated at least 6 cm down the agarose gel. Once the electrophoresis was completed, the gel was carefully removed from the gel box and placed into the UVidoc box and visualised.

3.9 Statistical analysis

All analyses done in this study were performed on duplicate samples and Analyses of variance (ANOVA) were performed with SPSS® 21.0 (SPSS® Inc., Chicago, Illinois, USA) to test and compare the effects of vitamin production with regards to broth culture, freeze-dried culture fermentations, pH and sampling times (1 d and 3 d). Vitamin concentration means and standard deviations were compared using the Duncan multiple comparison Post-hoc test, where significant differences were found in terms of the main effects. Univariate analyses of variance was performed with SPSS® 21.0 to calculate the corresponding p-values between vitamin B_{12} and folate levels in the kefir beverage.

3.10 Results and discussion

3.10.1 Linearity

In order to determine the response variation with the variation in concentration of vitamin B_{12} and the folate vitamers linearity curves were construed (Figs. 3.2 and Fig. 3.3). For vitamin B_{12} the curve represented acceptable linearity between the vitamin B_{12} peak area and concentration of the injected analyte over a concentration range between 0.01 $\mu g.mL^{-1}$ and 20 $\mu g.mL^{-1}$ (Fig. 3.2 and Table 3.3), the correlation coefficient (R) was 0.99989 and multiple correlation coefficient (R^2) = 0.99972 (Fig. 3.1). The LOD for vitamin B_{12} was 0.0054 $\mu g.mL^{-1}$ and the LOQ was 0.018 $\mu g.mL^{-1}$ (Table 3.3).

For folate, the linearity curves of the calibrant mixture consisting of the four folate vitamers were construed over a concentration range of 0.001 – 5 $\mu g.mL^{-1}$ (Table 3.4). The multiple correlation coefficients (R^2) of the vitamers ranged between 0.99738 – 1.00 and the correlation coefficients (R) ranged between 0.99869 – 1.00 (Fig. 3.3). The LOD for THF, 5-CH_3-THF, 5-CHO-THF were between 0.00015 $\mu g.mL^{-1}$ and 0.00025 $\mu g.mL^{-1}$, while the LOQ was between 0.00050 $\mu g.mL^{-1}$ and 0.00083 $\mu g.mL^{-1}$. The LOD and LOQ for PGA was detectable at 0.00021 $\mu g.mL^{-}1$ and 0.00068 $\mu g.mL^{-1}$, respectively (Table 3.4).

The calibration procedures for both vitamin B_{12} and folate were performed according to the AOAC method (Anon., 2002). In both cases, the results confirmed that a suitable concentration range over 12 points (vitamin B_{12}) and 9 points (folate), having equal spacing, were suitable calibration patterns. High multiple correlation

coefficients (R^2) and correlation coefficients (R) of >0.99 were evidence of a good linear fit (Anon., 2002).

The aforementioned results compare favourably with published results. According to the study done by (Zafra-Gómez *et al*. (2006), the multiple correlation coefficient for vitamin B_{12} and folate were similar to this study, namely 0.9999 and 0.9989, respectively. The higher LOQ of 0.04 $\mu g.mL^{-1}$ for both vitamin B_{12} and folate (Zafra-Gómez *et al*., 2006) and those reported by Van Wyk (2002) (LOQ of 0.005 $\mu g.mL^{-1}$ for vitamin B_{12} and Van Wyk & Britz (2012) (0.002 – 0.05 $\mu g.mL^{-1}$ for the folate vitamers), confirmed the sensitivity of the assays in this study. Hence, comparing these previously reported HPLC methods used to analyse vitamin B_{12} and folate, the LOD and LOQ of the present method was more sensitive than the methods optimized by previous researchers. The correlation coefficient and multiple correlation coefficient for both vitamin B_{12} and folate were 0.99 and 0.99 – 1.00 respectively, indicating a strong linear relationship between the concentration of the analytical standards and the response, while the proximity to one of the regression coefficients shows that the regression line fits the data. The HPLC method was, therefore, sufficiently sensitive to be used as an analytical tool.

3.10.2 Accuracy

The recovery values for vitamin B_{12} reported in Table 3.5 ranged from 97 to 102% overall, with the RSD ranging from 0.0033 to 7.33%. The recovery values for folate reported in Table 3.6 ranged from 95 to 100% overall, with RSD ranging from 0.19 to 4.13%. All the values were within the acceptable range of recovery limits according to the AOAC (Anon., 2002), confirming the quantitative validity or accuracy for both assay methods. According to similar research reported by Van Wyk (2002), the percentage recovery for vitamin B_{12} ranged between 98 and 103% overall, and the RSD ranged between 2.68 and 6.79%. Other researchers (Gómez *et al*., 2006), reported recovery values for vitamin B_{12} ranging between 95 to 102%. The percentage recovery for the folate analysed by Van Wyk & Britz (2012), ranged between 80 and 108% overall, with the %RSD ranging between 0.79 and 9.75%. Due to the close correlation between the results obtained by the aforementioned studies and the present results, it can be concluded that the analytical procedure and treatment ‘regimes’ achieved complete dissolution of vitamin B_{12} and folate in the

Table 3.3 The limits of detection (LOD), quantification (LOQ) and linear range of vitamin B_{12} standard solutions

Parameter	Vitamin B_{12}
Linear range	0.01 – 20 µg.mL^{-1}
LOD	0.0054 µg.mL^{-1}
LOQ	0.018 µg.mL^{-1}

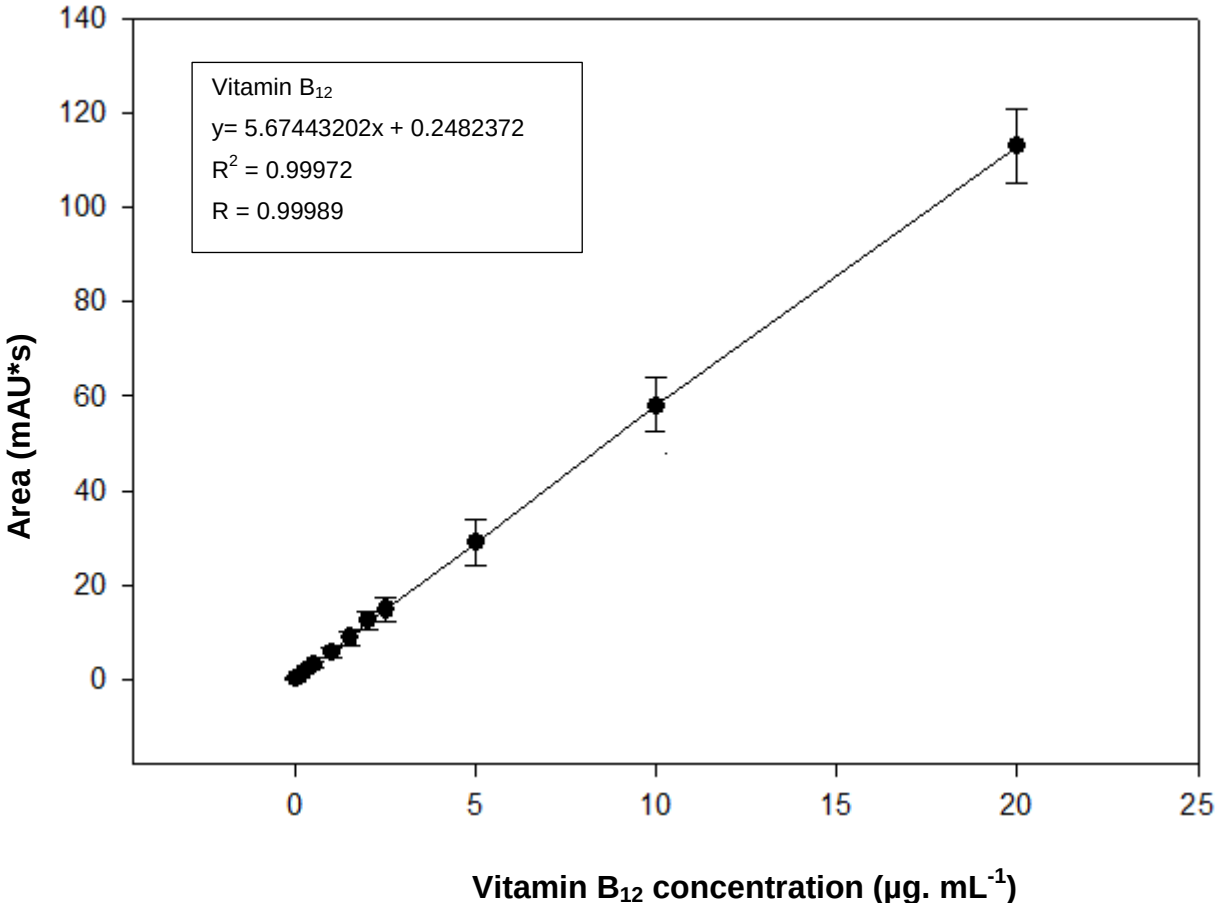


Figure 3.2 The linearity curve of the vitamin B_{12} standard solutions.

Table 3.4 The Limits of detection (LOD), quantification (LOQ) and linear range of folate vitamers

Parameter	THF[1]	5-MeTHF[2]	5-CHO-THF[3]	Folic acid Neat[4] (PGA)
Linear range	0.001 – 5 µg.mL^{-1}	0.001 – 5 µg.mL^{-1}	0.001 – 5 µg.mL^{-1}	0.001 – 5 µg.mL^{-1}
LOD	0.00015 µg.mL^{-1}	0.00016 µg.mL^{-1}	0.00025 µg.mL^{-1}	0.00021 µg.mL^{-1}
LOQ	0.00050 µg.mL^{-1}	0.00052 µg.mL^{-1}	0.00083 µg.mL^{-1}	0.00068 µg.mL^{-1}

[1]THF – Tetrahydrofolic acid; [2]5-MeTHF – 5-Methyltetrahydrofolic acid; [3]5-CHO-THF – 5-Formyltetrahydrofolic acid; [4]PGA – Pteroyl-glutamic acid

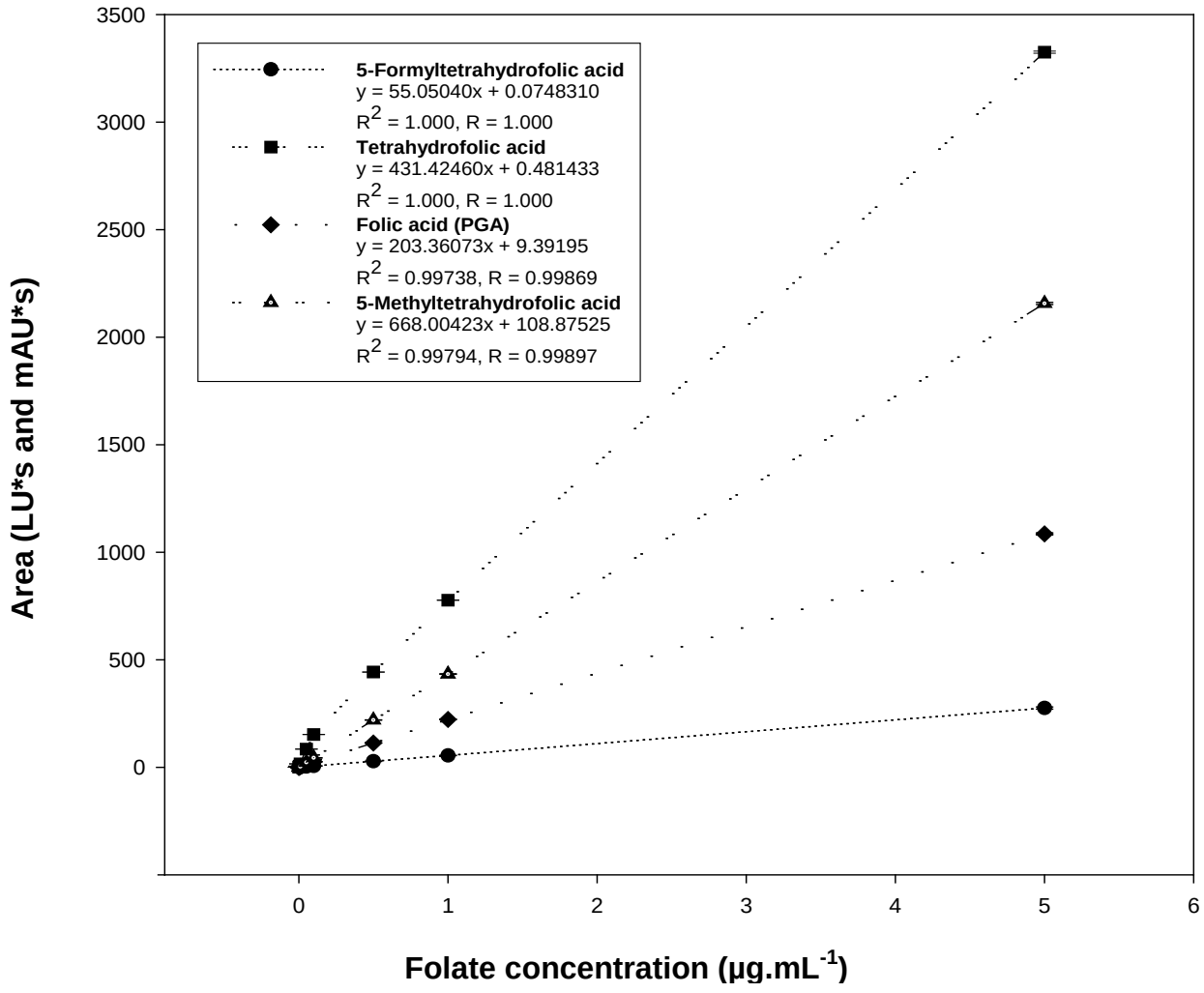


Figure 3.3 The linearity curves for the four folate vitamers.

beverage samples analysed in this study.

3.10.3 Precision

The HORRAT values were reported as the Horwitz criteria parameter of acceptance for precision studies (HORRATr) (Chen & Eitenmiller, 2007). Repeatability precision of intra-day and inter-day assay for vitamin B_{12} and folate are shown in Tables 3.7 and 3.8, respectively. The HORRATr values of intra-day assay and inter-day assay precisions for vitamin B_{12} ranged between 0.38 – 0.42 and 0.36 – 0.69, respectively. The HORRATr values of intra-day and inter-day assay precisions for folate vitamers in the calibrant mixture ranged between 0.41 –1.11 and 0.44 – 0.93, respectively. The results were within the acceptable range of 0.3 to 1.3 according to the AOAC guidelines (Anon., 2002). These results, therefore, indicate that the methods were precise.

