AN ABSTRACT OF THE THESIS OF

SOOKJA LIM RO for the degree of DOCTOR OF PHILOSOPHY

in Foods and Nutrition presented on June 2, 1978

Title: VITAMIN B_{12} AND ASCORBIC ACID IN KIMCHI WITH PROPIONIBACTERIUM SHERMANII

Redacted for privacy

Abstract approved: Margy Woodburn

Vitamin B_{12} reached a maximum of 47 ng/100 g during the fermentation at 4°C of kimchi, a Korean style fermented Chinese cabbage with salted shrimp and spices. Inoculation of the kimchi with Propionibacterium shermanii (ATCC 13673) increased the vitamin production to a maximum of 102 ng/100 g at 1 week of fermentation. Soy flour (0.5%) or beef extract (0.05%), which were regarded as protein sources, added to the inoculated kimchi further increased the vitamin B_{12} activity to 197 and 203 ng/100 g at 1 week of fermentation. After this point, the vitamin in all of the kimchi products decreased continuously to the end of the 5 weeks of fermentation. After 5 weeks of fermentation, no further decrease was observed.

The change in ascorbic acid content during kimchi fermentation did not differ significantly between the control and inoculated kimchi with or without soy flour or beef extract. The fresh, unfermented kimchi contained

16.8 mg/100 g of ascorbic acid. The content of ascorbic acid decreased continuously during the first 5 weeks of fermentation. The correlation coefficient, r, for the ascorbic acid levels and the fermentation time was significant at the 1% level.

It was found that lactobacilli are important in kimchi fermentation. By 3 weeks of fermentation, the lactobacilli (2.3×10^8 per g) constituted most of the anaerobic microorganisms (2.7×10^8 per g).

Propionibacterium shermannii, which was inoculated into kimchi, did not increase in numbers but survived during the fermentation. This species was not found in the control kimchi. Five species of propionibacteria were identified from the control kimchi, including P. freudenreichii, which has been known to produce vitamin B_{12}.

All of the kimchi samples were optimally fermented after 3 weeks with a pH of 4.8 and total acidity of 0.35% as lactic acid.

In triangular tests, no signficant difference in flavor was found by 9 Korean panel members between the control and inoculated kimchi with added soy flour or beef extract.

Canning of kimchi at the end of 1 week of fermentation gives promise in preliminary tests of reducing the loss of vitamin B_{12}.

Vitamin B_{12} and Ascorbic Acid in Kimchi with *Propionibacterium shermanii*

by

Sookja Lim Ro

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed June, 1978

Commencement June, 1979

APPROVED:

Redacted for privacy

Professor of Foods and Nutrition
in charge of major

Redacted for privacy

Head of Department of Foods and Nutrition

Redacted for privacy

Dean of Graduate School

Date thesis is presented June 2, 1978

Typed by Deanna L. Cramer for Sookja Lim Ro

ACKNOWLEDGEMENTS

Sincere gratitude is expressed to Dr. Margy Woodburn, Head of the Department of Foods and Nutrition, for her wonderful guidance, good suggestions, and continous encouragement during my graduate studies at O.S.U.

Special appreciation is given to Dr. William E. Sandine, professor of the Department of Microbiology, who allowed me to work in his laboratory and provided unfailing guidance in the laboratory work. My warm thanks is expressed to the graduate students in the Dairy Microbiology laboratory for their giving me a nice and comfortable atmosphere to work and sharing good knowledges.

Special thanks is extended to my husband, In Kyu, for his sound support, understanding, and encouragement throughout my study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Tables -- continued

VITAMIN B_{12} AND ASCORBIC ACID IN KIMCHI WITH *PROPIONIBACTERIUM SHERMANII*

INTRODUCTION

Vitamin B_{12} (cyanocobalamin) is known to be a dietary requirement for the growth of humans, a variety of animals, and microorganisms. In the human, it has been shown to be essential for normal blood formation, protein synthesis and thus for normal growth, maintenance of neural function, and for some metabolic processes in which the vitamin acts as a coenzyme (Wagner and Folkers, 1964).

Vitamin B_{12} is essential in the human diet as evidenced by the development of pernicious anemia with a deficiency of the vitamin. This generally occurs due to the inability to absorb the vitamin. However, subclinical deficiency levels of serum vitamin B_{12} (45-193 pg/ml with an average value of 111 pg/ml) have been found in adults who have subsisted on vegetarian diets for extended periods (Wokes *et al.*, 1955). Parenteral administration of the vitamin increased the levels to normal.

Since the vitamin was first isolated in crystalline form from liver in 1948 by Rickes *et al.* (1948a) and by Smith, it has been found in most animal tissues and muscles, milk, and eggs. It appears to be absent from most of the higher plants, although some plant roots show small amounts which may derive from contamination with microorganisms in the soil (Ungley, 1955). The vitamin is not synthesized by

animals, but it is produced by bacterial or fungal fermentation within the ruminants' own digestive tract and then absorbed. Humans are unable to utilize the intestinally synthesized vitamin. This exclusively microbial production of the vitamin is unique among the known vitamins (Maugh, 1973).

Fermented products of soybean or fish have been found to contain vitamin B_{12} (Lee et al., 1958). Bacillus megaterium inoculated during soybean fermentation increased the vitamin level (Choe et al., 1963 and Ke et al., 1963). Nutritionally significant amounts of vitamin B_{12} were also found in the Indonesian fermented products, ontjom and tempeh (Liem et al., 1977).

From dietary surveys and national food balance sheets, it has been reported that persons living in Korea are ingesting a high level of grains and vegetables and very low levels of animal foods (Haw et al., 1968 and Yun, 1968). The average daily protein intake of Korean people surveyed in several rural and urban areas was found to be only 64% of the recommended amount (Lee et al., 1968). Of the total protein intake only 12.5% was contributed by animal protein, including seafoods. Very poor families may eat little meat or fish (Ro, personal observation). Significantly lower serum levels and urinary excretion of vitamin B_{12} have been reported in Indian lactovegetarians as compared to non-vegetarians (Jather et al., 1975). Their low levels of

serum vitamin B_{12} were not due to defective absorption but to low dietary intake of this vitamin. Although there have not been reports on vitamin B_{12} intake among Koreans, it is very likely to be low.

Among vegetable products, kimchi, fermented vegetables with small amounts of beef, fish, or other seafoods, is the most popular dish in Korea. It had been found that during kimchi fermentation vitamin B_{12} is synthesized and reaches a maximum of 0.43 µg% (Lee et al., 1960); however, these data were not corrected for vitamin B_{12} analogs. Bacillus megaterium was isolated as the vitamin B_{12}-producing organism (Kim and Chung, 1962).

In this study, an attempt was made to increase the vitamin B_{12} production in kimchi. As kimchi is consumed very frequently, almost at every meal in considerable amounts, it would be a good source of the vitamin for Koreans.

REVIEW OF LITERATURE

Historical Background of Vitamin B_{12}

Vitamin B_{12}, the antipernicious anemia factor, was first isolated in 1948 in the crystalline form from liver almost simultaneously by Smith in England (at Glaxo Laboratories) and by Rickes et al. (1948a) in the United States (at Merck and Company). Rickes et al. (1948b) also found that vitamin B_{12} was a product of microbial fermentation; soon after that it became clear that many bacteria produce the vitamin. Various molds, such as Ustilago zeae, Aspergillus niger, Eremothecium species, and Neurospora species, synthesize vitamin B_{12} (Perlman, 1959).

In the industrial synthesis, the vitamin frequently has been a by-product of a fermentation for the production of antibiotics. Leviton (1956a, b) investigated a mixed fermentation in which lactobacilli were used in a lactose-containing medium. When lactic acid production was achieved, the appropriate propionibacteria were added. Mixtures of several organisms have also been used in the fermentation process (Hodge et al., 1952; Bambha et al., 1973).

The extraordinarily complex structure of the vitamin was determined in 1955 by Hodgkin et al. It was found to

have a molecular weight of 1355, being the largest vitamin yet discovered.

Twenty-five years after its isolation, the chemical synthesis was completed in 1973 through a joint effort by Woodword in the United States and Eschenmoser in Switzerland (Maugh, 1973).

Structure and Stability of Vitamin B_{12}

The structure of vitamin B_{12} (Figure 1) has two characteristic components. The first and most characteristic part of the molecule is a corrin ring system, the basic tetrapyrrole residue, which resembles a porphyrin but differs in that it lacks a methene bridge between two of the pyrroles. The second is a nucleotide-like structure, which contains the base, 5,6-dimethylbenzimidazole, in α-glycosidic linkage with D-ribose and a phosphate group at the 3' position. An atom of cobalt, an essential trace metal for growth, is coordinated to the four inner nitrogen atoms of the corrin ring. Linked to the cobalt at the center is a cyanide; this derivative is called cyanocobalamin and has the general term vitamin B_{12}. Vitamin B_{12} has no coenzyme activity itself, but its cyanide may be replaced with other anionic groups to be the coenzyme forms. One of the active coenzyme forms is 5'-deoxyadenosyl cobalamin. The 5'-deoxyadenosyl group is attached to the cobalt of vitamin B_{12} through its methylene group, replacing the cyanide of

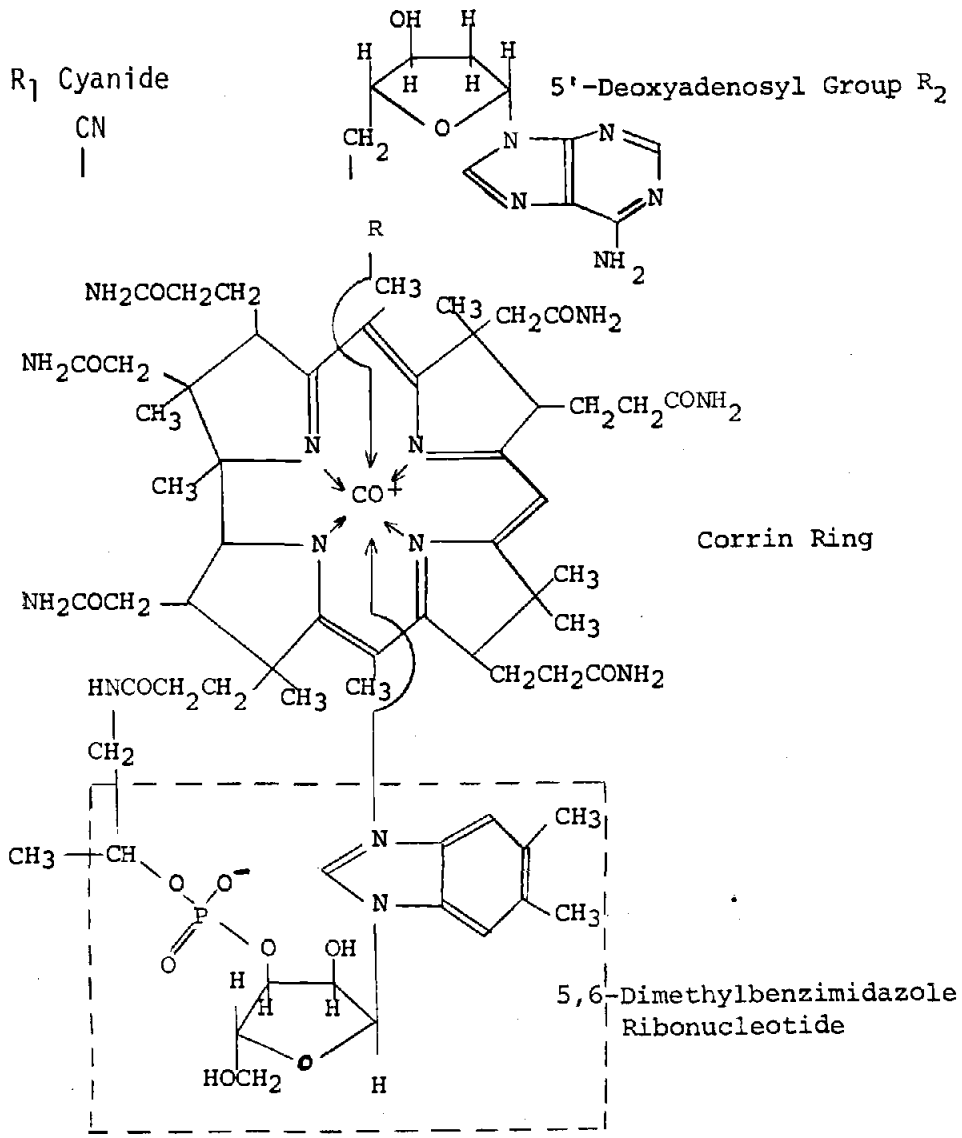

Figure 1. Structure of vitamin B_{12} (R_1) and 5'-deoxyadenosly cobalamin (R_2).

cyanocobalamin (Lehninger, 1970). Methylcobalamin is also an active coenzyme form and has a methyl group linked to the cobalt. Vitamin B_{12} also exists as hydrocobalamin, aquocobalamin and nitrocobalamin, in which a hydro group, a water molecule, or an ONO-group, respectively, links to the cobalt atom replacing the cyanide. Farquharson and Adams (1976) have found five forms of vitamin B_{12} in foods: adenosylcobalamin, hydroxocobalamin, methylcobalamin, cyanocabolamin, and sulphitocobalamin. Adenosylcobalamin and hydroxocobalamin were predominant.

Cyanocobalamin crystallizes as dark red needles or prisms; the color varies with the crystal size. It is darker at 210-220°C but does not melt below 300°C. The vitamin is hygroscopic to the extent of 10-20% and fairly soluble in water (about 1.2% at room temperature) and in the lower alcohols and aliphatic acids and in phenols, but not in many other organic liquids (Smith, 1965a). The aqueous solutions show a characteristic absorption spectrum with maxima at 278, 361, and 548 nm (Ellis et al., 1949a); these do not shift markedly with change in pH. It is relatively stable at pH 4.0 to 7.0 at normal temperature, but undergoes slight decomposition during autoclaving at 115°C for 30 min. Bartilucci and Foss (1954) found that the optimum pH for stability of cyanocobalamin in solution was between 4.5 and 5.0 and that there was no loss of vitamin activity after storage for 6 months at room temperature.

Hartley et al. (1950) reported from their pharmaceutical study that the vitamin was unstable in relatively acid (pH 2.0) or alkaline (pH 9.0) solutions and in the presence of oxidizing or reducing substances, such as hydrogen peroxide, sodium bisulphite, cysteine hydrochloride, hydroquinone, and thioglycollic acid.

However, Hoffmann et al. (1949) suggested that the addition of a reducing agent, such as thioglycollic acid, is necessary to stabilize the vitamin B_{12} against destruction during autoclaving the assay tubes. They also found that ascorbic acid or cysteine could replace thioglycollic acid as the reducing agent. Skeggs (1963) also reported that the incorporation of reducing agents, such as thioglycollic acid, thiomalic acid, or ascorbic acid, prevented the destruction of the heat-labile noncyanocabalamins during sterilization. This permits more accurate assay of total vitamin B_{12}. Hydroxocobalamin, for example, was found to be 20-100% as active as cyanocobalamin microbiologically and to be equal in biological activity in chicks. In contrast, Ford (1957) found that the addition of ascorbic acid or glutathione to milk or vitamin B_{12} solution in phosphate buffer increased the destruction of vitamin B_{12} by heating. Hydrogen peroxide liberated during oxidation of ascorbic acid to dehydroascorbic acid or oxidation of glutathione by copper was suspected to cause hydroxylation of the vitamin.

The incompatibility of vitamin B_{12} and ascorbic acid was first observed by Gakenheimer and Feller in 1949. From results of assay of solutions containing ascorbic acid (10 mg/ml) and vitamin B_{12} (15 µg/ml), of crystalline or concentrate forms, they observed more than 50% decomposition of vitamin B_{12} after storage at 28° to 30°C for 24 hr. The presence of 0.5% phenol did not significantly alter the rate of vitamin B_{12} decomposition. The control solutions containing no ascorbic acid showed no vitamin B_{12} decomposition. Further studies (Trenner et al., 1950) indicated that highly purified vitamin B_{12} showed a much higher degree of stability in pure ascorbic acid solution (about 1.5% loss of vitamin B_{12} activity at pH 2.5-3.0). On the other hand, vitamin B_{12a}, an analog of the vitamin, showed complete loss of color and activity in one day or less under the same conditions. The loss of its activity was found to be faster at higher pHs up to 7.0. On the contrary, maximum stability of a sample containing cyanocobalamin (10 ± 2 µg/cc) and ascorbic acid (50 ± 5 mg/cc) was shown at pH 6.3 in a study by Bartilucci and Foss (1954). Tetrasodium ethylene diaminetetraacetate, a stabilizing agent for ascorbic acid, showed a protective effect on cyanocobalamin in a solution acidified with sulfuric acid (pH about 5). No change in the vitamin content was found after storage for 2 months at room temperature. A 50% decomposition of the cyanocobalamin was observed in the unacidified

solution (pH 10.3) after 1 week at 40°C and 1 month at room temperature. A slight stabilizing effect of NaEDTA was noted after 60 days at 40°C in the mixture of the two vitamins; 55.3% retention of vitamin B_{12}, 72.3% of ascorbic acid with 0.05% NaEDTA; and 35.9% and 66.1%, respectively, without NaEDTA. A solution of the two vitamins in a vehicle composed of equal parts of propylene glycol and glycerine retained 80% of cyanocobalamin and 85% ascorbic acid after 6 months storage at 40°C.

The effect of ascorbic acid on the stability of the different forms of vitamin B_{12} was also observed by Hutchins et al. (1956). Cyanocobalamin, crystalline vitamin B_{12}, was shown to be markedly more stable in 1% ascorbic acid solutions buffered with 1 mole sodium acetate-acetic acid at pH 4.0 than cyanide-free cobalamins, such as chlorocobalamin, hydroxocobalamin, nitrocobalamin, and thiocyanatocobalamin. The presence of cobalamins other than cyanocobalamin in vitamin B_{12} concentrates thus seems to be a significant factor in the instability of such commercial concentrates of the vitamin. They also observed that the instability was temperature-dependent; at 30°C decomposition of the cobalamins was sufficiently rapid to cause difficulty in sampling, but the alteration was completely arrested during storage of samples at -10°C.

Herbert and Jacob (1974) reported the substantial destruction in vitro of vitamin B_{12} contained in foods by

"pharmacologic" doses of ascorbic acid, as would occur when ascorbic acid is eaten within an hour before or after a meal. Larger amounts of ascorbic acid had proportionally greater effects, with 0.1, 0.25, and 0.5 gm of ascorbic acid apparently destroying 43, 81, and 95% of the vitamin B_{12}, respectively, in the moderate vitamin B_{12}-content meals. The degree of destruction was also shown to be different for different meals, with the percentage loss inversely proportional to the vitamin B_{12} content in foods.

Preliminary results of another study by the same investigators (Herbert and Jacob, 1974) suggested that there was no destruction of vitamin B_{12} in food with the addition of 45 mg of ascorbic acid (the 1974 RDA for the adult), and that about one-third of vitamin B_{12} in human bile was destroyed by 0.5 g of ascorbic acid. Hines (1975) suggested that possibly 2-3% of subjects on a megadose regimen of ascorbic acid may risk vitamin B_{12} deficiency. From a group of 90 subjects taking more than 500 mg of ascorbic acid daily, three subjects, aged 50-60 years, showed subnormal serum vitamin B_{12} concentrations (65-100 pg/ml). These 3 subjects had been taking a minimum of 1,000 mg of ascorbic acid with each meal for more than 3 years. In one subject, the level of serum vitamin B_{12} increased from 100 to 180 pg/ml within 3 months after cessation of the megadose ascorbic acid ingestion.

Afroz et al. (1975) reported on the basis of studies of 10 male spinal cord injury patients that daily doses of more than 4 g of ascorbic acid for more than 11 months did not give evidence of destruction of vitamin B_{12}. The serum vitamin B_{12} levels were all well above the low normal value (300 pg/ml) and 3 were above the high normal (1,000 pg/ml). Large doses of ascorbic acid supplements, in the range of 200-1,100 mg daily, did not affect the plasma concentrations of vitamin B_{12} in 9 elderly women (Skinner, 1976). An in vitro experiment in which standard solutions of vitamin B_{12} and control samples of plasma had added ascorbic acid confirmed the fact; no effect of ascorbic acid on the vitamin B_{12} concentration.

It has been reported that iron acts as a stabilizer of vitamin B_{12} activity in liver extracts (Shenoy and Ramasarma, 1955). Mukherjee and Sen (1959) also found that ferric chloride has a protective effect at pH 4.0. Decomposition of vitamin B_{12} by aneurine, nicotinamide, cysteine, and H_2S was also prevented by ferric chloride (0.5 mg/ml).

Considerable destruction of vitamin B_{12} in some animal foods by cooking was observed by Banerjee and Chatterjea (1963). Pan frying of fish, goat meat, and milk at 110 and 120°C for 10 min followed by boiling in distilled water for 15 min decreased the vitamin activity (23.7-96.4% loss) much more than boiling in distilled water for 15 min.

Sterilization processing of liquid milk ("in-bottle" to 110°C for 20 min) caused a considerable destruction (67%) of vitamin B_{12} content (Ford, 1957). The loss was markedly increased by the addition of SH-compounds (more than 90% loss with 10 mg/100 ml of glutathione) or ascorbic acid (80% with 5 mg/100 ml). The addition of cyanide or bisulphite was found to prevent the destruction.