3.10.4 Peak identification and peak purity

The identification of vitamin B_{12} and folate was achieved by means of the retention time (t_R) and the quantification of the vitamers were done by measuring the peak area of the samples relative to that of the standards. The retention times of the vitamin B_{12} and folate vitamers (calibrant mixture) were determined by analysing the t_R of standard samples during three sessions (n = 3) and the means and standard deviations were calculated. The results of the retention time (minutes) obtained for vitamin B_{12} was 19.39 ± 0.05 (Fig. 3.4) and for the folate vitamers were: THF (16.29 ± 0.00); 5-CH_3-THF (17.11 ± 0.01); 5-CHO-THF (20.62 ± 0.04) and PGA (20.96 ± 0.01) (Fig. 3.5A and 3.5B). The low standard deviation of vitamin B_{12} and folate indicated that the HPLC methods were suitable and reliable tools for peak identification. Furthermore, purity tests were performed using the “Check purity” option of the Chemstation system. This option was used to determine if the purity factor of the peaks were within the threshold limit and therefore can be used to calculate the vitamin B_{12} and folate concentrations. The purity factors were > 990 and 1000, which indicated a perfect peak.

Table 3.5 Recovery results for HPLC determination of vitamin B_{12}

Vitamin B_{12} added to kefir sample (µg. mL^{-1})	Mean[1] ± SD[2]	%RSD[3]	Percentage (%) recovery[4]	Range[5] (%)
20	20.46 ± 0.004	0.02	102.29	95 – 102
50	48.77 ± 0.002	0.004	97.50	95 – 102
80	80.26 ± 5.88	7.33	100.40	95 – 102

[1,2]Measured vitamin B_{12} concentration (µg.mL^{-1}) expressed as mean ± standard deviation. [3]Relative standard deviation. RSD (%) = (SD/Mean) X 100. [4]% Recovery (percentage recovery of added standard) = (Cs – C_p/C_a) X 100. C_S, Mean vitamin B_{12} concentration in spiked sample; C_p, vitamin B_{12} concentration in the native sample and C_a, amount of vitamin B_{12} added. [5]AOAC range/limit for single laboratory validations (Anon., 2002).

Table 3.6 Recovery results for HPLC determination of folates

	Percentage (%) recovery[1]							
	Tetrahydrofolic acid (THF)		5-CH_3-THF[2]		5-CHO-THF[3]		Folic acid	
Concentration added calibrant mixture (µg.mL^{-1})	**Mean ± SD[4] (%RSD)[5]**	**Range[6] (%)**	**Mean ± SD (%RSD)**	**Range (%)**	**Mean ± SD (%RSD)**	**Range (%)**	**Mean ± SD (%RSD)**	**Range (%)**
50	99.3 ± 0.33 (4.13)	95 – 102	96.0 ± 0.11 (1.77)	95 – 102	98.2 ± 0.07 (0.58)	95 – 102	98.1 ± 0.04 (0.65)	95 – 102
80	95.9 ± 0.21 (2.25)	95 – 102	95.6 ± 0.11 (1.48)	95 – 102	99.2 ± 0.04 (0.19)	95 – 102	99.1 ± 0.10 (0.98)	95 – 102
100	97.4 ± 0.10 (1.01)	98 – 101	97.8 ± 0.21 (2.79)	98 – 101	98.9 ± 0.14 (0.56)	98 – 101	100.6 ± 0.11 (0.65)	98 – 101

[1]%Recovery (percentage recovery of added standard) = (C_s – Cp/Ca) X 100. C_S, [2]5-Methyl-THF, [3]5-Formyl-THF.Mean folate concentration in spiked sample; C_p, folate concentration in the native sample; C_a, amount of folate added. [4]Measured folate concentration (µg.mL^{-1}) expressed as mean and standard deviation. [5]Relative standard deviation reported in brackets. RSD (%) = (SD/Mean) X 100. [6]AOAC range/limit for single laboratory validations (Anon., 2002).

Table 3.7 Repeatability precision of intra-day and inter-day assay of vitamin B_{12} standards

	Session 1				Session 2			
Intra-assay[1]	**Mean[3]**	**SD[4]**	**RSDr (%)[5]**	**HORRATr[6]**	**Mean**	**SD**	**RSDr (%)**	**HORRATr**
Vitamin B_{12} (n = 7)	73.5	0.3	0.41	0.38	73.3	0.21	0.29	0.42
	Day 1				**Day 2**			
Inter-assay[2]	**Mean**	**SD**	**RSDr (%)**	**HORRATr**	**Mean**	**SD**	**RSDr (%)**	**HORRATr**
Vitamin B_{12} (n = 14)	73.5	0.28	0.38	0.36	73.1	0.18	0.25	0.69

Repeatability between sessions on the same day[1] and between days[2]; [3]Mean vitamin B_{12} concentration in µg.100 mL^{-1} measured with the HPLC method; [4]Standard deviation; [5]Relative standard deviation observed. RSDr observed (%) = (SD/Mean) X 100. [6]HORRATr = RSDr observed / RSDr predicted. RSDr predicted = $2/3 \times 2^{(1-0.5\log C)}$ (with C the analyte concentration in mass fraction in $g.g^{-1}$).

Table 3.8 Repeatability precision of intra-day and inter-day assay of folate standards

	Session 1				Session 2			
Intra-day assay[1]	**Mean[3]**	**SD[4]**	**RSDr (%)[5]**	**HORRATr[6]**	**Mean**	**SD**	**RSDr (%)**	**HORRATr**
THF (n = 7)	7.63	0.44	5.81	0.69	7.25	0.68	9.1	0.54
5-MeTHF (n = 7)	6.09	0.69	11.8	0.68	5.97	0.46	7.6	0.41
5-CHO-THF (n = 7)	13.46	1.55	11.47	0.74	12.19	2.21	17.3	1.11
Folic acid (PGA) (n = 7)	5.54	0.78	14.31	0.82	5.35	0.86	15.6	0.88
	Day 1				**Day 2**			
Inter-day assay[2]	**Mean**	**SD**	**RSDr (%)**	**HORRATr**	**Mean**	**SD**	**RSDr (%)**	**HORRATr**
THF (n = 14)	7.51	0.55	7.38	0.44	7.68	0.59	7.73	0.46
5-MeTHF (n = 14)	6.09	0.57	9.68	0.56	5.97	0.68	11.62	0.67
5-CHO-THF (n = 14)	13.05	1.88	14.34	0.93	13.15	1.84	13.96	0.91
Folic acid (PGA) (n = 14)	5.54	0.79	14.41	0.82	5.54	0.72	13.16	0.75

Repeatability between sessions on the same day[1] and between days[2]; [3]Mean folate concentration in µg.100 mL^{-1} measured with the HPLC method; [4]Standard deviation; [5]Relative standard deviation observed. RSDr observed (%) = (SD/Mean) X 100. [6]HORRATr = RSDr observed / RSDr predicted. RSDr predicted = $2/3 \times 2^{(1-0.5\log C)}$ (with C the analyte concentration in mass fraction in $g.g^{-1}$).

3.10.5 Complete deconjugation of $PteGlu_3$ to PGA

The deconjugation protocol was performed to determine the effectiveness of the deconjugation treatment. Complete deconjugation was indicated by the complete conversion of Pteroyltri-L-γ-glutamic acid ($PteGlu_3$) to folic acid (PGA) by the deconjugase enzyme present in human serum. This meant that the presence of the PGA peak where the $PteGlu_3$ was added before heat denaturation of the deconjugase contrasts with the absence of the PGA peak where the $PteGlu_3$ was added after denaturing the deconjugase. In this case, the $PteGlu_3$ peak replaced the PGA peak indicating complete deconjugation by the enzyme.

3.10.6 pH, vitamin B_{12} and folate

In comparison with untreated grains (control sample), co-inoculation treatments of the grains with the broth culture, freeze-dried culture as well as the grains directly co-inoculated with reconstituted freeze-dried PAB culture (henceforth referred to as the "direct culture") were used to determine which regime produced the highest levels of vitamin B_{12} and folate at 1 d and 3 d. In addition to 1 d and 3 d, pH was measured immediately after inoculation (0 d) as well, since it relates to acidity which is a critical sensory attribute of kefir (Simova *et al*., 2002). The results indicated that the pH (Table 3.9), vitamin B_{12} and folate levels (Table 3.10) differed significantly ($p < 0.05$) between 1 d and 3 d for all the aforementioned sample treatments. However, the sample treatments did not affect the vitamin B_{12} levels significantly ($p > 0.05$) (Table 3.10). In contrast, the folate levels between sample treatments differed significantly overall ($p < 0.05$) with folate levels between freeze-dried and direct PAB culture treatments not significantly different from each other, but both significantly lower than the control samples ($p < 0.05$), which in turn were significantly ($p < 0.05$) lower than the broth samples (Table 3.10). All pH levels measured at 0 d, 1 d and 3 d between sample treatments were numerically lower than the control sample ($p > 0.05$). However, the pH level for each sample treatment decreased significantly ($p < 0.05$) between each sampling time.

The pH values of the control sample at 0 d (6.56 ± 0.02), 1 d (6.56 ± 0.02) and at 3 d (4.61 ± 0.04) (Table 3.9), were numerically higher than the other sample treatments ($p > 0.05$). The pH at 0 d (6.55 ± 0.17) for the direct culture was the second highest and decreased to 5.78 ± 0.08 (1 d) and then to 4.27 ± 0.08 (3 d) ($p < 0.05$). The pH of the broth culture and freeze-dried culture measured at 0 d was 6.37

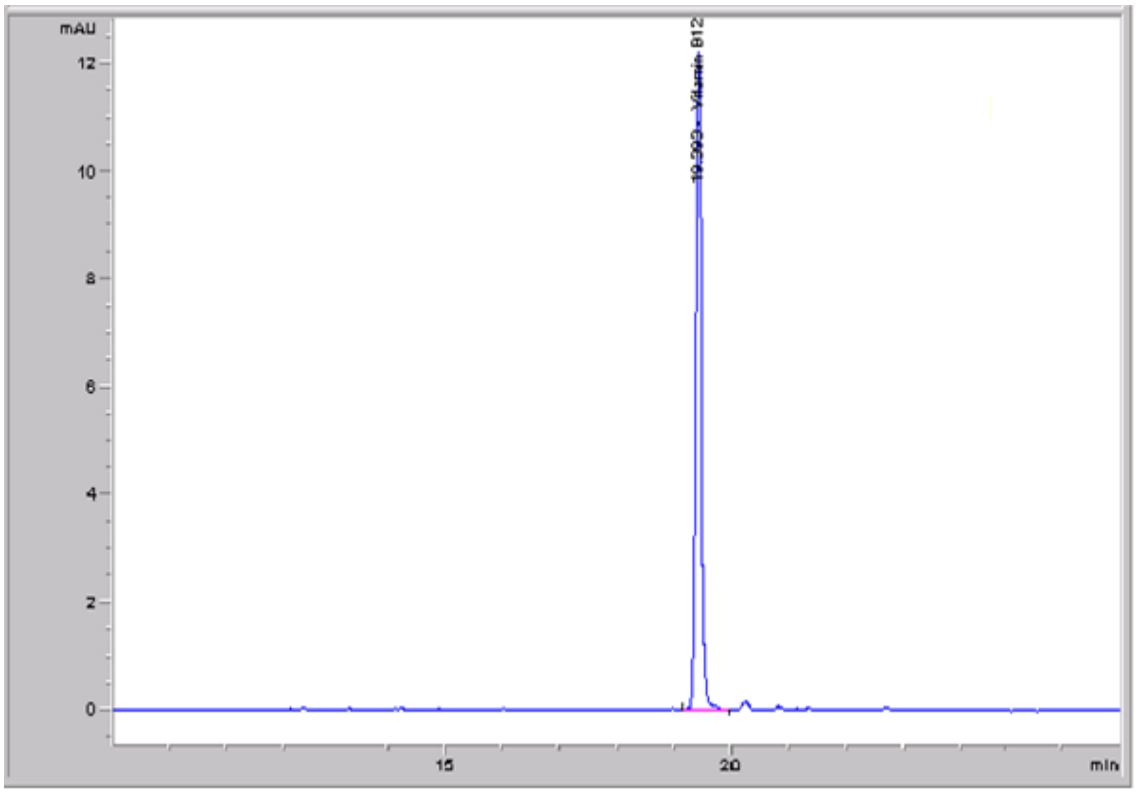


Figure 3.4 The elution of vitamin B_{12} detected using the DAD.