Microbiological Assay of Vitamin B_{12} in Foods

Vitamin B_{12} has been quantitated by measurement of growth of chicks and rats (Ott et al., 1948 and Lillie et al., 1954). The vitamin also promoted the growth of microbiological test organisms such as Lactobacillus lactis Dorner (Shorb, 1947), Lactobacillus leichmannii (Hoffmann et al., 1948 and Skeggs et al., 1948) and Escherichia coli (Coates et al., 1953), and of algae such as Euglena gracilis (Hutner et al., 1949) and Ochromonas malhamensis (Ford, 1953). Thus several types of assays have been developed for assessing vitamin B_{12} activity, but difficulties have been observed with each. The extraordinarily small amount of this vitamin found in foods is one problem in the assay. Assays based on the growth of chicks and rats are considered to be expensive and time consuming, and these have been used for only a few foods (Orr, 1969). Lactobacillus leichmannii and Ochromonas malhamensis have

been widely used as the most appropriate methods for the assay of vitamin B_{12} in various complex products.

Lichtenstein et al. (1959) reported that the L. leichmannii assay is better suited to routine practice and, in several modifications, the assay has been more widely accepted because of its rapidity, uniformity of response, and precision of results. Comparative data on the vitamin B_{12} content of foods of animal origin as assayed by the L. leichmannii and O. malhamensis methods showed the same order of magnitude in 15 of the 27 samples tested. Where the t-tests indicated significantly divergent mean values, the mean O. malhamensis values generally were higher than the L. leichmannii values, the mean differences being 13% or more. Only one sample, yogurt, had a significantly higher value in the L. leichmannii assay than in the O. malhamensis assay, the difference being 26%. Food products with bacterial fermentation and organ meats tested in the experiment contained no significant quantities of nonspecific activity to interfere with the L. leichmannii method. One explanation for the significantly higher values in the O. malhamensis assay is that compounds other than vitamin B_{12} which are stimulatory to O. malhamensis were present in the foods analyzed. Although there was no evidence by which either assay could be judged to yield more correct vitamin B_{12} values, the L. leichmannii method was considered preferable for the assay of foods of animal origin,

on the basis of greater precision and ease of completion (Lichtenstein et al., 1961).

A number of other cobalt-containing compounds have been shown to be present in natural materials and to differ from vitamin B_{12} in lacking the 5,6-dimenthylbenzimidazole group. These compounds are characteristically inactive for animals but active biologically in microbiological assays. Ford et al. (1953) reported that the vitamin B_{12} activity for microorganisms of a variety of natural materials was contributed by one or more of 5 compounds: factors A (vitamin B_{12m}), B, and C, and pseudovitamin B_{12}, as well as cyanocobalamin. Natural materials subjected to bacterial fermentation and various bacterial species have been found to contain these vitamin B_{12}-like compounds, generally in large amounts, along with cyanocobalamin. From an experiment in which tests with O. malhamensis, L. leichmannii, Bacterium coli, and Euglena gracilis, were compared for the assay of the cyanocobalamin in the presence of the vitamin B_{12}-like compounds, Ford (1953) concluded that the Ochromonas was capable of responding specifically to cyanocobalamin, even in crude extracts and in the presence of vitamin B_{12}-like compounds.

Williams et al. (1956) presented the results from comparative determinations of vitamin B_{12} activity in 40 samples of feed supplements and other natural products, in which they concluded that pseudocobalamins do not occur

commonly to a large extent in feed supplements. Four methods, L. leichmannii, O. malhamensis, E. coli pad plate, and chick assay, were compared in the study. The chick assay yielded in general the lowest values and E. coli pad plate resulted in the highest values. A close agreement was found between L. leichmannii and O. malhamensis methods except in those samples containing pseudocobalamin. Certain fermentation materials (Streptomyces fermentation) contained substances which are toxic to chick growth, so that the chick assay method could not be used for these.

It has been found that growth of L. leichmannii occurs in the presence of thymidine or other deoxyribosides (Hoffmann et al., 1948). Thus, in vitamin B_{12} assay with L. leichmannii, it appears to be necessary to separate vitamin B_{12} from the deoxyribosides. Hoffmann et al. (1949) found that vitamin B_{12} was destroyed by heating with 0.2N NaOH at 100°C for 30 min while the deoxyribosides of thymine, guanine, and hypoxanthine were not affected under the same conditions. Strohecker and Henning (1965) also recommended this alkali treatment (pH 11.0) to determine the level of this group of substances in the L. leichmannii assay. However, Newmark et al. (1976) found less than 20% of the vitamin B_{12} activity as measured by L. leichmannii to be nonspecific growth stimulating factors from the meals that were tested.

The extraction step in the assay procedure may also be a source of problems. Most of the noncyanocobalamins (coenzyme forms of cyanocobalamin) present in natural substances are less stable than cyanocobalamin and are readily destroyed by steaming or heating during the microbiological assay. The effect of cyanide in the preparation of assay solutions has been widely utilized to convert these unstable noncyanocobalamins to the more stable cyanocobalamin form (Loy et al., 1952; Volcani et al., 1961; and Rosenthal, 1968). Vitamin B_{12} activity from the cells of organisms is released by the cyanide solution. Practically, the cobamides are retained in the cells in fermentation processes (Wuest and Perlman, 1968). For the L. leichmannii assay procedure Loy et al. (1952) recommended the metabisulfite treatment for the extraction of vitamin B_{12} from complex materials, such as fermentation broths, or those purified materials in which the vitamin may occur either loosely bound or in noncyano form. Tissues, such as liver, pose a problem in that the vitamin B_{12} is bound to protein and largely in the noncyano form requiring prior denaturation of protein to release the vitamin activity and stabilization of the noncyano form. Shenoy and Ramasarma (1954) devised a combined digestion and protection system in which papain and metabisulfite were used. Gregory (1954) also recommended papain treatment for the liberation of the bound vitamin B_{12} in milk samples.

As reviewed earlier, cyanocobalamin is decomposed in the presence of ascorbic acid unless a suitable stabilizer, such as a trace amount of iron is added (Skeggs, 1963 and Shenoy and Ramasarma, 1955). Skeggs (1963) also recommended the use of equal parts of propylene glycol and glycerol.

Estimation of vitamin B_{12} in foods and food supplements can also be done by colorimetric methods (Ellis et al., 1949b and Smith et al., 1962), isotope and isotope dilution methods (Rosenblum and Woodbury, 1952), and chemical methods (Fantes et al., 1950 and Boxer and Rickards, 1950). Each method has specific advantages as well as some limitations. Chemical methods seem to be faster to perform and are slightly more reproducible but markedly less sensitive than either biological or microbiological assay. Colorimetric assays are simple but show difficulties with interfering effects of colored impurities while the isotope assays are highly specific but require larger amounts of samples.

Microbial Production of Vitamin B_{12} in Foods

Vitamin B_{12} is unique among the vitamins in being produced exclusively by microorganisms. Wherever the vitamin is found in nature, its origin can be traced back to microorganisms growing in soil or water or in the rumen or intestine of some animal (Smith, 1965b). Robbins et al., 1950, found traces of vitamin B_{12} in the roots of some

plants, such as tomatoes, but concluded that these traces were absorbed from soil. Traces of vitamin B_{12} have been found from some samples of turnip greens and comfrey by Gray and Daniel, 1959, apparently because of uptake, since vitamin B_{12} could be absorbed from relatively strong solutions by undamaged roots of comfrey and translocated to the leaves. The vitamin is synthesized by nodule bacteria and may possibly be concerned in nitrogen-fixing activities of root nodules of legumes (Kliewer and Evans, 1962). Recently, trace quantities of vitamin B_{12} activity were detected from spinach, amaranth and lucerne leaves (Jather et al., 1975).

Bacterial fermentation has been used for industrial production of the vitamin (Table 1). Leviton and Hargrove (1952) tried to produce vitamin B_{12} with Propionibacterium freudenreichii in several different types of laboratory-applied fermentations. Yields under anaerobic conditions were higher (1.4 mg/l) than under aerobic conditions. Employment of Lactobacillus casei and Propionibacterium freudenreichii as an inoculum to utilize the effect of their symbiosis in a lactose medium resulted in higher production of the vitamin.

Propionibacterium species, as well as being widely used in industrial production of vitamin B_{12}, have been recommended for the fortification with the vitamin for some dairy products. Karlin (1961a) determined the vitamin

Table 1. Some organisms used for industrial production of vitamin B_{12}.

Organism	Medium	Yield B_{12} (mg/l)	Reference
Streptomyces griceus	Glucose Soybean meal Cobalt salts	0.3	Rickes *et al.*, 1948b
Streptomyces olivaceus	Glucose Soybean meal Distiller's solubles Cobalt salts Inorganic salts	3.3	Hall *et al.*, 1953
Streptomyces species	Soybean meal Glucose K_2HPO_4 $CoCl_2$	5.7	Pagano and Greenspan, 1954
Bacillus megaterium	Beet molasses Ammonium PO_4 Cobalt salts Inorganic salts	0.45	Garibaldi *et al.*, 1953
Propionibacterium freudenreichii	Glucose Casein hydrolysate Yeast extract Cobalt salts Lactic acid	3.0 4.5	Leviton and Hargrove, 1952 Sudarsky and Fisher, 1957
Propionibacterium shermannii	Cornsteep liquor Glucose Cobalt NH_4OH, pH 7.0	23.0	Speedie and Hull, 1960

B_{12} content of yoghurt, a similar product made from raw milk, and milk plus 2% yeast extract with or without P. shermannii, and in kefir made from raw milk or milk plus 0.5% peptone with or without P. shermannii. The usual decrease in vitamin B_{12} content observed for yoghurt and a similar product was reduced for raw milk from 86.4% to 25.5% with the addition of P. shermannii; vitamin B_{12} in kefir was increased 2 to 3 times if P. shermannii were added. Thus he (Karlin, 1961b) tried to fortify kefir with vitamin B_{12} by the addition of Propionibacterium to the kefir grains and found that the use of cultures increased the vitamin content from 1.95-2.02 mμg/g in the original milk to 2.50-3.02 mμg/g after 24 hr incubation and to 2.94-3.80 mμg/g after 3 days incubation and 4 days refrigerated storage. The addition of 0.5% peptone in the presence of the P. shermannii further increased the vitamin content of the product. Kruglova (1963) prepared enriched curds from pasteurized cow's milk by fermentation with equal parts of lactic acid and propionic acid bacteria culture (2.5% each). The curds had approximately 10 times more vitamin B_{12} than when produced in the usual way with lactobacilli only. He also mentioned that there was no loss of flavor, aroma, or desired consistency. The curds, with 130 μg vitamin B_{12}/kg, were recommended for therapeutic diets.