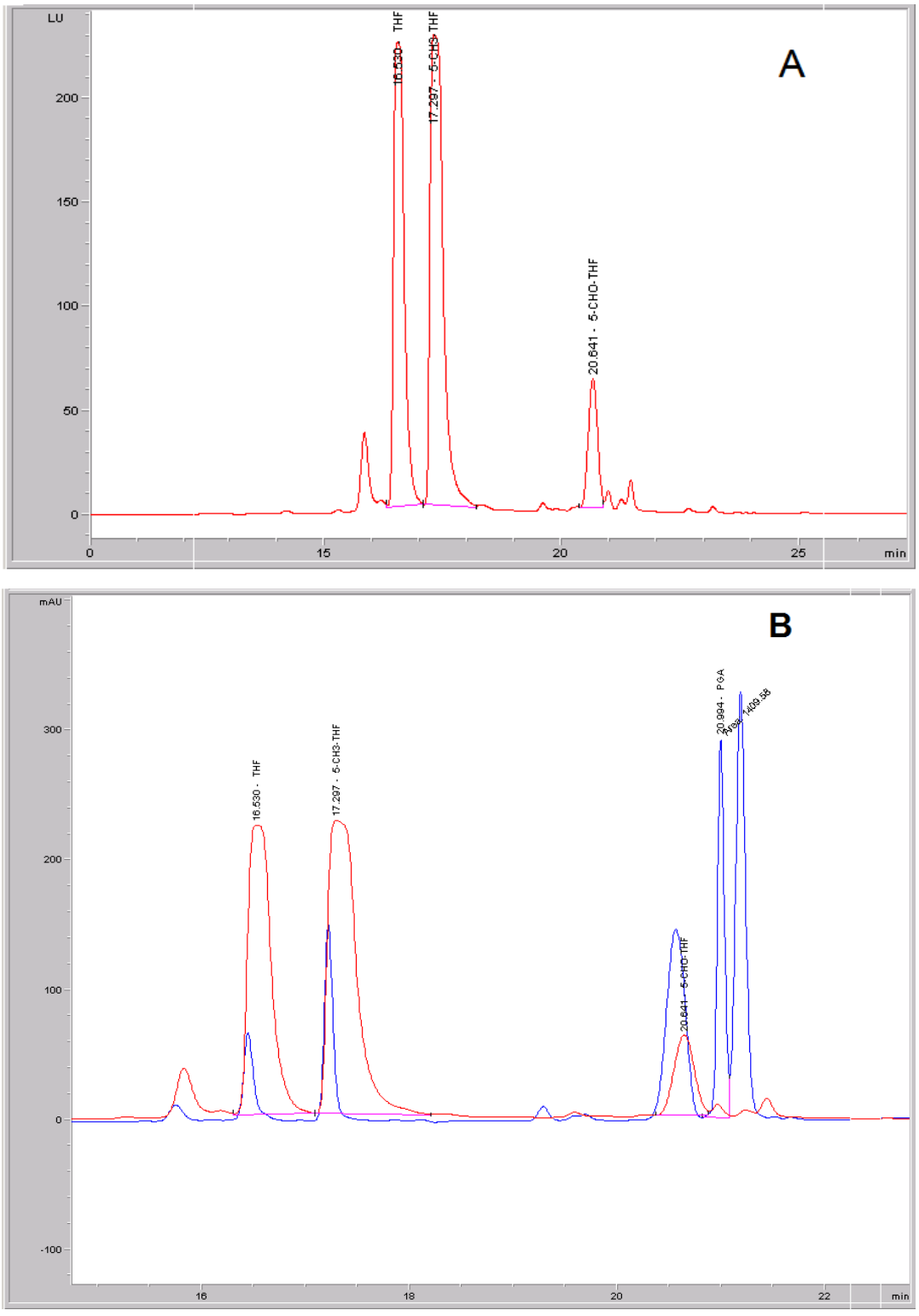


Figure 3.5A and 3.5B In Fig. 3.5A, the folate vitamers eluted and was detected in the order of: THF, 5-CH_3-THF and 5-CHO-THF using the FLD and the PGA was detected using the DAD (Fig. 3.5B)

± 0.09 and 6.12 ± 0.57, respectively, while it decreased to 5.61 ± 0.07 (broth culture) and 5.24 ± 1.15 (freeze-dried culture) at 1 d ($p > 0.05$) and to 4.09 ± 0.20 (broth culture) and 4.07 ± 0.92 (freeze-dried culture) at 3 d. Hence, at 1 d, the pH ranged from 5.24 – 6.19, while at 3 d the pH ranged from 4.07 – 4.61 (Table 3.9). While the lower end of the pH range at 1 d compared favourably with the pH range at 1 d reported by Affane (2012) (4.81 – 5.23), the pH range at 1 d was still higher than the typical pH of kefir (4.1 – 4.6) (Garrote *et al*., 1998; Farnworth, 2005; Powell, 2006; Păucean & Socaciu, 2008). Moreover, the high pH range at 1 d may be attributed to the grains possibly not being in a sufficiently active state (Affane, 2012) and could also relate to different groups of microbes present in the grains being active at different stages of the fermentation (Koroleva, 1982). Moreover, Collar (1996) reported that lactic acid bacteria multiply and produce lactic and acetic acid much slower when inoculated with a mixture of yeasts than with a pure culture.

The vitamin B_{12} and folate level for each sample treatment increased significantly ($p < 0.05$) between each sampling time. While the presence of PAB in the grains lead to numerically higher vitamin B_{12} levels, vitamin B_{12} was also measured in the control samples. This can be ascribed to the reported ability of the organisms in kefir grains to synthesize vitamin B_{12} and folate in the kefir beverage (Kneifel & Mayer, 1991; Ötles & Cadingi, 2003; Irigoyen *et al*., 2005). The freeze-dried culture that numerically produced the highest vitamin B_{12} concentration at 1 d ($p > 0.05$) yielded the second highest vitamin B_{12} level at 3 d ($p > 0.05$) (Table 3.10). The direct PAB culture produced the second highest vitamin B_{12} level at 1 d, but the second lowest at 3 d ($p > 0.05$). The control kefir sample produced the lowest vitamin B_{12} concentration at both 1 d and at 3 d compared to the other treatments ($p > 0.05$) (Table 3.10). The kefir grains treated with the PAB broth culture resulted in a numerically higher vitamin B_{12} level although there was no significant difference when compared to the other treatments at 3 d fermentation (Table 3.10). Van Wyk *et al.* (2011), reported an increase in vitamin B_{12} and folate when fermenting kefir with *Propionibacterium freudenreichii*. The findings of these authors concur with the results of this study indicating an increase in vitamin B_{12} during kefir fermentation when co-inoculated with PAB. However, despite the significant difference in vitamin B_{12} concentrations between sample times ($p < 0.05$), it is important to note that the different treatments of the grains with the PAB inoculum in this study did not have a significant effect ($p > 0.05$) with regards to the vitamin B_{12} concentration.

Table 3.9 pH of kefir samples at different sampling times and sample treatments

	pH		
Sample treatments	**Day 0[1] (mean ± SD)[2]**	**Day 1 (mean ± SD)**	**Day 3 (mean ± SD)**
Control (n = 2)[3]	6.56 ± 0.02[a]	6.19 ± 0.03[b]	4.61 ± 0.04[c]
Broth culture (n = 2)[4]	6.37 ± 0.09[a]	5.61 ± 0.07[b]	4.09 ± 0.20[c]
Freeze-dried culture (n = 2)[4]	6.12 ± 0.57[a]	5.24 ± 1.15[b]	4.07 ± 0.92[c]
Direct PAB culture (n = 2)[4]	6.55 ± 0.17[a]	5.78 ± 0.08[b]	4.27 ± 0.08[c]

[1]The pH of Day 0 samples was measured immediately after inoculation. [2]Results were reported as the mean ± standard deviation (SD). Univariate Analyses of Variance (ANOVA) for each sample tested the difference between the main effects i.e. sample type and sampling time (day 0, 1 and 3). Sample treatments was not significant ($p > 0.05$), while sampling time was ($p < 0.05$). Duncan's multiple comparison post-hoc test was performed and different letter superscripts in each row denote significant differences ($p < 0.05$) between individual means. [3]Kefir grains as is and grains co-inoculated with PAB.[4]

Table 3.10 Comparison of the vitamin B_{12} and folate results in kefir beverage samples at day 1 and day 3[1]

		cfu.mL^{-1} (mean ± standard deviation)[3]	Total vitamin B_{12} (µg.100 mL^{-1}) (mean ± standard deviation)		Total folate (µg.100 mL^{-1}) (mean ± standard deviation)	
Chapter 3	**Inoculum**		**1 d**	**3 d**	**1 d**	**3 d**
Control (n = 4)	None	N/A	8.16 ± 4.33^{a}	29.81 ± 3.90^{b}	458.45 ± 34.91^{b}	655.87 ± 15.2^{e}
Broth culture (n = 4)	PAB	5 X10^{6} ± 0.87^{x}	11.80 ± 2.18^{a}	23.73 ± 8.74^{b}	104.20 ± 0.15^{c}	111.69 ± 1.14^{f}
Freeze-dried culture (n = 4)	PAB	2 X 10^{5} ± 0.70^{x}	8.73 ± 0.78^{a}	22.24 ± 4.16^{b}	114.13 ± 3.36^{c}	124.45 ± 5.51^{f}
Direct PAB culture[2] (n = 4)	PAB	1 X 10^{3} ± 0.61^{x}	8.16 ± 4.33^{a}	29.81 ± 3.90^{b}	458.45 ± 34.91^{b}	655.87 ± 15.2^{e}

[1, 3]Univariate Analyses of Variance (ANOVA) determined differences between samples means based on Duncan's multiple comparison post-hoc test where different superscripts in each column as well as each row indicate significant differences ($p < 0.05$) between different samples for each assay. The total folate is the sum of individual folate vitamers (THF, 5-CH_3-THF, 5-CHO-THF and PGA). [2]Direct co-inoculation with reconstituted freeze-dried PAB culture.

The folate level produced by the broth culture was higher than the other treatments when sampled at 1 d (458.45 ± 34.91 µg.100 mL^{-1}) and at 3 d (655.87 ± 15.2 µg.100 mL^{-1}) (Table 3.10). The control sample produced the second highest folate level at 1 d (276.80 ± 19.6 µg.100 mL^{-1}) and at 3 d (608.80 ± 0.42 µg.100 mL^{-1}). These folate concentrations were significantly higher ($p < 0.05$) than the freeze-dried culture and the direct PAB culture, while at both sampling times, both the direct culture and the freeze-dried culture showed no significance ($p > 0.05$). In recent reports it was suggested that milk contains between 0.2 – 0.5 µg.100 mL^{-1} folate (Hugenholtz & Smid, 2002), while fermented milk was reported to contain even higher amounts (Alm, 1980), with up to 142 $mg.L^{-1}$ detected in yoghurt (Smid *et al*., 2001). LeBlanc *et al.* (2010), reported that the folate concentration in kefir was 50 $\mu g.L^{-1}$, yoghurt was 80 $\mu g.L^{-1}$ and buttermilk was 90 $\mu g.L^{-1}$. While these folate concentrations were lower than that of the control sample and the other sample treatments in this study, it proves that LAB, present in bacterial cultures, produce folate. As with folate, the overall vitamin B_{12} levels between the freeze-dried culture and the direct culture at 1 d and at 3 d were also not significant ($p > 0.05$). Hence, in this study, the broth culture was the best treatment with regards to vitamin B_{12} and folate levels.

The factors that may have affected the total vitamin B_{12} and folate production of the PAB inoculum co-inoculated with the kefir grains were the inoculum form (broth and freeze-dried cultures); concentration of PAB in the multiple PAB culture treatments with the kefir grains; and the relative PAB activity between the broth and freeze-dried cultures. The inoculum form of the PAB culture used for the multiple treatments of the kefir grains were used to investigate the effectiveness of PAB inclusion into the kefir grains. However, since the PAB cell concentration in the original inoculum did not differ significantly among treatments (Table 3.10), when comparing the vitamin B_{12} and folate concentrations among sample treatments, it is clear that the PAB cell concentration in the original inoculum did not directly translate into higher vitamin concentrations. This is especially evident when looking at the folate concentrations which showed a significant difference for the broth culture treatment (Table 3.10). When comparing the multiple treatments with the single treatment of PAB culture with the grains, it is evident that the multiple treatments yielded superior vitamin B_{12} and folate levels at 3 d. In this case, the multiple treatments of the kefir grains with the broth culture produced higher vitamin B_{12}

(numerically) and folate levels than the control, freeze-dried culture and direct culture. Hence, multiple additions using the broth culture was the best treatment with regards to vitamin B_{12} and folate levels. According to Regulation No. R. 146 under section 15(1) of the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972), the nutrient reference values (NRV) for individuals 4 years and older for vitamin B_{12} and folate are 2.4 µg and 400 µg per day, respectively (Anon., 2003). When comparing the aforementioned results of vitamin B_{12} and folate levels (per 100 mL serving of the kefir beverage) at 1 d with the NRV, the results were: the broth culture sample contained 340% (vitamin B_{12}) and 115% (folate) and the control sample contained 254% (vitamin B_{12}) and 69% (folate) of the NRV. The freeze-dried culture and direct PAB culture samples contained 492% and 364% for vitamin B_{12}, respectively, while the folate levels for the freeze-dried culture and direct PAB culture samples were 28% and 31% of the NRV per serving, respectively. Hence, according to these results the broth culture was the best treatment with regards to optimum levels of vitamin B_{12} and folate in the resultant kefir beverage.

3.10.7 DNA and PCR analysis

The DNA extraction and PCR assays were performed according to both manufacturers' guidelines, namely Promega and Kapa Biosystems, respectively, to confirm the presence of PAB in the kefir grains and kefir beverage of the PAB treated samples. Prior to the DNA extraction of the samples, DNA was initially extracted from 2 aliquots of the PAB bacterial broth culture (serving as a positive control) before analysing the 24 treatment samples using the Wizard Genomic DNA Purification kit (Promega). The quality and quantity of the DNA present in the control and PAB-treated samples were evaluated spectrophotometrically, using the NanoDrop ND1000 followed by agarose gel electrophoresis. Subsequently, the PCR analysis using *Propionibacterium freudenreichii* 16s rDNA specific primers (Prop 1 F 5'-GATACGGGTGACTTGAGG-3' and Prop 2 R 5'-GCGTTGCTGATCTGGATTAC-3') (Whitehead Scientific, South Africa) was performed for all samples using the Kapa HiFi™ PCR kit (Kapa Biosystems). The PCR products were subjected to Sanger sequencing and analysed against the positive control for identification.

The presence of *Propionibacterium freudenreichii* was observed in all PAB-treated samples, both kefir grains and kefir beverages (Table 3.11). This was evident by the DNA fragment present in all of the treated samples (indicated by a yellow

rectangle), as well as in the positive control (PAB bacterial culture-red arrows) (Fig. 3.6). However, several non-specific PCR bands were also observed indicating the possibility of other species such as lactic acid bacteria. The primers used were targeted to 16S RNA genes and therefore possibly amplified lactic acid bacterial strains present in the kefir grains and beverage and not just *Propionibacterium freudenreichii.* The major 750 bp DNA fragment was then excised from the gel and sequenced using the BioEdit and ClustalW (Ibis Biosciences, California, USA) to determine whether the these DNA fragments originated from *P. freudenreichii*. However, the 750 bp DNA fragment present in the control kefir grain samples as well as the control kefir beverage samples was positively identified as originating from *Lactobacillus kefiranofaciens*. These samples, therefore, did not contain *Propionibacterium freudenreichii*, while all the PAB-treated samples did (Table 3.11). The treatment of the PAB with the kefir grains using the various treatment regimes (Fig. 3.1) resulted in the presence of PAB in the grains and beverage when inoculated into milk. Hence, the treatments were successful regarding the incorporation of the PAB in the kefir grains.

3.10.8 Sensory analysis

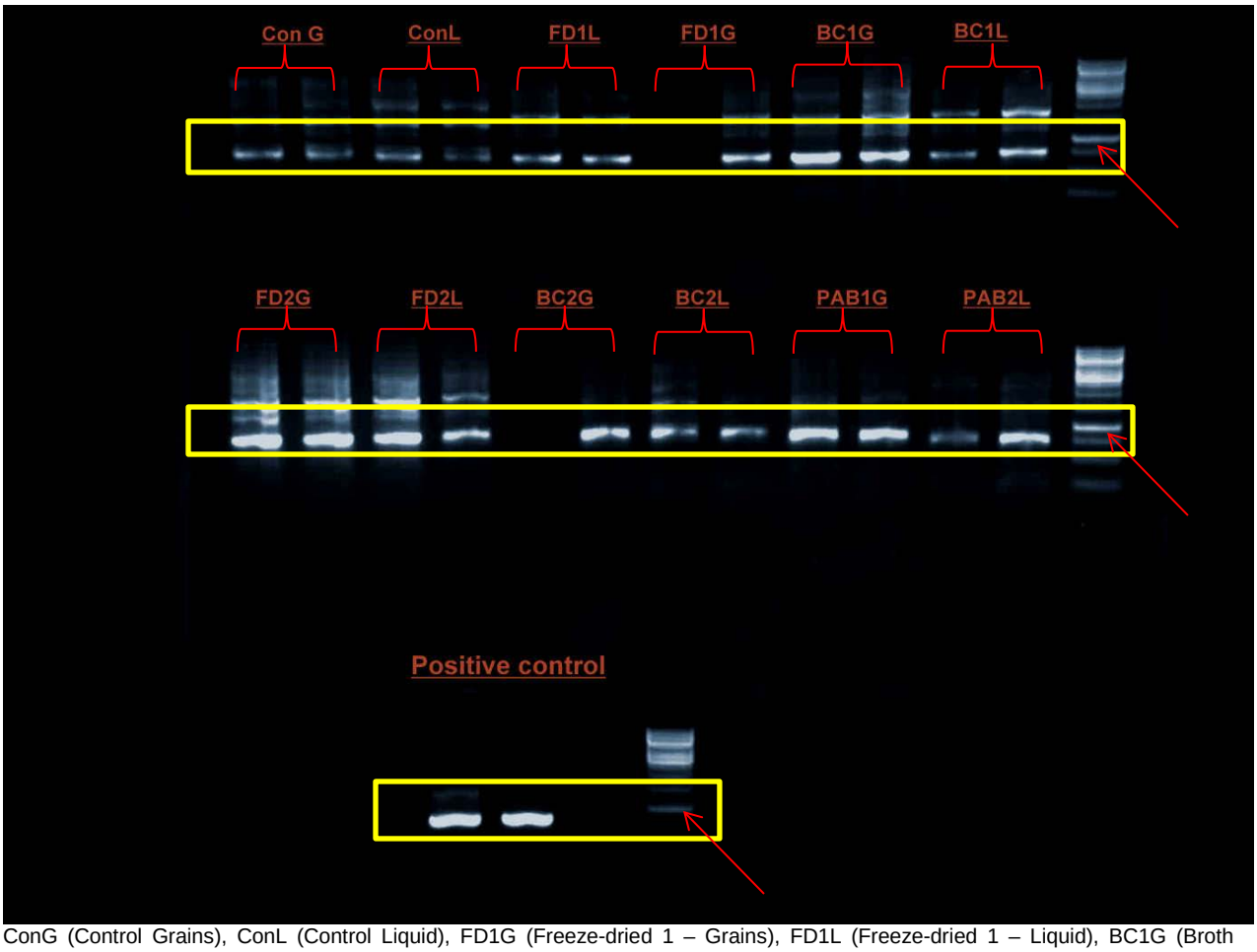
The major factors determining the quality of a kefir beverage are generally taste and overall acceptability. The overall taste and specifically the sour taste are attributed to the pH and the microbial profile of the final beverage, usually affected by the fermentation time, which has an effect on the sensorial quality of the beverage (Garrote *et al*., 1998). In this study, the changes in pH and the acid content affected differences in the sour taste and overall taste of the kefir beverage at 1 d and 3 d between the treated and control samples (Fig. 3.7). The sample treatment of the grains (freeze-dried, broth culture and direct culture of PAB) did not have a significant effect, while the kefir fermentation time had a significant impact on the results of the sensory evaluation (Fig. 3.7).

According to the sensory panel, the sour taste of the kefir beverage treated with the freeze-dried culture sampled at 3 d was numerically higher than that of all the other samples (Fig. 3.7). The sour taste of the samples treated with the direct

Table 3.11 The PCR assays performed on the DNA extract from the sample treatments included kefir grains and beverage samples. Both DNA and PCR assays were performed in duplicate.

	Day 3	
Sample treatment	**Grains**	**Kefir beverage**
Control (n = 2)	-	-
PAB broth culture (n = 2)	+	+
PAB freeze-dried culture (n = 2)	+	+
PAB direct culture (n = 2)	+	+

A positive result (+) indicated that PAB was present in detectable numbers, while a negative result (-) indicated the absence of PAB in the sample.



ConG (Control Grains), ConL (Control Liquid), FD1G (Freeze-dried 1 – Grains), FD1L (Freeze-dried 1 – Liquid), BC1G (Broth culture 1 – Grains), BC1L (Broth culture 1 – Liquid), PAB1G (Direct culture – Grains) and PAB1L (Direct culture – Liquid)

Figure 3.6 *Propionibacterium freudenreichii* 750 bp DNA fragment present in all of the treated samples.

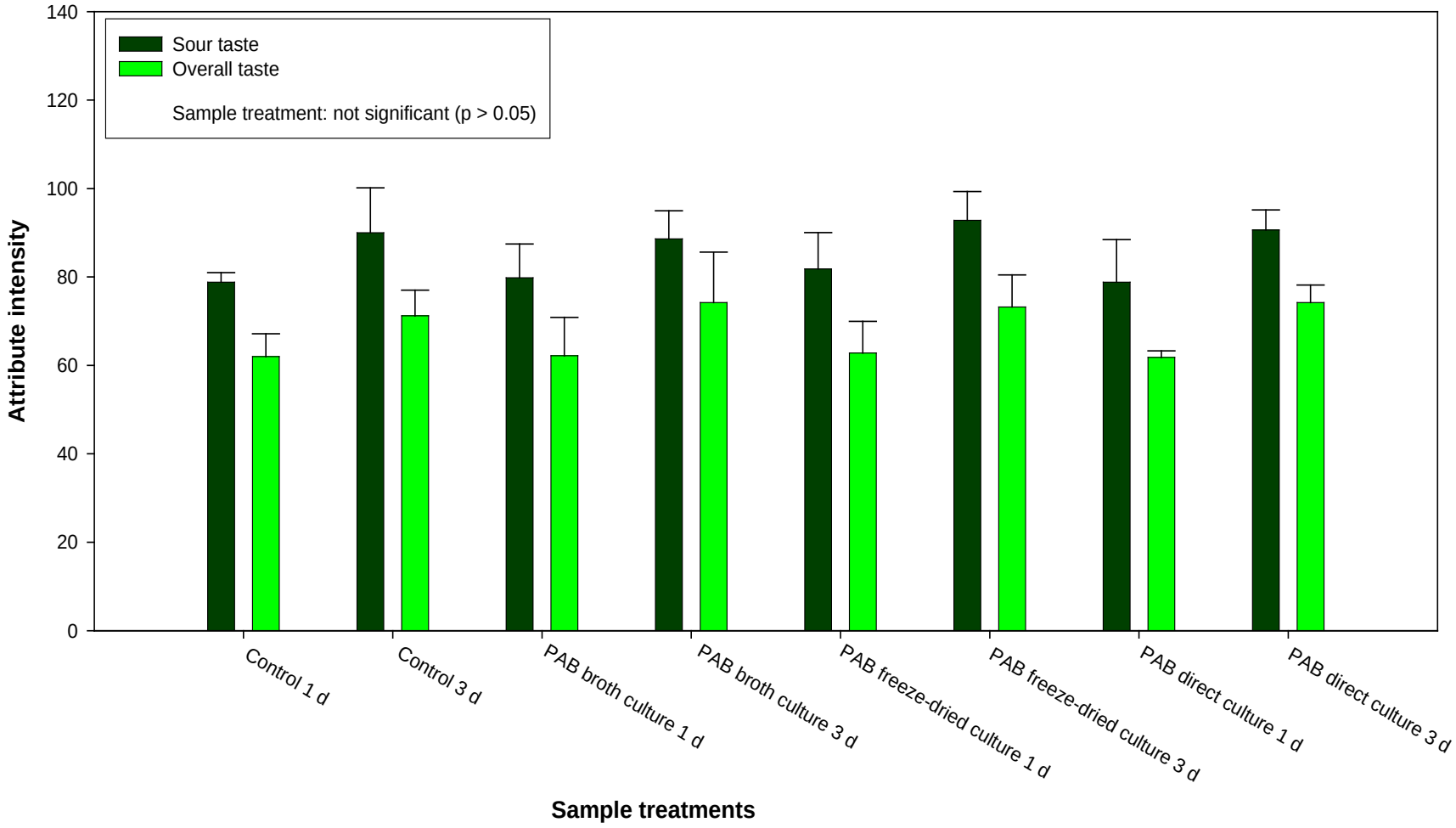


Figure 3.7 Sensory evaluation of the kefir beverage sample.

culture at 3 d was scored the second highest, followed by the control samples and the broth culture at 3 d.

As expected, the sour taste of the samples at 1 d was less intense than at 3 d (Fig. 3.7). This was in agreement with the high pH at 1 d (Table 3.9) which may be attributed to the grains not being sufficiently active, leading to low yields of lactic acid which is essentially responsible for the acidic taste of the kefir (Affane, 2012). At 3 d the panel scored the sour taste of samples higher (Fig. 3.7) due to an increase in acidification, judged by the low pH that was observed (Table 3.9).

The overall taste of the samples were not significantly different ($p > 0.05$), with the panel scores for all samples around 60 out of 100 (Fig. 3.7). At 3 d the beverage of the kefir grains treated with the direct culture was numerically the highest, while the treated sample with the broth culture scored the second highest for overall taste followed by the freeze-dried culture and control sample, respectively ($p > 0.05$) (Fig. 3.7). It is interesting to note that, contrary to expectation, the scores for the sour taste and overall taste did not agree at 1 d.

Hence, while previous authors reported that the distinctive acid taste is a major contributor to overall taste (Güzel-Seydim *et al*., 2000; Yüksekdağ *et al*., 2004), this direct relationship is attributed to the effect of the pH after fermentation. However, the overall taste of the kefir beverage samples was more desirable at 3 d fermentation than at 1 d.

Based on the overall results of the samples at 1 d, the broth culture showed ideal vitamin B_{12} and folate levels in terms of the %NRV. In terms of sensory analysis, the untreated kefir grains (control) had the best sour taste and overall taste at 3 d. However, at 1 d sensorially there was no significant difference ($p > 0.05$) between the samples, with the broth culture-treated sample scoring 80 for “sour taste” and 60 for “overall taste”. The presence of the PAB was also confirmed in all treated samples. Hence, with an improvement in the taste, the broth culture sample will be the best treatment suitable for a B-vitamin enriched kefir beverage. However, since the 340% NRV for vitamin B_{12} in this sample exceeded 100% considerably, there is merit in investigating all three treatments in a further study where PAB and LAB are combined with the kefir grains.

3.11 Conclusions

The treatment of the PAB with the kefir grains resulted in the inclusion of PAB within the kefir grains. The elevated concentration of vitamin B_{12} and folate at 1 d and 3 d in the kefir beverage sample, as well as the PCR assay results, confirmed that the treatments were successful.

The overall concentration of the PAB inoculum ($cfu.mL^{-1}$) between culture types did not have a significant effect ($p > 0.05$) with regards to vitamin B_{12} levels, but the broth culture sample showed a significantly higher ($p < 0.05$) folate level (Table 3.10). Therefore, the vitamin B_{12} and folate levels between the culture types were numerically different and not significantly different ($p > 0.05$).

Based on these results, the vitamin B_{12} and folate produced in terms of fermentation time and concentration, the best results were obtained at both 1 d and 3 d and was achieved when reacting the broth culture with the kefir grains. However, since the typical kefir fermentation is 24 h (Lengkey & Balia, 2014), the results at 1 d was favoured. This allowed a viable manner in which the PAB can be preserved and remain active with the grains. Moreover, the results also showed that this type of co-inoculation may have the potential to enrich the kefir with B-vitamins resulting from a natural biosynthetic process.

When considering the B-vitamin levels and the PAB inoculum concentration, the increased B-vitamin levels were expected to occur since the grains were reacted multiple times. However, the direct PAB culture was treated once with the grains and produced comparable vitamin levels. Hence, this is a viable regime to increase vitamin B_{12} and folate levels in the kefir samples. However, the 340% NRV for vitamin B_{12} in this sample exceeded 100% considerably, while the folate in the other samples were much lower than 100% of the NRV. Hence, pre-treatment of the grains with an organism that will enhance folate levels was also indicated.

The sensory analysis of the kefir beverage in terms of sour and overall taste amongst the samples showed no significant difference. However, the panel preferred the overall taste of the beverage fermented at 3 d opposed to 1 d, while the sour taste was preferred at the fermentation time of 1 d. Hence, more work is required to improve

this, for example, more effective reconstitution and an increase in inoculum concentration.

In conclusion, the treatments of the inocula used to produce the kefir beverages enabled the development of technology-based beverages with the potential to produce and expand the range of fermented dairy products on the market with enhanced nutritional value.

3.12 References

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CHAPTER 4

THE DETERMINATION OF VITAMIN B_{12} AND FOLATE CONCENTRATION IN THE KEFIR BEVERAGE INOCULATED WITH A FREEZE-DRIED AND BROTH CULTURE OF *PROPIONIBACTERIUM FREUDENREICHII* J19 AND *STREPTOCOCCUS THERMOPHILUS* ATCC 19258

4.1 Abstract

In this study, two treatment regimes regarding the inclusion of *Propionibacterium freudenreichii* and *Streptococcus thermophilus* in combination with the kefir grains were performed. These regimes were used to produce kefir grains with the capacity to biosynthesize higher levels of vitamin B_{12} and folate in the kefir beverage. The success of the treatment was measured in terms of (1) the vitamin B_{12}, folate and pH levels in the kefir beverage; (2) the sensorial quality of the kefir beverage; and (3) the confirmed presence of PAB in the kefir grains and the kefir after fermentation. Multiple treatments of the kefir grains with PAB and LAB broth culture and freeze-dried culture, respectively, resulted in elevated vitamin B_{12} and folate levels compared to control samples. At 1 d and 3 d fermentation time PCR assays confirmed the presence of PAB in all treated kefir grains and beverages samples.