From an experiment with 10 cultures of lactic acid bacteria and one culture of P. shermannii in the production

of dahi (fermented milk), Bambha et al. (1973) found that the vitamin B_{12} content was reduced in all cases with lactic acid bacteria and that Propionibacterium shermannii was the only organism which increased the vitamin content. A mixed culture of S. lactis, S. cremoris, L. acidophilus, P. shermannii and Candida species increased all the B-vitamins tested. Propionibacterium shermannii, when grown alone, produced off flavors and bitter taste; however, in the mixed cultures no such undesirable effect was noticed.

The production of vitamin B_{12} by 21 strains of mycobacteria was examined by Antonelli (1962), with the medium of Sauter or a similar one with 0.7% Tween 80, or potato infusion with glycerol and peptone. Two strains produced no vitamin B_{12}, 4 synthesized less than 100 µg/l medium, and other strains produced up to 700 µg/l. Among them, Mycobacterium lacticola strain Milch produced 500 µg and when grown for 30 days the yield rose to 2150 µg/l of vitamin B_{12}.

The ability of mesophilic strains of Bacillus megaterium to produce vitamin B_{12} has been known from studies of Garibaldi et al. (1953) and Goodwin (1963). A greater ability to synthesize the vitamin was observed in a thermophilic strain of the Bacillus (Gajcy, 1973). An increase in vitamin B_{12} content in the biomass occurred only during the lag phase of growth. He also found that the addition of sulfathiazole partly inhibited growth but increased the

efficiency of vitamin biosynthesis in the culture.

Vitamin B_{12} production by a newly isolated, unidentified organism, which is pink-pigmented and capable of utilizing methanol as a sole source of carbon and energy, was studied by Toraya et al. (1975). The maximal yield of the vitamin, 2.6 mg/l of medium, was attained by increasing the methanol concentration and adding L-methionine. The yield was still lower than the values obtained by industrial-type microorganisms cultivated on carbohydrate media (23 mg/l by P. shermannii, 5.7 mg/l by Streptomyces species, as reported by others).

Microbial production of vitamin B_{12} in kimchi, Korean fermented vegetables, has been reported (Lee et al., 1958, and Kim et al., 1960). The unique strain producing the vitamin in the fermentation was identified as Bacillus megaterium (Kim and Chung, 1962). Lee et al. (1960) studied the variation in vitamin levels during kimchi fermentation and found that the B-vitamins including B_{12} increased to a maximum level, twice the content of the original, at the maturation point. After this time, there was a sharp decrease in the vitamins and a marked increase in acidity. Lee et al. (1960) were analyzing winter kimchi which contains fish in addition to vegetables. Thus the initial content of vitamin B_{12} was not zero.

In the microbiological assays using L. leichmannii, it was found that most of the results in the references

were not corrected for the non-vitamin B_{12} activity. Therefore, the actual values of vitamin B_{12} are likely to be lower than the reported values.

EXPERIMENTAL PROCEDURE

Preparation of Kimchi Sample

Kimchi, a group name given to fermented vegetables, has long been a tradition in Korea. There are many procedural variations in its production depending on seasons and regions. The flavor also varies with the different processing methods and ingredients. A typical winter kimchi was studied in this research, as it is the major type which is most popular among Koreans. The ingredients and their distribution in the kimchi are shown in Table 2.

Table 2. Ingredients of kimchi studied and their distribution.

Ingredients	Distribution %
Korean cabbage (Chinese cabbage)	81
Radish roots (white)	9
Salt (Morton's pickling salt)	3.5
Green onion	2.7
Salted shrimp (imported from Korea)	1.8
Red pepper powder (dried)	0.8
Garlic (fresh)	0.7
Ginger (fresh)	0.5

Washed cabbages were cut into 4 to 6 pieces longitudinally and brined with three-quarters of the total salt.

No additional water was used and the cabbages were held overnight in a cool room. Radish roots were sliced into very thin pieces and mixed with all other minced or sliced ingredients and spices, including the other quarter of the weighed salt. This mixture was then sandwiched in between the salted cabbage leaves. The stuffed cabbages were packed tightly into a 2-gallon glass jar and pressed with a weight (a bottle of water) to exclude air. The cabbages were covered with the juice without additional water. The jar was kept in a refrigerator at 4°C, covered with a lid. Fifteen pounds of kimchi were made for each sample and the sample was fermented for 10 weeks. Vitamin B_{12} was assayed at 0 day; 4 days; 1, 2, 3, 4, 5 and 10 weeks of fermentation. The 10 week sample was also cooked for 13 min and vitamin B_{12} was determined. Soy flour (0.5%, hammer milled, Star flour, Eugene, Oregon) or beef extract (0.05%, BBL, Division of BioQuest, Cockeysville, Maryland) was added in some experiments to the stuffing mixture. Ascorbic acid, pH and total acidity, and microbial counts were determined at the same time as vitamin B_{12} was assayed. Three replications were done for the studies.

In a preliminary study, the effect of added cobalt, a structural constituent of the vitamin B_{12} molecule, on the production of vitamin B_{12} was studied. One ppm of Co^{++}. ($Co(NO_3)_2 \cdot 6H_2O$, Baker Chemical Co., Phillipsburg, New Jersey) was added to the kimchi with the stuffing mixture.

Vitamin B_{12} production was compared with the control kimchi. Two supplemental studies were also done. Vegetarian kimchi was prepared to test the effect of the salted shrimp on vitamin B_{12} production. Vitamin B_{12} was determined in commercial kimchi available in Corvallis and compared with the prepared kimchi. The brands available were: Kings Kimchi, Joe Kim's Kim Chee and Kohala Won-Bok Kim Chee. Kimchi at the end of 1 week of fermentation was canned and stored at room temperature for 4 weeks then compared with the product fermented for 5 weeks. Canning was done in pint glass jars by the method in Making Pickles and Relishes at Home (1970) for sauerkraut: a hot pack and 10 min processing in the boiling water bath. Before canning the kimchi juice was adjusted to pH 4.5 with white vinegar.

Inoculation with Vitamin B_{12}-Producing Organism

To determine the possibility of increasing the production of vitamin B_{12} during the kimchi fermentation, P. shermannii was chosen for this study as an inoculum at the beginning of the fermentation. A culture of P. shermannii (ATCC No. 13673) in dehydrated form was obtained from the American Type Culture Collection and rehydrated with 10 ml of sodium lactate broth (Vedamuth and Reinbold, 1967). The broth culture was incubated at 30°C for 48 hr and transferred into sodium lactate agar stabs for stock cultures

grown at the same temperature. For inoculation of the organism into a kimchi sample, the stab culture was again transferred into the broth, grown at 30°C for 48 hr, and a dilution to give 10^6 cells (according to optical density measurements standardized with cell counts) was inoculated per g of the sample at the time the kimchi was made.

Determination of Vitamin B_{12}

Vitamin B_{12} produced in kimchi fermentation was determined by a microbiological assay method with Lactobacillus leichmannii as the test organism. The assay procedure followed the Official Methods of Analysis of A.O.A.C. (1975, p. 839-843), except for the buffer substitution described below.

Test Organism

Lactobacillus leichmannii (ATCC No. 7830) was obtained from the American Type Culture Collection and transferred into 10 ml of liquid culture medium (Difco Lactobacilli broth for AOAC microbiological assays). The organism was grown at 37 ± 0.5°C for 24 hr and transferred into 10 ml of agar culture medium (Difco Lactobacilli broth plus 1.5% agar) and then incubated at 37 ± 0.5°C. The stab cultures of agar medium were stored in a refrigerator at 7°C after having grown for 18-20 hr. Before using the new culture in the assay, successive daily transfers of the stab culture

were made in a 2-week period at the incubation temperature of 37 ± 0.5°C for 18-20 hr. Better growth was obtained by daily rather than every-other-day transfer and so this was the procedure followed. To make an inoculum, the cells were transferred from the stab culture into 10 ml of liquid culture medium and incubated as for the stab culture. The cells were then centrifuged at 6,000 rpm for 10 min and decanted aseptically. The cells were washed 3 times with 10 ml portions of sterile suspension medium, resuspended in 10 ml of the same medium, and diluted with the medium to give a percent transmittance (%T) equivalent to that of a dried cell weight of 0.50-0.75 mg/tube (as described in calibration of the Spectronic 20) when read against the suspension medium set at 100%T. The cell suspension so obtained was used as the inoculum and it was made fresh for each assay.

Calibration of Spectronic 20

To read %T of each assay tube, a Spectronic 20 (Bausch and Lomb Inc., Rochester, New York) was used and the photometer was calibrated according to the AOAC method to determine the respective assay limits expressed as mg dried cell weight per tube. Using the inoculum of L. leichmannii grown in sterile suspension medium with standard cyanocobalamin (100 ng/300 ml), a curve was drawn relating %T at 660 nm to mg dried cell weight (Figure 2) and this curve

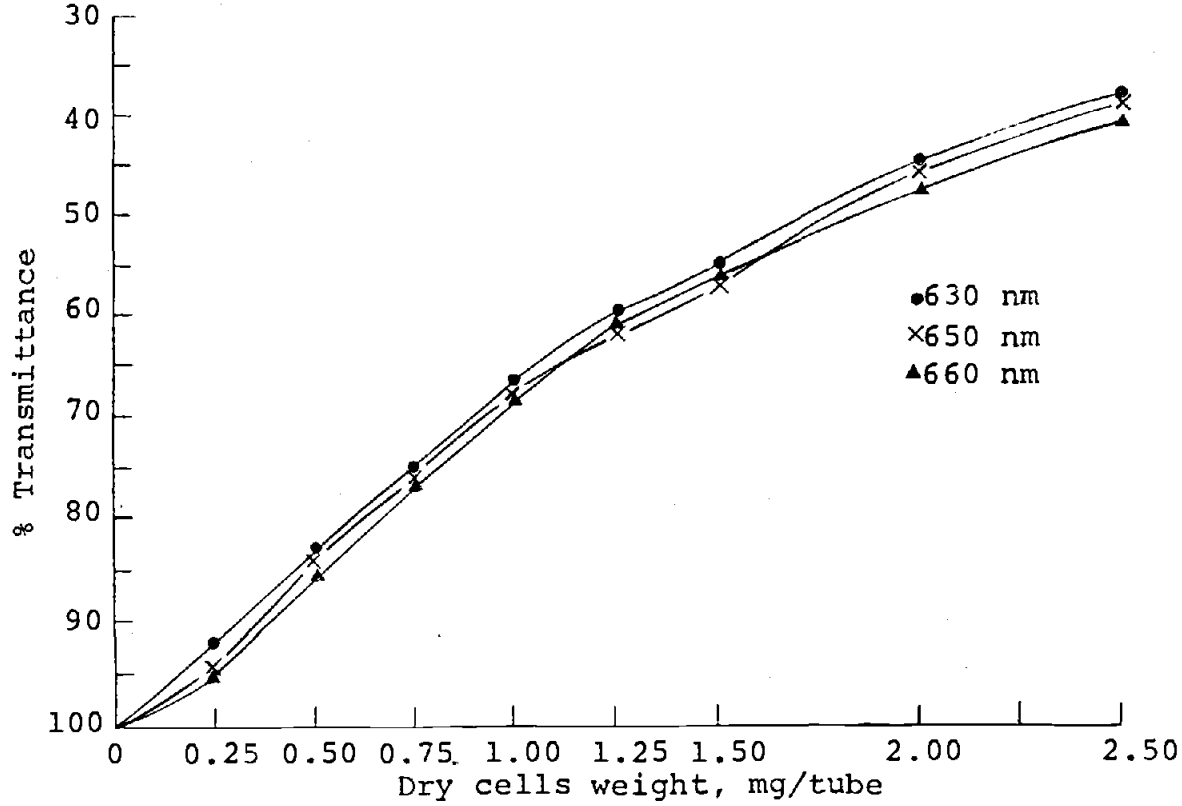

Figure 2. Calibration of Spectronic 20.

was used to determine the concentration of inoculum for each assay.

Cyanocobalamin Standard Solution

To prepare a stock solution, crystalline cyanocobalamin (ICN Pharmaceuticals, Inc., Cleveland, Ohio) was dissolved in 25% ethanol and diluted with additional 25% ethanol to make the cyanocobalamin concentration exactly 100 ng/ml. The solution was stored in the dark at 7°C. Intermediate standard solution was prepared with 1.0 ml of stock solution diluted with 25% ethanol to 100 ml. The cyanocobalamin concentration was 1 ng/ml. This was stored in the dark at 7°C. The working standard solution was made with 1.5 ml of the intermediate standard solution diluted with sterile water to 50 ml. The final concentration was 0.03 ng cyanocobalamin/ml. This working standard solution was used for determination of the standard curve.

Media and Buffer Solutions

Basal medium stock solution was prepared from vitamin B_{12} assay medium U.S.P. (Difco, Detroit, Michigan) and used as the basal medium for the assay. Suspension media were prepared for stock cultures. Lactobacilli broth A.O.A.C. (Difco, Detroit, Michigan) was used to make liquid culture medium and agar culture medium with the addition of 1.5%

agar. Basal medium diluted with an equal volume of water was prepared to use to wash the culture. All of the prepared media were cooled rapidly in a cold water bath to prevent color formation after sterilization and stored in the dark at 7°C.

Metabisulfite-buffer solution was recommended as the vitamin B_{12}-extracting solution in the A.O.A.C. method. However, preliminary studies showed that kimchi samples extracted with water had higher values of vitamin B_{12} activity than the samples extracted with metabisulfite-buffer solution (Table 3). The vitamin in kimchi may be in the

Table 3. Effects of buffer solutions and heat treatments on vitamin B_{12} extraction of kimchi.

		Replication				
		1	2	3		
Solution	Heat Treatment	Total ng/g	Total ng/g	Total ng/g	B_{12} analog ng/g	B_{12} ng/g
H_2O	15 min steaming	2.45	3.68	3.66	3.44	0.22
S_2O_5-solution	15 min steaming	2.80	3.10	3.21	3.03	0.18
S_2O_5-solution	10 min autoclaving[a]	2.35	2.96	2.54	2.49	0.05
CN-solution	15 min steaming	2.39	3.61	NT[b]	NT[b]	NT[b]
CN-solution	10 min autoclaving[a]	2.43	3.83	3.78	3.34	0.44

[a]Autoclaving was at 121°C.
[b]Not tested.

free form rather than bound. Metabisulfite probably inhibits the growth of the test organism. Cyanide-buffer solutions have been recommended to convert the less stable

noncyanocobalamins to the more stable cyanocobalamin (Volcani et al., 1961; Rosenthal, 1968). Cyanide solution is also active in releasing vitamin B_{12} retained in the cell materials of the organism (Wuest and Perlman, 1968). A cyanide-buffer (pH 6.0) solution was used in preliminary tests and resulted in higher values of vitamin B_{12} as compared to those obtained from samples extracted with metabisulfite-buffer or water (Table 3). The cyanide-buffer solution is formulated with 3.25 g Na_2HPO_4 (dibasic sodium phosphate), 1.5 g citric acid·H_2O and 10 mg KCN per 250 ml.

Assay Procedure

The assay procedure followed the principles of the AOAC method. Assay tubes and other necessary glassware were meticulously washed with sodium lauryl sulfate, U.S.P., and carefully rinsed in water and distilled water. All glassware and tubes were then sterilized by dry heat for 2 hr at 250°C.

Assay samples were prepared with kimchi juice and the extracting solution. Each 10 ml of kimchi juice was transferred into a flask containing 50 ml of the freshly made cyanide-buffer solution. The 10 ml of juice were taken from 5 different parts of the kimchi sample. The sample was then extracted by autoclaving 10 minutes at 121°C. The flask was cooled rapidly by shaking in a cold water bath. The extracted sample was diluted with sterile water to 100

ml and centrifuged 10 min at 6,000 rpm to settle the undissolved particles. Five ml of the diluted sample solution were transferred into a beaker containing 40 ml of sterile water, adjusted to pH 6.0 with 1N-NaOH solution aseptically, and diluted further with additional sterile water to 50 ml. The final dilution was 1:100, designated the assay solution.

The results of the preliminary tests indicated that assay values for 1 ml (1 g) of juice or 1 g of solids plus liquid were equivalent. Samples of solids plus liquid were steamed 10 min (to an end-point temperature of 85°C) before blending and compared to products blended without a prior heat treatment.

Sample and standard tubes were filled, according to the AOAC procedure, in duplicate with the assay solution and the working standard solution, respectively. After autoclaving 5 min at 121°C, 5.0 ml of sterile basal medium stock solution were added to the tube of uninoculated blank. Then, freshly prepared inoculum was added to the rest of the sterile basal medium stock solutions, 1 drop of inoculum with a 10 ml pipette for each 5.0 ml of the stock solution, and mixed well. Five ml of the stock solution plus inoculum were added to each tube, except the uninoculated blank tube. After thorough shaking, all the tubes were incubated 22-24 hr at 37 ± 0.5°C.

The growth of the test organism in the assay tubes was read in percent transmittance (%T) by using the

Spectronic 20. It was found in the preliminary tests that the most definitive standard curve was obtained at 660 nm with the photometer. The assay tubes were placed in the Spectronic 20 set at a wave length of 660 nm, and %T was read at 30 sec. The reading scale was adjusted with uninoculated blank set at 100%T and the inoculated blank was read. (If this reading was higher than that of dried cell weight of 0.6 mg/tube, the results of the assay were not used.) Then it was readjusted with the inoculated blank set at 100%T.

A standard concentration-response curve (Figure 3) was prepared for each assay by plotting %T readings against amounts of reference standard in respective tubes. The amount of vitamin B_{12} for each level of assay solution was determined from the standard curve and the duplicates were averaged. The concentration was then multiplied by the dilution factor.

Effect of Ascorbic Acid on Vitamin B_{12} Assay

Preliminary tests were conducted to study the effect of ascorbic acid on vitamin B_{12} assay in kimchi. Various amounts of ascorbic acid were added to kimchi samples and the resultant assay values were compared with those of the control sample without additional ascorbic acid. Protective effects of iron and a mixture of propylene glycol and glycerine also were studied. In order to see the effect

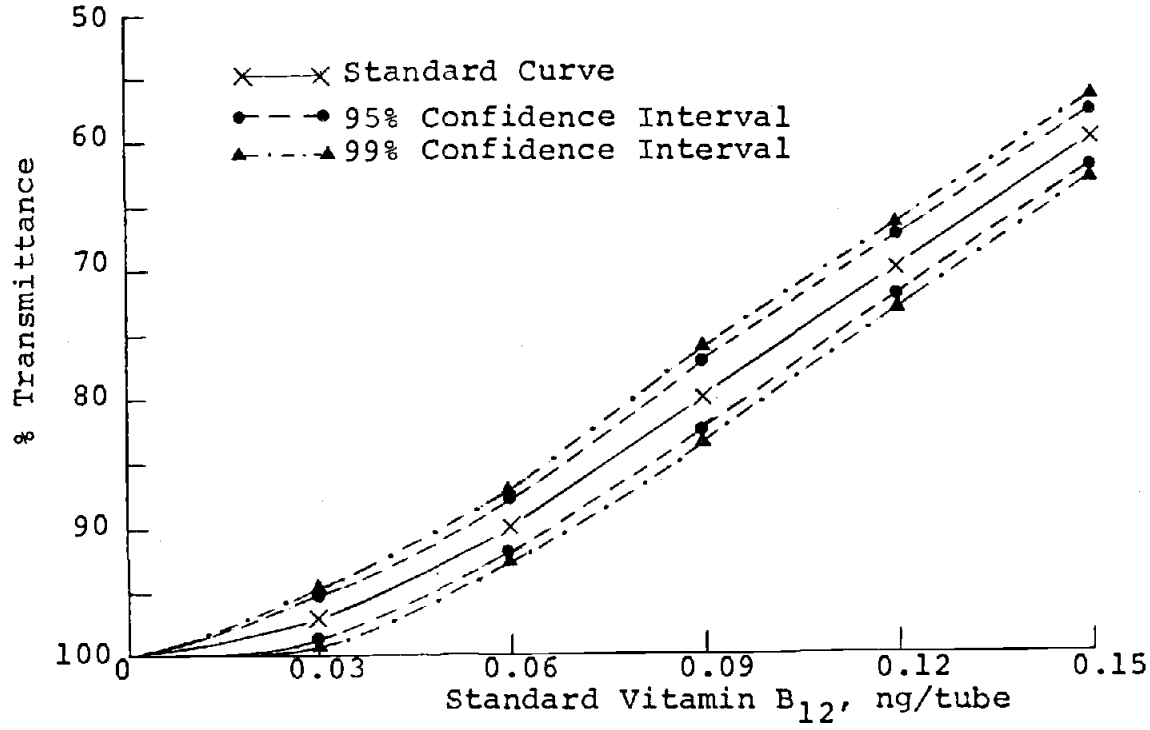

Figure 3. Standard curve based on values of 20 replications.

of ascrobic acid and determine the validity of the assay results for kimchi samples, standard cyanocobalamin and ascorbic acid (Baker Chemical Co., Phillipsburg, New Jersey) were added to samples which had first been autoclaved 30 min at pH 12.0 to destroy native vitamin B_{12} present and the recovery of the added vitamin B_{12} was determined.