In terms of the %NRV for vitamin B_{12} and folate levels at 1 d, the freeze-dried treatment samples contained vitamin B_{12} (5.03 ± 1.89 µg.100 mL^{-1}) levels that did not excessively exceeded the NRV at 110%, while the folate (412.89 ± 0.00 µg.100 mL^{-1}) level was on par with the NRV daily requirements (103%).

The desired beverage according to the sensory evaluation with regards to the sour taste and overall taste was the freeze-dried culture sample at 1 d and the control sample at 3 d. However, since the typical kefir fermentation is 24 h, the treatment results at 1 d were favoured. Hence, the results confirmed that this type of biosynthetic process resulting from inoculation of milk with the freeze-dried cultures with PAB and LAB incorporated into kefir grains have the potential to enrich the kefir with B-vitamins.

4.2 Introduction

Kefir is a beverage produced by the synergistic action of lactic acid bacteria (LAB), yeasts, and acetic acid bacteria in milk. The bacteria present in kefir grains are homofermentative and heterofermentive *Lactobacillus* (*Lb*.), *Lactococcus* (*L*.) and *Leuconostoc* (*Leuc*.) (Simova *et al*., 2002; Santos *et al*., 2003; Yüksekdağ *et al*., 2004). The main end-product of homofermentative LAB is the conversion of carbohydrates to lactic acid, while heterofermentative LAB produce lactic acid, ethanol, acetic acid and carbon dioxide (Jay, 2000; Halasz, 2009). The complex consortium of microorganisms present in kefir produces a distinctive fermented milk product with unique properties. However, fermentation with substrates such as milk has become a widespread method of preservation, preferred to other preservation procedures such as canning and pasteurization to extend shelf-life. This is due to the majority of South Africans with low income living conditions lacking in refrigeration facilities (Van Wyk *et al*., 2002). Kefir has been produced using milk from cows, goats, and buffalo (Ismail *et al*., 1983; Mann, 1985).

Fermented foods are of great significance since it provides nutritious foods in a wide diversity of flavours, aromas and textures, which enrich the human diet. Traditionally, LAB have been used as probiotic cultures in the fermentation of dairy products, as a way of preservation of such foods (Shah, 2007). Probiotics are live microorganisms which are beneficial to human beings (Brown & Valiere, 2004). Probiotics have beneficial characteristics as it minimizes gastrointestinal (GI) infections, protects the immune system, has anti-cancer and anti-diarrheal properties, decreases inflammatory bowel disease such as Crohn's disease and restores the microbial population in the small and large intestine. All these beneficial outcomes have been proven by the addition of selected microbial strains to food products (Nomoto, 2005; Imasse *et al*., 2007). Dairy products are considered as the most convenient vehicle for delivering probiotics to the human gut. Yoghurt, followed by cultured butter milk, kefir, cheeses and ice-cream are considered as ideal dairy products which contain probiotics (Tamine *et al*., 2005; Soccol *et al*., 2010). According to Kumar et al. (2012), probiotics are applied to enhance the nutritional status and/or the health of the host. Hence, probiotic organisms can be utilized to deliver the probiotic effect exogenously or

endogenously. Endogenous effects are achieved when the probiotic microorganisms colonise the GI tract. However, since humans lack the biosynthetic capacity to produce most vitamins, these vitamins may be provided via exogenous activity of probiotics. The exogenous probiotic effect is usually achieved during the fermentation of various foods leading to enhanced levels and bioavailability of nutrients with extended shelf-life of the food product (Van Wyk, 2002). The selection of the probiotic organisms which enhance the nutrient profile is based on specific properties desired, namely the propensity to synthesize specific vitamins. This effect has been reported as the result of the LAB and PAB fermentation in yoghurt, cheeses and other fermented foods (Patel *et al*., 2013).

Folate and vitamin B_{12} deficiency are the most common B-vitamin deficiencies in humans and may occur as a result of deficient intake. Early symptoms of folate deficiency may appear within 1 – 4 weeks whereas megaloblastic anaemia develops within 4 – 5 months at the start of a folate deficiency. A deficiency of folate early in pregnancy, before the neural tube has been formed, may be directly related to NTDs in the fetus (Robertson *et al*., 1997). However, the deficiency of vitamin B_{12} have also been known to be one of the factors implicated in causing neural tube defects (NTDs). Studies have shown that increased levels of methyl-malonic acid in women carrying NTD-affected fetuses and deranged vitamin B_{12} absorption may be the pathophysiological mechanism involved in development of NTDs (Mamabolo *et al*., 2006). Although NTDs are among the most common congenital malformations, little is known about the underlying developmental mechanisms in humans. Neural tube formation is a multifactorial process, determined by both extrinsic and intrinsic factors.

Therefore, it may be assumed that the cause is from a multifactorial origin of both genetic and environmental factors, including maternal nutritional status (Czeizel & Dudas, 1992). In addition, the existence of micronutrient malnutrition have also been known to have implications for economic development and productivity, such as potentially high public health costs and the loss of human capital formation (Anon., 2011).

Fermented milk products are a well-known source of folate. Fermented milk products, especially yoghurt, are reported to contain high amounts of folate of up to 150 $\mu g.L^{-1}$ (Alm, 1980). The high levels of additional folate are produced by the LAB present

in the yoghurt. The two common LAB species present in yoghurt, namely *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, have been reported to produce folate (Mousavi *et al*., 2013). Recently, some other food-grade bacteria were observed to produce folate during milk fermentation (Rao *et al*., 1984). *Propionibacterium freudenreichii* species is a dairy culture which has the ability to produce vitamin B_{12} and has the capability of producing folate (Hugenholtz *et al*., 2001). However, there are differences between production levels of folate with regards to different strains and different species. According to Smid *et al*. (2001), the levels of folate produced by the propionibacteria may be equally as high as the levels produced by the well-known folate producer *S. thermophilus*. However, there is a difference in the ability to excrete folate between various dairy propionibacteria. In certain strains, all the folates are retained intracellularly, while in other strains almost complete excretion of the folate is observed (Hugenholtz & Smid, 2002). This may be due to the different forms of folate that are produced by these propionibacteria such as the nature of the bound C1 moiety and the presence and length of the polyglutamate molecular structure.

Moreover, inasmuch some of the PAB-treated kefir cultures produced folate levels exceeding 100% of the NRV (Chapter 3 of this study), in previous studies in this laboratory with the same organisms, folate levels well below 100% NRV were found (38.04 ± 0.64 µg.100 mL^{-1}, Van Wyk *et al*., 2011). In addition, the sensory acceptability of the kefir beverage was not ideal (Chapter 3 of this study). Hence, to ensure consistently high levels of folate, a specific LAB (*S. thermophilus*) was selected due to its ability to produce folate. This organism was co-inoculated with PAB which are high producers of vitamin B_{12} to produce novel fermented foods with increased nutritional values, namely a kefir beverage with vitamin B_{12} and folate concentration not less than 100% NRV per serving, yet does not contain excessively high vitamin levels.

The aim of this study was to measure the vitamin B_{12}, folate and pH levels, to confirm the presence of PAB in the kefir grains, as a function of the form of the initial inoculum, the state of the preservation of the grains and the fermentation time in order to determine which procedure results in the optimum yield of the B-vitamins and sensorial acceptability in the enriched kefir beverage and the presence of PAB and LAB in the kefir grains.

4.3 Materials and methods

4.3.1 Chemical reagents and other chemicals

Unless otherwise specified, all the chemicals used in this study were of Analar grade and chemical reagents were prepared according to standard analytical procedures. Vitamin B_{12} and the four folate vitamers were used as external standards during the HPLC assays as described under section 3.3.2.1 and 3.3.3.1 in Chapter 3.

4.3.1.1 Growth media

4.3.1.1.1 Trypticase soy broth/agar (TSB or TSA) and vitamin B_{12} medium (VBM)

The Trypticase soy broth/agar (TSB or TSA) and vitamin B_{12} medium, was prepared as described in Chapter 3, Section 3.3.1.2.1 and 3.3.1.2.2, respectively.

After preparation, all media (i.e. TSA, TSB and VBM) were autoclaved for 15 min at 121°C. To ensure that sterilization was achieved, the media were kept at room temperature for one week and inspected for absence of growth before use.

4.3.1.1.2 De Man Rogosa Sharpe (MRS) medium

The MRS growth medium was prepared by adding 62 $g.L^{-1}$ of the MRS powder (Merck) to Milli-Q water.

After preparation, all media were autoclaved for 15 min at 121°C. To ensure completely sterile media, the media was kept at room temperature for one week and inspected for growth before use.

4.3.1.2 Microbial cultures

Freeze-dried cultures of *Propionibacterium freudenreichii* subsp*. shermanii* strain J19 was obtained from the Department of Food Science and Technology, Cape Peninsula University of Technology. The kefir grains were obtained from the Department of Food Science, University of Stellenbosch. The freeze-dried culture of *Streptococcus thermophilus* ATCC 19258 was obtained from the American Type Culture Collection, Manassas, VA, USA.

4.3.1.3 Reconstitution of freeze-dried cultures

The reactivation of the freeze-dried PAB and LAB occurred in the vial containing the original culture by adding 1 mL of sterile Milli-Q water using a sterile precision pipette, thus rehydrating the contents in the vial.

4.3.1.4 Freeze-drying of cultures and cell enumeration

The reconstituted freeze-dried cultures of the *Streptococcus thermophilus* ATCC 19258 and PAB were inoculated at a concentration of 200 µL.100 mL^{-1} into MRS broth and VBM, respectively. The *S. thermophilus* (LAB) was incubated for 3 d at 45°C and the PAB was incubated for 7 d at 32°C. Broth cultures were then transferred into two separate sterile 250 mL centrifuge tubes and centrifuged for 10 min at 10 000 X *g* at 4°C and the supernatant discarded. This was followed by the addition of 10 mL sterile Ringer's solution to each culture followed by resuspension of the pellet by shaking the capped tube, followed by centrifugation as before. The Ringer's solution was discarded and the pellet was resuspended in 10 mL sterile milk/lactose mixture which was used as the medium for freeze-drying. The milk/lactose mixture contained 12% $m.v^{-1}$ endospore free skim milk powder (Merck) and 5% $m.v^{-1}$ lactose monohydrate (Merck) (Joubert & Britz, 1987). Two millilitre aliquots of the cell suspension was dispensed into sterile freeze-dryer vials. In order to determine the cell concentration in the inocula, serial dilutions of reconstituted LAB and PAB (i.e. the contents of each freeze-dried vial) were carried out in sterile Ringer's solution according to the method of Malik (1994). The dilutions were made from 10^{-1} to 10^{-7} and plated using the relevant growth medium for viable cell enumeration. This was performed to establish the inoculation concentration in colony forming units (cfu) per mL. PAB was enumerated on TSA and LAB on MRS agar. The plates were incubated anaerobically at 30°C for 5 d for PAB and for LAB at 45°C for 3 d. The cell counts were performed in duplicate and mean values were expressed. Aseptic protocol was strictly adhered to throughout.

4.3.1.5 Reaction of the kefir grains with LAB and PAB culture

The treatment entailed inoculation of reconstituted grains with the LAB broth culture and freeze-dried cultures, respectively (Fig. 4.1). The LAB broth culture was centrifuged for

10 min at 10 000 X *g* at 4˚C, the supernatant was decanted and the reconstituted pellet was used for the co-inoculation, whereas the 1 mL reconstituted freeze-dried LAB culture was co-inoculated directly with the kefir grains. This procedure was applied to 2 X 10 g grain samples for each inoculum type, i.e. two replicates per treatment were prepared. The inoculum concentration was 6 X 10^6 (variation of 5% at final count) (broth culture) and 4.3 X 10^5 (variation of 5% at final count) (freeze-dried culture). The kefir grains plus LAB inoculum preparations were allowed to react for 4 d at 30˚C, with gentle agitation on the Orbital shaker at a speed set at level 3. A 100 mL aliquot of full cream UHT milk was then added and the samples were agitated for a further 4 d at 30˚C before straining each sample using a sterile strainer. The latter was repeated twice.

This was followed by reaction of the grains with PAB inocula. The inoculation of the PAB broth and freeze-dried cultures were performed as described in Chapter 3, Section 3.3.1.8. The PAB inoculum volume was 200 µL per 100 mL broth, resulting in a inoculum concentration of 3.5 X 10^6 (variation of 5% at final count) for the broth culture, while the freeze-dried culture inocula contained 1.05 X 10^5 (variation of 5% at final count). After the aforementioned sample treatments, all sample preparations (kefir grains reacted with LAB and PAB) were then inoculated into 100 mL of UHT milk each and incubated at 30˚C.

4.3.1.6 Sampling

Kefir beverage samples were collected at day 1 and 3 for the following analyses: the measurement of the extracellular folate derivatives by HPLC; measurement of vitamin B_{12} by HPLC; pH measurement; and DNA/PCR analysis. At day 3, kefir grains samples were also collected for DNA/PCR analysis (Fig. 4.1).

4.3.2 **Vitamin B_{12} and folate analysis**

4.3.2.1 Standard solutions and calibration method of vitamin B_{12} and folate

The preparation of the vitamin B_{12} and folate standard solutions as well as the linearity analyses of the vitamin B_{12} and folate using the HPLC were performed as described in Chapter 3 of this thesis.