Correction for Vitamin B_{12}-Analogs

Pseudovitamin B_{12} and deoxyribosides were determined, in the preliminary studies, to be present in large quantities in kimchi. Hydrolysis of food extracts at pH 12.0 for 30 min at 121°C was used to destroy vitamin B_{12} (Shenoy and Ramasarma, 1955). The microbiological activity remaining after this hydrolysis was considered to be growth factors for the assay organism that are not cobalamins. Therefore, the difference in the results before and after the alkali treatment is the activity of vitamin B_{12} assayed in the samples. The non-vitamin B_{12} activity was determined and used as a correction factor in this study with kimchi.

Determination of Ascorbic Acid

During the kimchi fermentation, ascorbic acid and dehydroascorbic acid were determined by the microfluorometric method recommended in Official Methods of Analysis of the AOAC (1975, p. 829-831). Sampling was done on 50 g

of kimchi juice and 150 g of solids, blended 5 min in an Osterizer (Oster Corporation, Milwaukee, Wisconsin). The solid sample was taken from 1 piece of kimchi without head part and the juice was taken from 5 different parts of the sample. The vitamin in the samples was oxidized to dehydroascorbic acid by acid-washed Norit (Pfanstiehl Chemical Company, Waukegan, Illinois) and was reacted with O-phenylenediamine to produce a fluorophor having an activation maximum at 350 nm and fluorescence maximum at 430 nm. To correct for nonspecific fluorescence, boric acid solution was added to form H_3BO_3-dehydroascorbic acid with the derivative of the vitamin prior to the addition of the diamine solution. The remaining fluorescence was due to the unrelated materials and this was used as the "blank." Total ascorbic acid content was quantitated by comparison of the corrected fluorescence reading for the sample with that of a standard solution similarly oxidized and treated. The fluorescence was read on the Aminco-Bowman Spectrophotofluorometer (American Instrument Company, Inc., Silver Spring, Maryland).

Microbial Counts

During the fermentation of kimchi, total anaerobic microorganisms, total lactic acid bacteria, and total propionibacteria were counted. To determine the total number of anaerobic microorganisms, lactic agar (Elliker

et al., 1956) was prepared and diluted samples of liquid were incubated with the medium anaerobically in Case anaerobic jars (GasPak Anaerobic Jar, BBL, Division of Bio-Quest, Cockeysville, Maryland) at 37 ± 0.5°C for 48 ± 2 hr. All colonies were counted at this time. A selective isolation and enumeration medium, LBS agar (Rogosa et al., 1951), was chosen for the count of total lactic acid organisms. After incubation of the sample with the LBS agar medium in Case anaerobic jars at 37 ± 0.5°C for 48 ± 2 hr, total cell numbers were determined. Sodium lactate agar, a selective medium for propionibacteria, was formulated by Vedamuth and Reinbold (1967) and was used in this study to determine the numbers of total propionibacteria. Pour plates of samples were incubated anaerobically using the Case anaerobic jar at 30 ± 0.5°C for 6 days. All colonies, including P. shermannii in the inoculated samples, were counted.

Identification of Propionibacteria

Selected colonies from the sodium lactate agar plates were studied to identify the individual Propionibacterium species growing in kimchi. After incubation for 24 hr at 30 ± 0.5°C in sodium lactate broth in Case anaerobic jars, gram staining, colony consistency, and the following tests were studied: hydrolysis of gelatin and esculin; acid formation from mannitol, lactose, trehalose, maltose and ribose;

reduction of nitrate; and production of indole. These identification criteria were assessed for speciation by reference to Holt (1977). Propionibacterium shermannii was used as the reference organism for the identification.

pH and Total Acidity Measurements

During the fermentation of kimchi, pH of the juice was measured on each sample by a Research pH meter (Corning Scientific Instruments, Model 12, Medfield, Massachusetts). Total acidity also was determined by titration with 0.0990 N NaOH solution and expressed in percent as lactic acid. A Fisher Titrimeter, Model 36 (Fisher Scientific, Chicago, Illinois) was used.

Sensory Evaluation

The difference in flavor between the control and inoculated kimchi samples with added soy flour or beef extract was judged by 9 Korean people (6 women and 3 men) who lived in Corvallis, Oregon. All were graduate students attending Oregon State University except for two student wives. For the sensory evaluation, a triangle test (Amerine et al., 1965) was used and the panel members were trained with several different kimchi samples. The samples were cut into small pieces and were served with juice in paper muffin cups. On each tray there were three randomly 3 digit-numbered samples: two were the same and one was

different. Two trays with different samples were given, one after the other, to each panelist, who was then asked to identify the odd sample on each tray. Although the panel was too small to give other than a preliminary assessment, members were asked to judge the acceptability and identify any flavor differences which were found. Two different fermentation stages, 1 week (under-fermented) and 3 weeks (optimum-fermented), were evaluated for each replication. Judging was done under incandescent electric light in a separate room in the Foods and Nutrition Department. The numbers correctly identified for the three replications within each treatment were obtained and then analyzed using a significance table for triangular tests ($p = 1/3$) (Roessler et al., 1948).

Statistical Analysis

An analysis of variance was done in order to measure the effects of fermentation time and treatments with culture and soy flour or beef extract. To determine the relationship between the decrease in ascorbic acid content and fermentation time, the correlation coefficient, r, was calculated and a linear equation was derived. The sensory evaluation data were also analyzed using a significance table for triangular tests as described above.

RESULTS AND DISCUSSION

Development of Assay Method

The vitamin B_{12} assay procedure followed the Official Methods of Analysis of A.O.A.C. (1975, p. 839-843) with some minor modifications determined experimentally. Increased destruction of vitamin B_{12} by addition of ascorbic acid was observed in kimchi samples extracted with water or metabisulfite-buffer solution (Table 4). The addition of iron in levels of 12 and 17 ppm with the same extracting solutions showed a protective effect for vitamin B_{12} (Tables 4 and 5). The addition of equal volumes of propylene glycol and glycerine, which was recommended by Bartilucci and Foss (1954), did not exhibit any protective effect. Added vitamin B_{12} (1 ng/ml) was fully recovered from samples extracted with a cyanide-buffer solution (Table 5). A cyanide-buffer solution at pH 6.0 (Rosenthal, 1968) replaced the metabisulfite-buffer solution to extract vitamin B_{12} in kimchi samples. The cyanide solution resulted in higher values of vitamin B_{12} assay and showed no inhibitory effect on the growth of the test organism.

Blending kimchi samples for 5 min with an Osterizer mixer destroyed vitamin B_{12}. However, steaming the kimchi 10 min before blending (end-point temperature of 85°C) decreased this loss (Table 6). The 10-min steaming

Table 4. Effect of added ascorbic acid and iron on vitamin B_{12} assay in kimchi.

Extraction Solution	pH of Kimchi	Added Ascorbic acid mg/g	Protector Added	Vitamin B_{12} Level Found[a] %
Water	4.40	0.13	-	87
Water	4.60	0.25	-	89
Water	4.60	0.50	-	83
Water	4.60	-	Fe^{++}[b], 10 ppm	100
Water	4.40	-	Fe^{++}[b], 12 ppm	101
Water	4.40	-	Fe^{++}[b], 17 ppm	104
Water	4.35	-	Fe^{++}[b], 17 ppm	103
Water	4.40	-	Pg-gly[c], 0.1 ml/ml	76
Water	4.35	-	Pg-gly[c], 0.2 ml/ml	69
Metabisulfite-buffer (10^{-1} dil.)	4.35	13.0	-	77
Metabisulfite-buffer (10^{-1} dil.)	4.35	25.0	-	71
Metabisulfite-buffer (10^{-1} dil.)	4.35	50.0	-	66

[a]Vitamin B_{12} content of control sample with no additional ascorbic acid or protective agents was used as 100% base.

[b]$FeSO_4$ was used.

[c]Mixture of equal volumes of propylene glycol and glycerin.

Table 5. Recovery of vitamin B_{12} added to kimchi samples.

Extraction solution	pH of kimchi	Vitamin B_{12} added ng/g	Ascorbic acid added mg/g	Recovery of added vitamin B_{12} %
water	5.55	2	0.13	68
water	5.55	2	0.25	36
water	5.55	2	0.25 + Fe^{++}[a], 17 ppm	56
Cyanide-buffer	4.49	1	-	110

[a] $FeSO_4$ was used.

Table 6. Effects of blending and steaming before blending of kimchi on vitamin B_{12} content.

Sample	Treatment	Total ng/100g	Analogs[a] ng/100g	B_{12} ng/100g
Kimchi juice	None	383	298	85
Kimchi juice	Steamed and blended	375	305	70
Kimchi juice + solids	Blended	356	345	11
Kimchi juice + solids	Steamed and blended	389	339	50

[a] Values obtained after hydrolysis of the kimchi extracts at pH 12.0 for 30 min at 121°C.

apparently destroyed the oxidases. In laboratory tests with added vitamin B_{12}, none of the products of hydrolysis of the vitamin served to stimulate growth of the assay organism. From the results of hydrolysis of kimchi at pH 12.0 for 30 min at 121°C, vitamin B_{12}-analogs, which would be pseudovitamin B_{12} and deoxyribosides, were found in

large quantities in the samples. The alkali-treatment of kimchi samples was sufficient to destroy the vitamin B_{12} and permit the detection of analogs with the same assay organism.

Production of Vitamin B_{12} in Kimchi Fermentation

The optimum fermentation of kimchi was reached by 3 weeks of fermentation when the pH was at 4.5-5.8. The panel members also agreed that the kimchi was at the optimum stage. Lee et al. (1960) reported that kimchi has a good taste at pH higher than 4.5 and is overfermented when the pH is below 4.3.

Table 7 and Figure 4 show the production of vitamin B_{12} in kimchi fermented at 4°C. Vitamin B_{12} was found at a maximum concentration of 47 ng/100 g in control kimchi, which had not been inoculated with Propionibacterium shermannii. Inoculation of kimchi samples with the Propionibacterium increased the vitamin production to 102 ng/100 g. The addition of soy flour (0.5%) or beef extract (0.05%) further increased the vitamin content to 197 and 203 ng/100 g, respectively. Maximum production of vitamin B_{12} was achieved, in all samples, at 1 week of fermentation and, after this point, the vitamin activity was reduced markedly by 2 and 3 weeks of fermentation. After 3 weeks, the vitamin decreased gradually to the 5 weeks of fermentation; no further decrease was seen at 10 weeks of

Table 7. Content of vitamin B_{12} in control kimchi and kimchi inoculated with *Propionibacterium shermannii*, with and without added soy flour or beef extract, and fermented at 4°C.

Fermentation Time	Replication	Vitamin B_{12}, ng/100 g			
		Control	Inoculated	Inoculated plus Soy Flour[a]	Inoculated plus Beef Extract[b]
0 day	1	7	29	40	35
	2	12	25	39	41
	3	17	45	49	55
	Ave.	12	33	43	44
	S.D.	5.0	10.6	5.5	10.3
4 days	1	13	34	105	99
	2	16	50	106	105
	3	23	68	118	122
	Ave.	17	51	110	109
	S.D.	5.1	17.0	7.2	11.9
1 week	1	59	113	193	200
	2	33	90	192	195
	3	50	103	206	213
	Ave.	47	102	197	203
	S.D.	13.2	11.5	7.8	9.3
2 weeks	1	33	73	95	102
	2	27	67	122	128
	3	36	87	149	161
	Ave.	32	76	122	130
	S.D.	4.6	10.3	27.0	29.6
3 weeks	1	5	43	88	98
	2	18	43	90	109
	3	28	70	130	140

Table 7 (continued)

Fermentation Time	Replication	Vitamin B12,ng/100 g			
		Control	Inoculated	Inoculated plus Soy Flour[a]	Inoculated plus Beef Extract[b]
	Ave.	17	52	103	116
	S.D.	11.5	15.6	23.7	21.8
4 weeks	1	15	39	83	90
	2	12	38	80	96
	3	22	60	119	126
	Ave.	16	46	94	104
	S.D.	5.1	12.4	21.7	19.3
5 weeks	1	11	36	77	80
	2	8	33	74	85
	3	18	51	114	121
	Ave.	12	40	88	95
	S.D.	5.1	9.6	22.3	22.4
10 weeks	1	14	33	76	85
	2	12	39	80	90
	3	26	50	116	125
	Ave.	17	41	91	100
	S.D.	7.6	8.6	22.0	21.8
10 weeks, cooked	1	12	25	70	75
	2	11	33	72	76
	3	24	41	86	108
	Ave.	16	33	76	86
	S.D.	7.2	8.0	8.7	18.8

[a]Hammer Milled, Star Flour, Eugene, Oregon.

[b]BBL, Division of Bioquest, Cockeysville, Maryland.

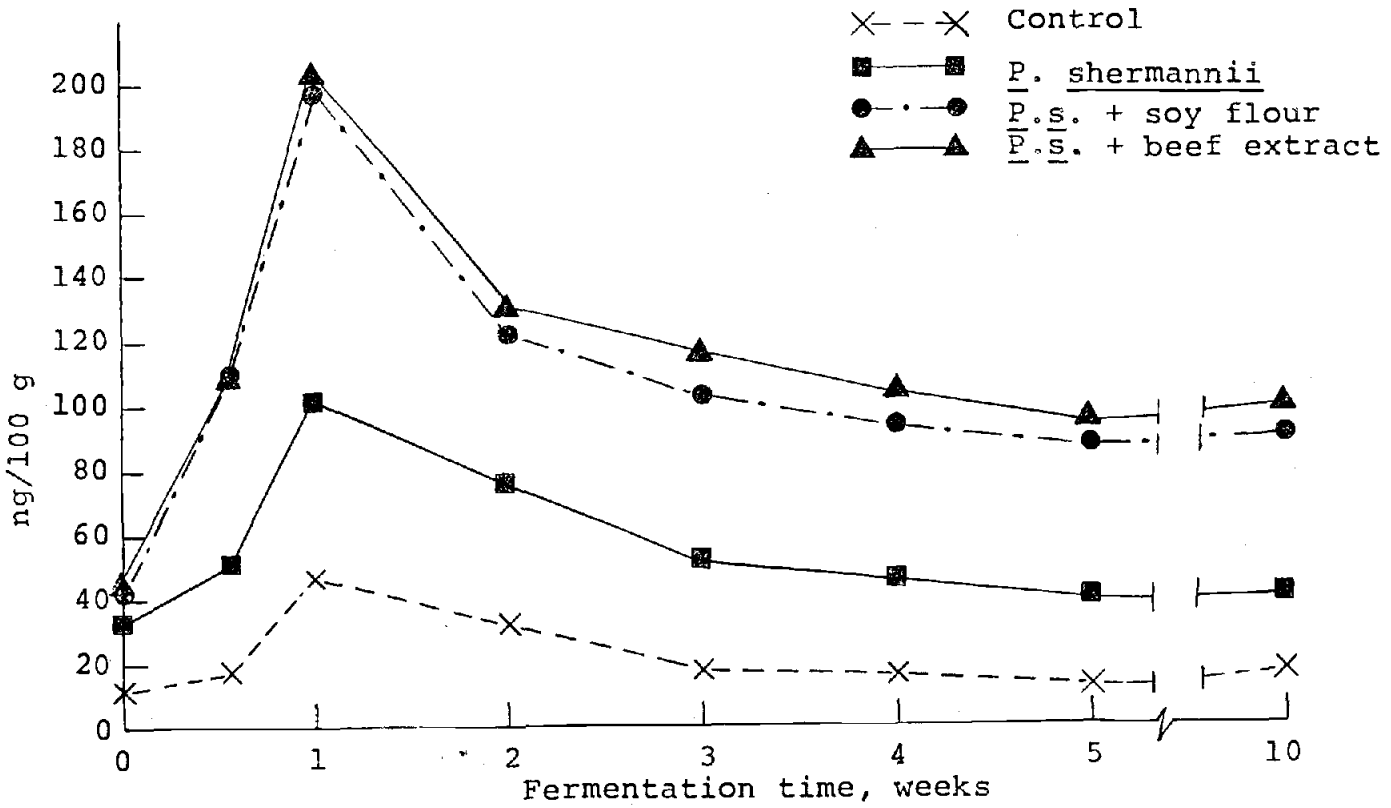

Figure 4. Content of vitamin B12 in control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

fermentation. After 5 weeks of fermentation, the vitamin content, 12 ng/100 g, was the same as that of the unfermented, 12 ng/100 g in control kimchi. However, the vitamin activity was slightly higher in the inoculated kimchi after 5 weeks of fermentation, 40 ng/100 g, than the original activity, 33 ng/100 g. Kimchi samples with soy flour or beef extract added with the inoculum showed much higher amounts of the vitamin after 5 weeks of fermentation, 88 and 95 ng/100 g, respectively, than the fresh, unfermented kimchi products, 43 and 44 ng/100 g, respectively. Cooking of the kimchi for 13 min after 10 weeks of fermentation lowered the vitamin content in all samples. Kimchi is usually cooked if it is overfermented and is consumed as a delightful dish with the addition of meats.

The exact process by which vitamin B_{12} decreased is not known. Vitamin B_{12} is known to be relatively stable at pH 4.0 to 7.0. Hartley et al. (1950) reported that the vitamin is unstable in acid (pH 2.0) or alkaline (pH 9.0) solutions and in the presence of oxidizing or reducing substances. Destruction of vitamin B_{12} by ascorbic acid has been reported in a number of references. The vitamin may also be metabolized by the microorganisms involved in the kimchi fermentation.

Added cobalt (1 ppm Co^{++} as $Co(NO_3)_2 \cdot 6H_2O$) did not increase the vitamin B_{12} production. Addition of cobalt to the inoculated kimchi with soy flour increased the

production but not significantly, less than 4%. Hendlin and Ruger (1950) had found that supplementation of the medium with as little as 1 to 2 ppm of cobalt increased the vitamin B_{12} activity in fermentation by various microorganisms.

From an analysis of variance (Table 8), the F values for the effects of fermentation time and inoculation with

Table 8. ANOVA table for vitamin B_{12} production in kimchi.

Source of Variation	d.f.	Mean Square	F value
Total	31		
Fermentation time	7	3,656	12.6**
Inoculation	3	15,079	52.1**
Control vs. Inoculation	1	29,400	101.7**
Among inoculation types	2	7,918	27.4**
Error	21	289	

**Indicates significance at the 1% level.

P. shermanii were significant at the 1% level. The difference between the effects of added soy flour or beef extract was not significant at the 5% level (test for LSD).

The per capita consumption of kimchi in Korea ranges from 200 to 300 g (about 1 to 1 1/2 C) per day in the winter season (Kwon, 1972). Three hundred grams of the inoculated kimchi with added soy flour or beef extract will provide about 0.6 µg of vitamin B_{12} at 1 week of

fermentation: that is 30% of FAO-WHO's daily requirement, 1970. The same amount of the inoculated kimchi will provide 0.3 µg, while that of the control kimchi will give 0.14 µg.

Content of Ascorbic Acid in Kimchi

The content of ascorbic acid gradually decreased during the kimchi fermentation (Table 9). The variation in ascorbic acid content was not significantly different between the control and inoculated kimchi with or without soy flour or beef extract. The average content of the vitamin in the fresh, unfermented samples was 16.8 mg/100 g and after 5 weeks of fermentation it had decreased to 7.7 mg/100 g. No further decrease was observed between the 5 and 10 weeks of fermentation. Lee et al. (1960), reported that the ascorbic acid content of kimchi fermented at 2-7°C decreased during the first week and then slightly increased until the end of 2 weeks and again decreased gradually as the fermentation continued. The temporary increase was not noticed in this study. Figure 5 shows the ascorbic acid content in log mg/100 g in the kimchi. A linear relationship between the ascorbic acid content and the fermentation time to 5 weeks was studied and the negative correlation coefficient, r, was significant at the 1% level. A regression equation, $Y = 1.207 \text{ mg} - 0.069 X$, was derived: Y as the content of ascorbic acid in log mg/100 g and X as the

Table 9. Ascorbic acid content of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

Fermentation Time	Replication	Ascrobic Acid, mg/100 g				
		Control	Inoculated	Inoculated plus Soy Flour	Inoculated plus Beef Extract	Average
0 day	1	15.9	15.7	15.9	17.4	
	2	18.1	19.1	17.8	19.3	
	3	16.1	15.4	16.6	14.4	
	Ave.	16.7	16.7	16.7	17.0	16.8
	S.D.	1.3	2.0	0.9	2.5	
1 week	1	14.4	13.2	13.1	16.1	
	2	15.5	14.6	13.3	15.7	
	3	12.3	12.6	13.1	11.4	
	Ave.	14.1	13.5	13.7	14.4	13.8
	S.D.	1.6	0.9	0.1	2.6	
2 weeks	1	10.4	10.5	10.4	10.8	
	2	13.3	13.1	12.8	13.2	
	3	10.2	10.6	10.4	9.7	
	Ave.	11.3	11.4	11.2	11.3	11.3
	S.D.	1.7	1.9	0.9	1.8	
3 weeks	1	8.7	6.9	8.9	9.4	
	2	10.8	11.4	10.6	10.8	
	3	9.3	9.1	8.9	8.8	
	Ave.	9.6	9.1	9.5	9.7	9.5
	S.D.	1.1	2.3	0.9	1.0	
4 weeks	1	7.7	6.6	7.8	9.0	
	2	9.5	10.3	9.7	8.9	
	3	8.8	8.4	7.6	8.2	