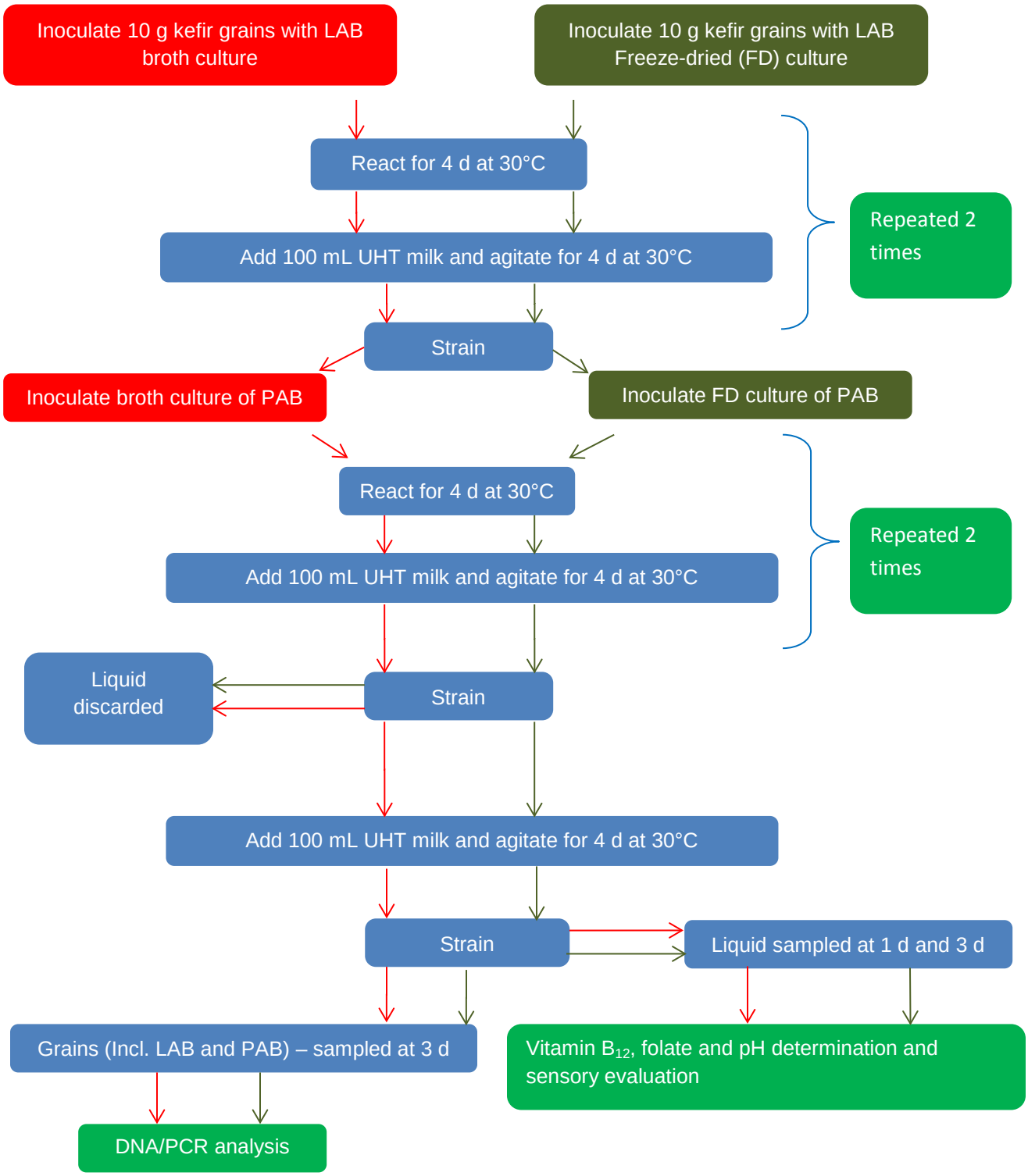

Figure 4.1 Illustration of the inclusion of LAB and PAB in the form of freeze-dried (FD) and broth cultures into kefir.

4.3.2.2 Sample extraction and purification of vitamin B_{12} and folate

The extraction and purification of vitamin B_{12} and folate were conducted as described in Chapter 3, Sections 3.3.2.2 and 3.3.2.3 (vitamin B_{12}) and Sections 3.3.3.4 and 3.3.3.5 (folate), respectively. In brief, the extraction of the vitamin B_{12} samples was performed using a KCN-acetate buffer, while the folate was extracted using the tri-enzyme method. Prior to sample elution, the sample extracts were filtered using a 0.45 µm syringe filter eliminating denatured proteins. The purification of the vitamin B_{12} and folate samples were performed by eluting the supernatant through the Chromabond C_{18} and strong anion exchange (SAX) SPE column, respectively. Once extraction, centrifugation and purification was completed the samples were then filtered through a 0.22 µm syringe filter into amber sampling vials and flushed with nitrogen gas to prevent oxidation labile folates. All samples were stored at -20˚C prior to HPLC analysis.

4.3.2.3 HPLC determination of vitamin B_{12} and folate

The HPLC separations of the standards and samples were performed using an Agilent 1100 HPLC system as described in Chapter 3, Sections 3.3.2.5 and 3.3.3.6. The HPLC system was equipped with the same analytical components as well as analytical methodology used to quantify vitamin B_{12} and folate as described in Chapter 3.

4.3.2.4 Complete deconjugation of PGA to $PteGlu_3$

The deconjugation of the samples indicated the complete conversion of folic acid (PGA) to Pteroyltri-L-γ-glutamic acid ($PteGlu_3$) by the deconjugase enzyme present human serum as described in Chapter 3, Section 3.3.3.4. This meant that the deconjugation of PGA to $PteGlu_3$ in the kefir sample inoculated with the PAB and LAB.

4.3.2.5 LOD and LOQ

The vitamin B_{12} and folate linearity curves were construed as described in Chapter 3, Sections 3.3.2.5 and 3.3.3.7, respectively. Briefly, the LOD and LOQ were calculated based on signal to noise ratios of 3 and 10 (S/N = 3 and 10), respectively. The standard deviation (SD) of the response and the slope (S) of the calibration curve were used to approximate the LOD and LOQ.

4.3.2.6 Precision

Repeatability precision of vitamin B_{12} and folate assays were conducted as described in Chapter 3, Sections 3.3.2.6 and 3.3.3.8, respectively, by analysing one sample numerous times. This includes intra-day and inter-day assays. The mean ± standard deviation (SD) and the relative standard deviation (RSD_r) were calculated. The Horwitz ratio of acceptance was used to determine repeatable precision.

4.3.2.7 Accuracy

The recovery analysis of vitamin B_{12} and folate was performed as described in Chapter 3, Sections 3.3.2.7 and 3.3.3.9, respectively, by using the standard addition method. The sample or placebo was divided into three aliquots each containing 4 mL. Ten mL of standard spiked solutions containing 20, 50 and 80 $\mu g.mL^{-1}$ cyanocobalamin and folate calibrant mixtures, respectively, were added separately to each sample aliquot. Both B-vitamins were determined in two replicate samples at each level of addition. The percentage recovery, the relative standard deviation (RSD_r), mean and standard deviation (SD) were calculated. The %Recovery was calculated taking the native cyanocobalamin and folate in a control sample into consideration.

4.3.2.8 Sensory Evaluation

The sensory evaluation and panel training for of the kefir beverage was conducted as described in Chapter 3, Sections 3.4 and 3.4.1, respectively. A descriptive panel of five judges which were recruited among a pool of undergraduates and staff from the Department of Food Science and Technology at the Cape Peninsula University of Technology. These judges were selected based on availability and product interest. Standard evaluation forms were used to evaluate the beverage in terms of the attributes of sour taste and overall taste. An unstructured linear scale (100 mm) was used with anchor terms, namely absent and intense (sour taste) and unpleasant and pleasant (overall taste). The panellist were asked to make a vertical slash on the scale to indicate their rating of the sample. The score was determined by measuring the distance from the origin in millimetres. The freshly made kefir beverage (fermented for 1 d) was stored at 4°C for not more than six hours before being evaluated.

4.3.2.9 DNA extraction of PAB in kefir samples

The DNA and PCR assays of the *Propionibacterium freudenreichii* strain J19 present in the kefir grains and beverage were performed in detail as described in Chapter 3, Section 3.5 and 3.6, respectively. The method used for both assays were performed in accordance to both manufacturers' guidelines, namely Promega and Kapa Biosystems, respectively. The DNA extraction of the PAB present in the kefir grains and beverage were performed using the Wizard Genomic DNA purification kit (Promega) and specific primers, namely Prop1 forward primer 5'GATACGGGTGACTTGAGG-3' and Prop2 reverse primer 5' GCGTTGCTGATCTGGATTAC-3' (Whitehead Scientific, South Africa) were used during the PCR assay.

4.3.2.10 Agarose gel preparation

The agarose gel was prepared as described in Chapter 3, Section 3.7. The 1% agarose gel was prepared by adding 0.5 g of agarose powder to 50 mL 1 X TAE and heated in the microwave to dissolve the agarose, followed by the addition of 10 µL of ethidium bromide.

4.3.2.11 Electrophoresis procedure

The electrophoresis procedure was performed as described in Chapter 3, Section 3.8. The comb was carefully removed from the solidified agarose gel and 250 mL of 1 X TAE (electrophoresis buffer) was poured over the gel. Once the buffer was poured over the gel the sample consisting of the loading dye was carefully pipetted and electrophoresed at 100 Volts for 45 min until the sample had migrated down the agarose gel. Once the electrophoresis was completed, the gel was carefully removed and placed into the UVidoc box and visualised.

4.4 Statistical analysis

All analyses done in this study were performed on duplicate samples and Analyses of variance (ANOVA) were performed with SPSS® 21.0 (SPSS® Inc., Chicago, Illinois, USA) as described in Chapter 3 to test and compare the effects of vitamin production

with regards to broth culture, freeze-dried culture fermentations, pH and sampling times (1 d and 3 d).

4.5 Results and discussion

4.5.1 Linearity

The results of the linearity curve with regards to the variation in concentration of vitamin B_{12} and folate were reported and discussed in detail in Chapter 3, Section 3.10.1. In summary, for vitamin B_{12} the peak area and concentration of the injected analyte over a concentration range between 0.01 µg.mL^{-1} and 20 µg.mL^{-1} showed acceptable linearity, the correlation coefficient (R) was 0.99989 and the multiple correlation coefficient (R^2) = 0.99972. The LOD for vitamin B_{12} was 0.0054 µg.mL^{-1} and the LOQ was 0.018 µg.mL^{-1}.

For folate, the peak area and concentration of the injected calibrant mixture consisting of the four folate vitamers were construed over a concentration range of 0.001 – 5.0 µg.mL^{-1} and showed acceptable linearity. The multiple correlation coefficients (R^2) of the vitamers ranged between 0.99738 – 1.00 and the correlation coefficients (R) ranged between 0.99869 – 1.00. The LOD for THF, 5-CH_3-THF, 5-CHO-THF were between 0.00015 µg.mL^{-1} and 0.00025 µg.mL^{-1}, while the LOQ was between 0.00050 µg.mL^{-1} and 0.00083 µg.mL^{-1}. The LOD and LOQ for PGA was 0.00021 µg.mL^{-1} and 0.00068 µg.mL^{-1}, respectively.

Based on the correlation coefficient (R) and multiple correlation coefficient approaching unity, as well as the low LOD and LOQ for vitaminB_{12} and all folate vitamers, it was evident that the HPLC method was, therefore, sufficiently sensitive to be used as an analytical tool.

4.5.2 Accuracy

The detail concerning the percentage recovery values for vitamin B_{12} and folate was reported in Chapter 3 (Tables 3.5 and 3.6). For vitamin B_{12}, it ranged from 97.5 to 102.29% overall, with relative standard deviation (RSD) ranging from 0.0033 to 7.33%, while the recovery values for folate ranged from 95.5 to 100.6% overall, with RSD ranging from 0.19 to 4.13%. All the values were within the acceptable range of recovery

limits according to the AOAC procedure (Anon., 2002), confirming the quantitative validity or accuracy for both assay methods.

4.5.3 Precision

The HORRAT values for vitamin B_{12} and folate were reported in Chapter 3 (Tables 3.7 and 3.8) as the Horwitz criteria parameter of acceptance for precision studies (HORRATr) (Chen & Eitenmiller, 2007). The repeatability precision of intra-day and inter-day assay for vitamin B_{12} ranged between 0.38 – 0.42 and 0.36 – 0.69, respectively and for folate it ranged between 0.41 – 1.11 and 0.44 – 0.93, respectively. The intra-day and inter-day assays for both vitamin B_{12} and folate showed no significant difference ($p > 0.05$), between sessions indicating that the methods were precise.

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4.5.4 Peak identification and peak purity

The identification of vitamin B_{12} and folate was achieved by means of the retention time (t_R) and the quantification of the vitamers was done by measuring the peak area of samples, relative to that of known concentrations of the reference standards (pure analytes) as described in Chapter 3, Section 3.10.4. The peak identification of vitamin B_{12} and folate vitamers were determined during three sessions ($n = 3$) and the means and standard deviations were calculated. The results of the retention time (minutes) obtained for vitamin B_{12} was 19.39 ± 0.05 and for the folate vitamers were: THF (16.29 ± 0.00); 5-CH_3-THF (17.11 ± 0.01); 5-CHO-THF (20.62 ± 0.04) and PGA (20.96 ± 0.01). The purity tests were performed using the “Check purity” option in the Chemstation system. The purity factors were > 990, with 1 000 indicating a perfect peak. Hence, the HPLC methods were suitable and reliable tools for analyte identification and quantification.

4.5.5 pH, vitamin B_{12} and folate

The pH for all sample treatments ranged from 5.61 – 5.82 immediately after inoculation, i.e. on day 0 (Table 4.1). When comparing the control sample with the treated samples, namely the broth culture, freeze-dried culture as well as the grains directly treated with reconstituted freeze-dried PAB-LAB culture (direct culture), the regime which resulted in

the lowest pH at day 1 was the direct culture, but the pH was not significantly lower than for any of the other treatments ($p > 0.05$) (Table 4.1). The pH of the broth culture, freeze-dried culture and control samples at day 1 ranged from 4.11 – 4.27 and compared favourably with the typical pH range of kefir (4.1 – 4.6) after 24 h of fermentation as widely reported (Farnworth, 2005; Powell, 2006; González-Sánchez *et al*., 2010; Glibowski & Kowalski, 2012). However, the pH of the direct culture (4.04 ± 0.04) was lower than the typical pH of kefir. On day 3 the pH for all sample treatments ranged from 2.71 – 3.86 (Table 4.1), similar to the results reported by Van Wyk *et al*. (2011). This excessive decrease in pH compared to 1 d is due to the extended fermentation time, resulting in the lactic acid bacteria producing an excess of lactic acid and lowering the pH in the beverage (Suriasih *et al*., 2012). The vitamin B_{12} and folate levels differed significantly ($p < 0.05$) between sampling times (Table 4.2). Hence, as expected and in agreement with the results reported in Chapter 3 of this study, increased fermentation time resulted in elevated vitamin B_{12} and folate levels. However, the sample treatments had no significant effect on the vitamin B_{12} levels ($p > 0.05$) (Table 4.2). In contrast, the folate levels between sample treatments differed significantly, both at 1 d and 3 d with the broth culture treatment resulting in a significantly higher folate level ($p < 0.05$) at 1 d than the other treatments; while the freeze-dried culture treatment resulted in the highest folate level ($p < 0.05$) at 3 d (Table 4.2).

The control kefir sample that numerically produced the third lowest vitamin B_{12} concentration at 1 d (5.82 ± 0.54), produced the lowest level at 3 d (8.85 ± 0.95) compared to the other treatments ($p > 0.05$) (Table 4.2). In numerical terms, the direct culture produced the highest vitamin B_{12} level ($p > 0.05$) at both 1 d and 3 d fermentation (Table 4.2), while the broth culture produced the second highest vitamin B_{12} level ($p > 0.05$) at both 1 d and 3 d. When comparing these results with the results reported in Chapter 3 of this study, the vitamin B_{12} level of all the sample treatments were lower. For the direct culture treatment, the vitamin B_{12} level was 1.3-fold and 1.7-fold lower than in Chapter 3 at 1 d and 3 d, respectively. The freeze-dried culture sample resulted in 2.3-fold and 2.5-fold lower vitamin B_{12} levels at 1d and 3 d, respectively. However, these results (Table 4.2) compare favourably with the vitamin

Table 4.1 pH variation between sampling times and sample treatments

	pH		
Sample treatments	**Day 0[1] (mean ± SD)[2]**	**Day 1 (mean ± SD)**	**Day 3 (mean ± SD)**
Control (n = 2)	5.61 ± 0.02[a]	4.27 ± 0.21[b]	3.86 ± 0.01[c]
Broth culture (n = 2)	5.80 ± 0.20[a]	4.11 ± 0.01[b]	2.71 ± 0.01[c]
Freeze-dried culture (n = 2)	5.68 ± 0.05[a]	4.21 ± 0.01[b]	2.86 ± 0.01[c]
Direct culture (n = 2)	5.82 ± 0.01[a]	4.04 ± 0.04[b]	3.84 ± 0.10[c]

[1]The pH of Day 0 samples was measured immediately after inoculation. [2]Results were reported as the mean ± standard deviation (SD). Univariate Analyses of Variance (ANOVA) for each sample tested the difference between the main effects, i.e. sample type and sampling time (day 0, 1 and 3). Duncan's multiple comparison post-hoc test was performed and different letter superscripts denote significant differences ($p < 0.05$) between individual means.

Table 4.2 Comparison of the vitamin B_{12} and folate results in kefir beverage samples at day 1 and day 3[1]

		cfu.mL^{-1} (mean ± standard deviation)[3]	Total vitamin B_{12} (µg.100 mL^{-1}) (mean ± standard deviation)		Total folate (µg.100 mL^{-1}) (mean ± standard deviation)	
Chapter 3	**Inoculum**		**1 d**	**3 d**	**1 d**	**3 d**
Control (n = 4)	None	N/A	8.16 ± 4.33^{a}	29.81 ± 3.90^{b}	458.45 ± 34.91^{b}	655.87 ± 15.2^{e}
Broth culture (n = 4)	PAB	5 X10^{6} ± 0.87^{x}	11.80 ± 2.18^{a}	23.73 ± 8.74^{b}	104.20 ± 0.15^{c}	111.69 ± 1.14^{f}
Freeze-dried culture (n = 4)	PAB	2 X 10^{5} ± 0.70^{x}	8.73 ± 0.78^{a}	22.24 ± 4.16^{b}	114.13 ± 3.36^{c}	124.45 ± 5.51^{f}
Direct culture[2] (n = 4)	PAB	1 X 10^{3} ± 0.61^{x}	8.16 ± 4.33^{a}	29.81 ± 3.90^{b}	458.45 ± 34.91^{b}	655.87 ± 15.2^{e}
Chapter 4	**Inoculum**		**1 d**	**3 d**	**1 d**	**3 d**
Control (n = 4)	None	N/A	5.82 ± 0.54^{a}	8.85 ± 0.95^{b}	345.57 ± 0.99^{a}	452.61 ± 3.45^{c}
Broth culture (n = 4)	PAB	3.5 X 10^{6} ± 0.5^{x}	6.14 ± 1.54^{a}	12.38 ± 1.05^{b}	444.97 ± 16.92^{b}	466.87 ± 5.88^{c}
	LAB	6 X 10^{6} ± 0.41^{x}				
Freeze-dried culture (n = 4)	PAB	1.05 X 10^{5} ± 0.52^{x}	5.03 ± 1.89^{a}	9.31 ± 1.06^{b}	412.89 ± 0.00^{a,b}	561.52 ± 35.06^{d}
	LAB	4.5 X 10^{5} ± 0.53^{x}				
Direct culture (n = 4)	PAB	2.5 X 10^{6} ± 0.5^{x}	6.65 ± 3.01^{a}	12.55 ± 1.52^{b}	378.29 ± 58.53^{a,b}	458.99 ± 4.91^{c}
	LAB	5 X 10^{6} ± 0.67^{x}				

[1, 3]Results were reported as the mean ± standard deviation (SD). Univariate Analyses of Variance (ANOVA) determined differences between samples means based on Duncan's multiple comparison post-hoc test where different superscripts indicate significant differences ($p < 0.05$) between different samples for each assay. The total folate is the sum of individual folate vitamers (THF, 5-CH_3-THF, 5-CHO-THF and PGA). [2]Direct co-inoculation with reconstituted freeze-dried PAB and LAB cultures.

B_{12} levels in kefir treated with *Propionibacterium freudenreichii* as reported by Van Wyk *et al*. (2011), namely 9.21 ± 0.37 µg.100 mL^{-1} vitamin B_{12} after 3 d fermentation.

The folate level produced by the control kefir sample was lower at both 1 d and 3 d compared to the other treatments (Table 4.2). The broth culture treatment resulted in the highest folate concentration at 1 d compared to the other treatments, but significant ($p < 0.05$) only compared to the control (Table 4.2). The freeze-dried culture produced the second highest folate level at 1 d ($p > 0.05$) and the highest folate level at 3 d (561.52 ± 35.06) ($p < 0.05$) compared to the other treatments (Table 4.2). In comparison to the results reported in Chapter 3, the folate level of the control sample was 1.2-fold higher and 1.3-fold lower at 1 d and 3 d, respectively. The folate level of the freeze-dried culture increased by 3.9-fold and 5.0-fold at 1 d and 3 d, respectively, while the direct culture increased by 3.3-fold and 3.7-fold at 1 d and 3 d, respectively, when compared to Chapter 3.

Hence, as hypothesized, the inclusion of *Streptococcus thermophilus* following the PAB inclusion when treating the kefir grains, resulted in higher folate levels than when only PAB treatment was used (Chapter 3). This was expected since *S. thermophilus* produce more folate than other LAB (Crittenden *et al*., 2003). Moreover, the highest folate levels measured at 1 d and 3 d (Table 4.2) were for the broth culture-treated sample at 1 d (444.97 ± 16.92 µg.100 mL^{-1}) and freeze-dried sample at 3 d (561.52 ± 35.06 µg.100 mL^{-1}) (Table 4.2), which exceeded the highest level measured by Van Wyk *et al*. (2011) at 3 d (38.04 ± 0.64 µg.100 mL^{-1}) more than 10-fold. These results confirm the hypothesis that the LAB co-inoculated with PAB used in this study will contribute a higher concentration of folates than when inoculating with PAB alone.

According to Regulation No. R. 146 under section 15(1) of the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972), the NRV for individuals 4 years and older for vitamin B_{12} and folate are 2.4 µg and 400 µg per day, respectively (Anon., 2003). Hence, when comparing the aforementioned results of vitamin B_{12} and folate levels (per 100 mL serving of the kefir beverage) at 1 d with the nutrient reference values (NRV), the results showed: the broth culture-treated sample contained 256% (vitamin B_{12}) and 111% (folate) and the control sample contained 243% (vitamin B_{12}) and 86% (folate) of the NRV. The freeze-dried culture and direct culture treated

samples contained 210% and 277% for vitamin B_{12}, respectively, while the folate levels for these samples were 103% and 95% of the NRV per serving, respectively. Hence, when considering the appropriate vitamin intake level regarding the NRV, the freeze-dried treated sample contained vitamin B_{12} levels that were not excessively higher than the NRV, while the folate level was on par with the NRV daily requirements. Therefore, the freeze-dried treated sample showed the best results for vitamin B_{12} and folate levels regarding the NRV. Hence, as hypothesized, the freeze-dried sample has the potential to produce kefir enriched with B-vitamins resulting from a natural biosynthetic process.

Moreover, as was observed in Chapter 3 of this study, the concentration of the initial inocula used to treat the kefir grains did not differ significantly ($p > 0.05$; Table 4.2). Furthermore, the vitamin B_{12} concentration at day 1 also did not differ significantly, but the numerical differences did not follow the same trend as did the numerical differences in PAB inoculum concentration ($p > 0.05$) (Table 4.2). However, since the presence of LAB clearly influenced the vitamin concentrations in the right direction, namely that vitamin B_{12} concentration decreased, while folate increased to values closer to 100% NRV, there is merit to standardise and control the microbial concentration. Moreover, there is merit in exploring the relationship between the original inoculum concentration and the relative vitamin concentration.

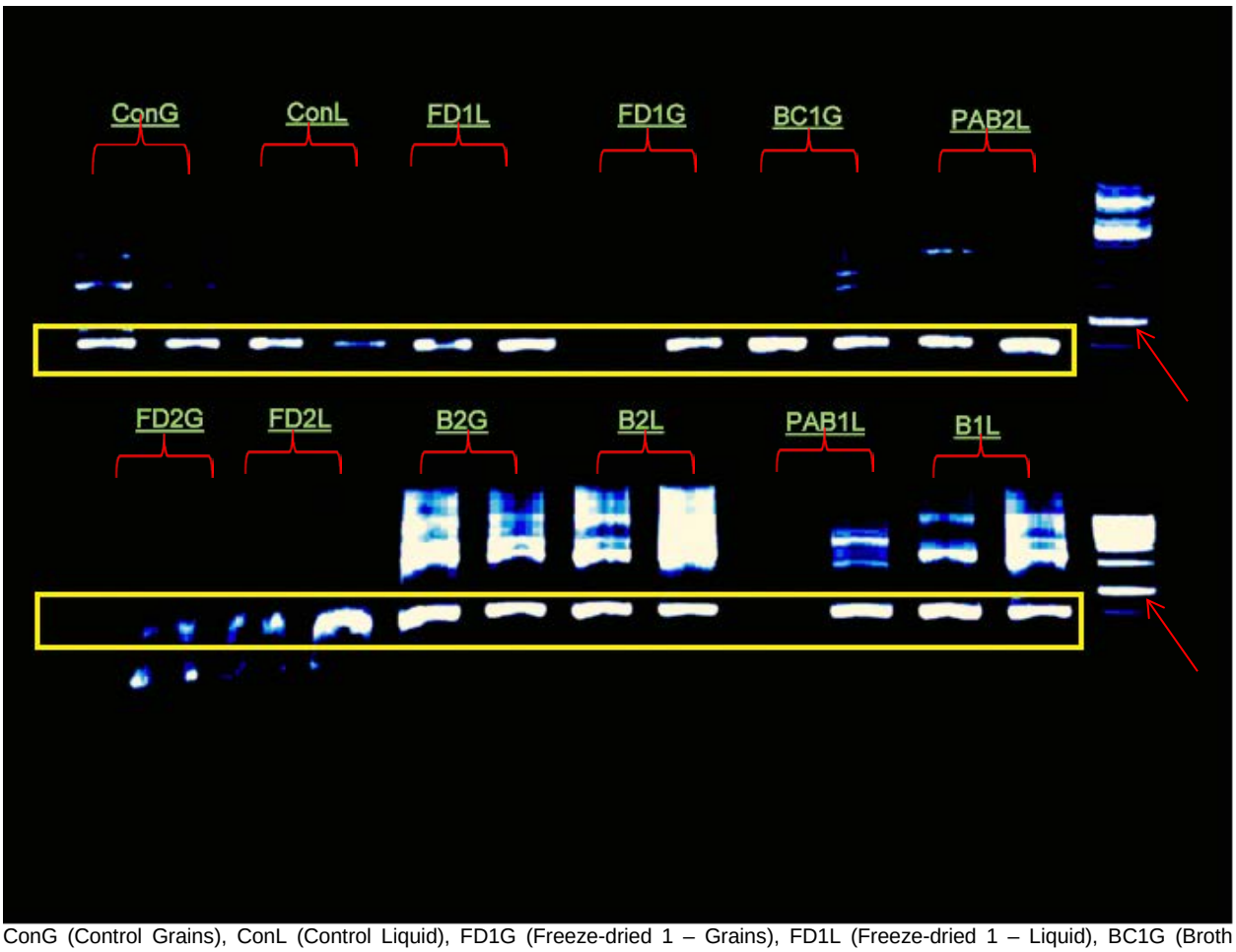
4.5.6 DNA and PCR analysis of Propionibacterium freudenreichii

In this study, the presence of *Propionibacterium freudenreichii* was observed in all PAB-LAB treated samples, both kefir grains and kefir beverages (Table 4.3 and Fig. 4.2). This was evident by the DNA fragment present in all of the treated samples (indicated by a yellow rectangle) as well as in the positive control (PAB bacterial culture) (Fig. 4.2). However, the presence of the *Streptococcus thermophilus* ATCC 19258 co-inoculated with the kefir grains was not determined due to its presence in the symbiotic consortium of LAB in the kefir grains (Simova *et al*., 2002; Gemechu, 2015). Moreover, since kefir grains contain *S. thermophilus*, it would be difficult to determine the exact strain of *S. thermophilus* ATCC 19258 co-inoculated with the kefir grains without specific primers using conventional culture-dependent methods. In addition, the resulting DNA and PCR

Table 4.3 The PCR assays performed on the DNA extracts for the different sample treatments included kefir grains and beverage samples. Both DNA and PCR assays were performed in duplicate.

Sample treatment	Day 3	
	Grains	Kefir beverage
Control (n = 2)	-	-
PAB broth culture (n = 2)	+	+
PAB freeze-dried culture (n = 2)	+	+
PAB direct culture (n = 2)	+	+

A positive result (+) indicated that PAB was present in detectable numbers, while a negative result (-) indicated the absence of PAB in the sample.



ConG (Control Grains), ConL (Control Liquid), FD1G (Freeze-dried 1 – Grains), FD1L (Freeze-dried 1 – Liquid), BC1G (Broth culture 1 – Grains), BC1L (Broth culture 1 – Liquid), PAB1G (PAB-LAB Direct culture – Grains) and PAB1L (Direct culture – Liquid)

Figure 4.2 *Propionibacterium freudenreichii* present in all of the treated samples.

assays in this study showed several non-specific PCR bands, indicating other species of lactic acid bacteria types. The use of non-specific primers would have targeted the 16S RNA genes and therefore possibly amplifying the aforementioned lactic acid bacterial strains as well as the *S. thermophilus* already present in the grains and beverage and not the specific *S. thermophilus* ATCC 19258. However, the increase in folate levels was a positive indication for the activity of this organism.

4.5.7 Sensory analysis of the kefir beverage

The sensorial quality of the kefir beverage was assessed according to the sour taste and overall taste, which was influenced by changes in pH which, in turn, decreased with increasing fermentation time. The PAB-LAB culture treatment and the fermentation time significantly ($p < 0.05$) affected the sensory evaluation results (Fig. 4.3). The variations in pH and acidity of the kefir beverage samples were reflected by differences in the taste panel scores for sour taste and overall taste of the beverage at 1 d and 3 d, as well as between the treated and control samples (Fig. 4.3). However, the sample treatments did not have a significant effect on the taste panel scores ($p > 0.05$), while the fermentation time had a significant effect ($p < 0.05$) (Fig. 4.3). The sour taste for the kefir beverage treated with the broth culture sampled at 1 d and 3 d, respectively, was scored numerically higher than that of all the other samples, with the freeze-dried culture scoring second highest (55) followed by the control sample (53 mm)and then the direct culture (35) (Fig 4.3). The sour taste of the kefir samples at 1 d was less intense than at 3 d (Fig. 4.3) due to the relatively low acidity level at 1 d (as confirmed by the higher pH than at 3 d) (Table 4.1).

The overall taste between treated samples were numerically different ($p > 0.05$) at 1 d and 3 d, with the panel scores for all samples ranging from 61 – 82 out of 100 (Fig. 4.3). At 1 d, the overall score for the control sample was numerically the highest (82), while the sample treated with the freeze-dried culture scored the second highest for overall taste (80) ($p > 0.05$) (Fig. 4.3). As expected, the overall taste of the treated samples was less desirable at 3 d than at 1 d. Since the scores for the sour taste was higher at 3 d than at 1 d, indicating that the sour taste and overall taste were

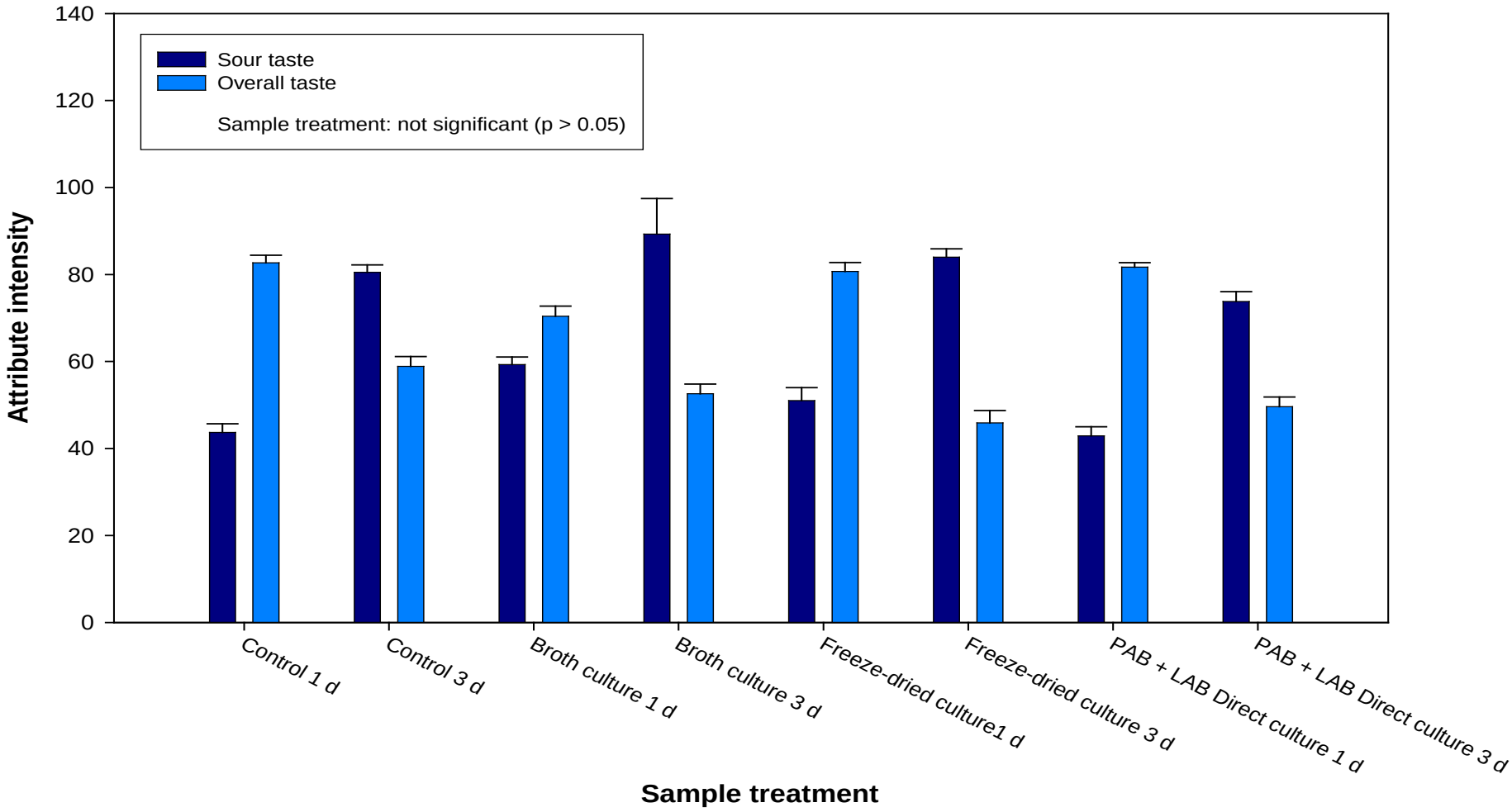


Figure 4.3 Sensory evaluation of the kefir beverage samples.

in agreement at both 1 d and 3 d. The sensory evaluation showed that the overall taste of the kefir beverage samples was more desirable at 1 d fermentation than at 3 d (Fig 4.3). Moreover, the treatment using the co-inoculation of PAB and LAB also achieved the objective of improved sensory acceptability. With the PAB-treatment alone, the freeze-dried culture resulted in unacceptably high acidity (panel score 87) and lower acceptability (panel score 62) (Chapter 3 of this study).

Hence, based on the overall results of the samples at 1 d, the freeze-dried culture resulted in ideal vitamin B_{12} and folate levels in terms of the %NRV. In terms of sensory analysis, the untreated kefir grains (control) had the best sour taste and overall taste at 3 d. However, at 1 d sensorially there was no significant difference ($p > 0.05$) between the samples, with the freeze-dried culture-treated sample scoring 50 for “sour taste” and 79 for “overall taste”. Furthermore, as mentioned, the presence of the PAB was also confirmed in all treated samples by PCR, while the elevated folate levels strongly indicated the presence of *S. thermophilus*. Hence, with an improvement in the taste, the freeze-dried culture sample will be the best treatment suitable for a B-vitamin enriched kefir beverage.

4.6 Conclusions

The PAB-LAB co-culture treatment regime developed in this study led to the simultaneous production of elevated folate and vitamin B_{12} levels via a biosynthetic process which could be employed to increase dietary folate and vitamin B_{12} intake.

The treatment of the PAB-LAB with the kefir grains resulted in elevated levels of vitamin B_{12} and folate at 1 d and 3 d in the kefir beverage samples, while the PCR assay result further confirmed that the PAB treatments resulted in inclusion in the kefir grains. The fact that the co-culture treatment, i.e. inclusion of the LAB resulted in significantly ($p < 0.05$) higher folate levels than when only PAB treatment was employed (Chapter 3).

In terms of the vitamin B_{12} and folate levels produced after 1 d fermentation time, the best results in terms of the %NRV were achieved when reacting the freeze-dried culture with the kefir grains.

The sensory analysis of the kefir beverage in terms of sour and overall taste amongst the treated samples showed no significant difference. However, the panel preferred the sour and overall taste of the beverage after fermentation for 1 d.

In conclusion, the PAB-LAB co-culture treatments of the kefir grains enabled the development of a technology-based beverage with the potential to expand the range of fermented dairy products on the market with enhanced nutritional value. Furthermore, additional research is required to improve the kefir beverage quality, i.e. improved reconstitution protocols and an increase in inoculum concentration.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Discussion

The South African population, like that of other developing countries, coexists with both poor and rich suffering from under- or over-nutrition. These malnutrition problems affect the country's economy adversely (Anon., 2008a). Although various national nutritional and health programmes were initiated in South Africa over the last decade, recent findings have indicated that child health has deteriorated (Schaay & Sanders, 2008). Of particular interest are those studies that have shown that vitamin B_{12} and folate deficiencies are prevalent among the poor in rural areas and, therefore, fortification of basic foodstuffs with folate and vitamin B_{12} is needed (Van Wyk *et al*., 2010).

The fortification of foods with micronutrients is a widely acceptable practice to reduce micronutrient malnutrition, especially in population groups who have limited access to existing food provisions, hence providing inadequate levels of the nutrients in the diet (Anon., 2008b). The success of the food fortification programmes is based on the selection of a suitable vehicle, economic sustainability, quality control and the deficiency prevalence. Milk and other dairy products, especially fermented milk products, are part of the daily diet in almost all countries and therefore provide a suitable vehicle for fortification to enhance B-vitamin intake (Anon., 2008b). Moreover, the shelf-life of fermented milk beverages is enhanced due to LAB activity leading to acidification and inhibition of the growth of spoilage and pathogenic microorganisms in these foods (Ananou *et al*., 2007).

Kefir is an example of a fermented milk beverage that typically contains viable LAB, as well as vitamins, minerals and essential amino acids (Farnworth & Mainville, 2003; Powell, 2006; Van Wyk *et al*., 2011). Moreover, LAB in kefir and kefir grains have probiotic characteristics, which are nutritionally beneficial and have shown to inhibit spoilage microorganisms (Păucean & Socaciu, 2008). The LAB present in kefir that was shown to be beneficial to human health include *Lactobacillus*, *Lactococcus* and

Streptococcus species (Gemechu, 2015). However, among these food-grade bacteria and dairy starters, *S. thermophilus* has been regarded as one of the best folate producers. This organism produces folate extracellularly in the milk products during fermentation (Iyer & Tomar, 2009), while other dairy cultures such as Propionibacteria has the ability to produce vitamin B_{12} and minute quantities of folate in milk products (Zárate, 2013).

In this study, the inclusion treatment of the PAB broth culture with the kefir grains resulted in elevated concentrations of vitamin B_{12} and folate during fermentation of kefir and produced 340% vitamin B_{12} (8.16 ± 4.33 µg.100mL^{-1}) and 115% folate (458.45 ± 34.91 µg.100mL^{-1}) of the NRV per 100 mL serving. Hence, to achieve both vitamin B_{12} and folate concentrations closer to the target of 100% of the NRV, a LAB-PAB co-culture treatment regime was employed. This led to the production of folate at 103% and vitamin B_{12} at 110%, i.e. the objective of approximately 100% NRV was achieved. The treatment that achieved the best results was reacting freeze-dried cultures with the kefir grains. During the subsequent fermentation the desired %NRV values were obtained after 1 d, the typical duration of a kefir fermentation.

The sensory analysis of the kefir beverage in terms of sour and overall taste amongst the samples showed that the panel preferred the sour taste and the overall taste of the beverage fermented at 1 d as opposed to 3 d.

Apart from the elevated B-vitamin levels in the kefir beverage samples fermented by treated kefir grains, PCR results also confirmed the effective inclusion of PAB into the grains.

In conclusion, the treatments of the inocula used to produce the kefir beverages enabled the development of a technology-based beverage with the potential to produce and expand the range of fermented dairy products on the market with enhanced nutritional value and sensorial acceptability.

5.2 Recommendations

Although the comparison between the broth and freeze-dried cultures of PAB inoculated into the kefir grains, as well as the co-culture of the PAB-LAB with the kefir grains produced ideal levels of vitamin B_{12} and folate, there did not appear to be a direct

relationship between the inoculum concentration and the B-vitamin concentration. However this was not one of the objectives of this study, but it is hypothesised that there is merit in performing further work to optimize the B-vitamin levels using various microbiological and processing techniques namely, the use of a controlled microbial cell concentration and spray-drying, respectively.

Studies reported by Golowczyc *et al*. (2010), have shown that spray drying is a suitable method to preserve microorganisms isolated from kefir grains. A high proportion of cells were still viable after 80 days of storage at refrigerated temperatures.

The alternative method used to increase the B-vitamin concentration, is accomplished by utilizing a bioreactor for the large-scale cultivation of microbial cells from bacterial and yeast populations. The metabolic and biochemical pathways are used to produce a specific B-vitamin (product) with the consumption of the carbon source. Alterations of the environmental conditions during fermentation may also have an influence on the PAB-LAB growth and vitamin B12 and folate production (Hugenschmidt *et al*., 2011). The B-vitamins are then secreted into the media and recovered by means of downstream processing. The production of a specific quantity of the B-vitamin can also be calculated over a period of time, as well as the amount of bacterial cells used during the bioprocess.

Further optimization of increased levels of folate and vitamin B_{12} produced by the LAB-PAB co-culture process could also include the addition of guanosintriphosphate (GTP) to the fermentation medium (Hugenschmidt *et al*., 2011).

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