Table 9 (continued)

Fermentation Time	Replication	Ascorbic Acid, mg/100 g				
		Control	Inoculated	Inoculated plus Soy Flour	Inoculated plus Beef Extract	Average
	Ave.	8.7	8.4	8.5	8.7	8.6
	S.D.	0.9	1.9	1.1	0.4	
5 weeks	1	6.9	4.5	7.3	7.2	
	2	8.7	9.2	8.9	8.1	
	3	7.9	8.5	7.9	7.8	
	Ave.	7.8	7.4	8.0	7.7	7.7
	S.D.	0.9	2.5	0.8	0.2	
10 weeks	1	6.8	4.8	7.5	5.4	
	2	8.9	8.8	8.7	7.5	
	3	7.7	8.6	7.9	7.6	
	Ave.	7.8	7.4	8.0	6.8	7.5
	S.D.	1.1	2.2	0.6	1.3	

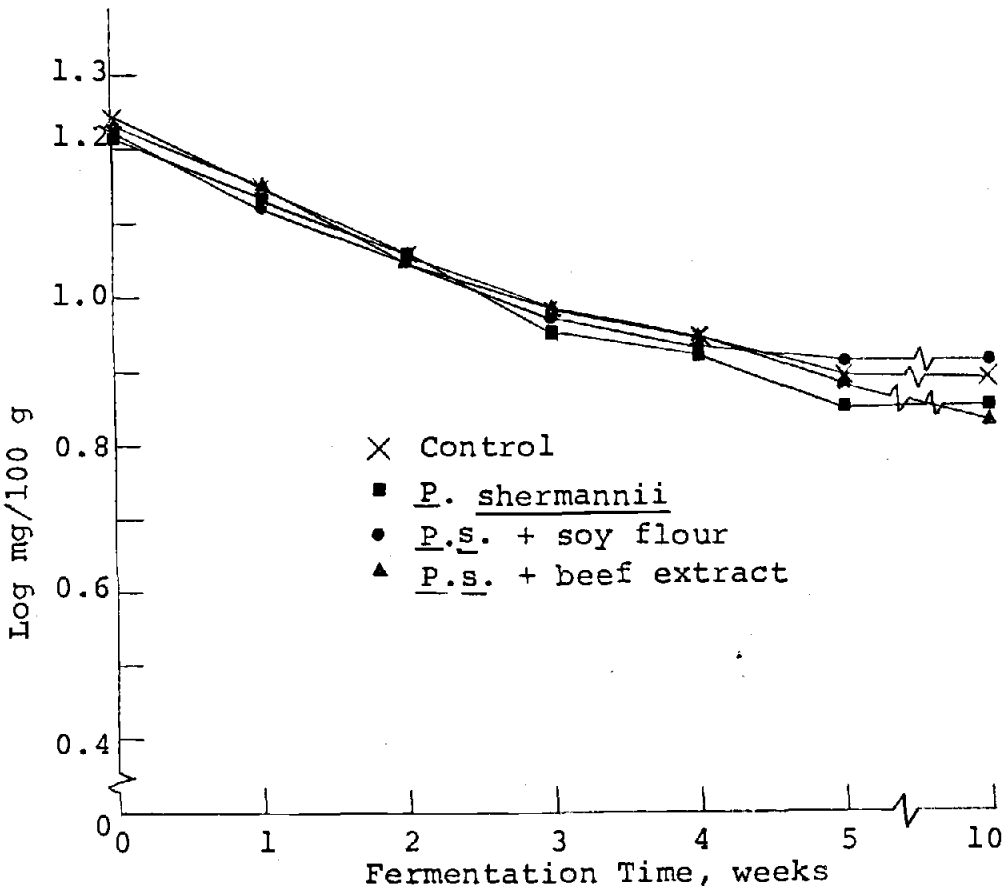

Figure 5. Ascorbic acid content of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

fermentation time in weeks.

Kimchi is regarded as an important source of ascorbic acid. From the results of this study, 300 g of fresh unfermented kimchi provides about 50 mg of ascorbic acid (FAO/WHO's daily requirement for the adult is 30 mg); the same amount of kimchi at optimum fermentation (3 weeks), 28 mg; and even that of over-fermented kimchi at 10 weeks provides about 23 mg.

Microbial Counts

Total counts of anaerobic microorganisms and lactobacilli are presented in Table 10 and Figures 6 and 7. No differences in the total counts were observed between the control and inoculated kimchi with or without soy flour or beef extract. Anaerobic microorganisms increased from average counts of 5.9×10^4 per g in the fresh, unfermented kimchi to the maximum number of 2.8×10^8 per g in the kimchi at the end of 4 weeks of fermentation. By 4 weeks of fermentation, the total number of lactobacilli increased sharply from an average of 1.8 x 10 per g in the fresh, unfermented kimchi to a maximum of 2.3×10^8 per g. From Table 10 and Figures 6 and 7, it appears that the lactobacilli participated actively in the kimchi fermentation; the total anaerobic microorganisms at 3 weeks of fermentation were almost all lactobacilli. Kim and Chung (1962) identified some of the anaerobic

Table 10. Total counts of anaerobic microorganisms and lactobacilli per gram of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

Fermentation Time	Replication	Control		Inoculated		Inoculated plus Soy Flour		Inoculated plus Beef Extract	
		Total Anaerobes	Total Lactobacilli	Total Anaerobes	Total Lactobacilli	Total Anaerobes	Total Lactobacilli	Total Anaerobes	Total Lactobacilli
0 day	1	2.0×10^{4}	2.0×10	1.2×10^{5}	1.6×10	1.4×10^{5}	1.8×10	1.1×10^{5}	2.0×10
	2	5.4×10^{4}	2.0×10	3.6×10^{4}	2.1×10	3.9×10^{4}	1.5×10	6.3×10^{3}	3.1×10
	3	3.7×10^{4}	1.3×10	6.8×10^{4}	1.8×10	2.9×10^{4}	1.7×10	2.0×10^{4}	1.0×10
	Ave.	3.7×10^{4}	1.8×10	7.5×10^{4}	1.8×10	6.9×10^{4}	1.7×10	4.5×10^{4}	2.0×10
1 week	1	2.0×10^{4}	3.0×10^{4}	2.2×10^{5}	1.0×10^{4}	4.0×10^{5}	1.1×10^{5}	1.0×10^{6}	9.0×10^{4}
	2	6.0×10^{4}	3.0×10^{2}	4.0×10^{4}	9.0×10^{2}	5.0×10^{4}	4.0×10^{2}	5.0×10^{4}	1.2×10^{3}
	3	4.1×10^{4}	6.0×10^{2}	7.9×10^{4}	8.1×10^{2}	3.1×10^{4}	7.2×10^{2}	2.7×10^{4}	3.2×10^{2}
	Ave.	4.0×10^{4}	1.0×10^{4}	1.1×10^{5}	3.9×10^{3}	1.6×10^{5}	3.7×10^{4}	3.6×10^{5}	3.1×10^{4}
2 weeks	1	6.0×10^{4}	2.1×10^{4}	4.9×10^{5}	1.9×10^{4}	9.5×10^{6}	5.9×10^{5}	6.2×10^{6}	4.5×10^{5}
	2	1.5×10^{5}	1.2×10^{4}	1.3×10^{5}	1.1×10^{4}	1.5×10^{5}	1.4×10^{4}	1.7×10^{5}	1.2×10^{4}
	3	2.0×10^{7}	3.3×10^{5}	2.8×10^{6}	3.1×10^{5}	1.0×10^{6}	3.0×10^{5}	9.9×10^{5}	2.8×10^{5}
	Ave.	6.7×10^{6}	1.2×10^{5}	1.1×10^{6}	1.1×10^{5}	3.6×10^{6}	3.0×10^{5}	2.5×10^{6}	2.5×10^{5}
3 weeks	1	1.3×10^{7}	7.3×10^{6}	1.4×10^{7}	8.2×10^{6}	3.0×10^{8}	9.5×10^{7}	4.6×10^{8}	9.0×10^{7}
	2	1.3×10^{6}	4.5×10^{6}	1.9×10^{6}	4.9×10^{6}	1.6×10^{6}	5.6×10^{6}	1.6×10^{6}	5.0×10^{6}
	3	1.9×10^{8}	1.7×10^{8}	1.5×10^{8}	1.3×10^{8}	7.1×10^{7}	5.7×10^{7}	1.8×10^{8}	1.8×10^{8}
	Ave.	6.8×10^{7}	6.1×10^{7}	5.5×10^{7}	4.8×10^{7}	1.2×10^{8}	5.3×10^{7}	2.1×10^{8}	9.2×10^{7}
4 weeks	1	5.0×10^{8}	4.2×10^{8}	5.2×10^{8}	4.0×10^{8}	6.7×10^{8}	6.2×10^{8}	6.8×10^{8}	6.1×10^{8}
	2	7.3×10^{6}	5.0×10^{5}	5.0×10^{6}	4.0×10^{5}	1.2×10^{7}	6.0×10^{5}	1.3×10^{7}	5.0×10^{5}
	3	1.8×10^{8}	1.3×10^{8}	1.8×10^{8}	1.2×10^{8}	2.4×10^{8}	2.1×10^{8}	2.8×10^{8}	2.1×10^{8}
	Ave.	2.3×10^{8}	1.8×10^{8}	2.4×10^{8}	1.7×10^{8}	3.1×10^{8}	2.8×10^{8}	3.2×10^{8}	2.7×10^{8}
5 weeks	1	1.9×10^{8}	1.8×10^{8}	2.0×10^{8}	1.1×10^{8}	2.4×10^{8}	1.2×10^{8}	2.5×10^{8}	1.8×10^{8}
	2	8.0×10^{6}	6.9×10^{5}	1.5×10^{7}	7.8×10^{5}	8.0×10^{6}	5.3×10^{5}	1.3×10^{7}	7.0×10^{5}
	3	2.0×10^{8}	1.7×10^{8}	1.7×10^{8}	1.6×10^{8}	3.2×10^{8}	2.7×10^{8}	2.7×10^{8}	1.8×10^{8}
	Ave.	1.3×10^{8}	1.2×10^{8}	1.3×10^{8}	9.0×10^{7}	1.9×10^{8}	1.3×10^{8}	1.6×10^{8}	1.2×10^{8}
10 weeks	1	2.2×10^{7}	1.9×10^{7}	2.0×10^{7}	1.7×10^{7}	2.5×10^{7}	2.2×10^{7}	2.5×10^{7}	2.2×10^{7}
	2	8.2×10^{6}	2.0×10^{6}	1.7×10^{7}	1.2×10^{6}	1.2×10^{7}	4.2×10^{6}	1.3×10^{7}	3.9×10^{6}
	3	5.2×10^{7}	4.8×10^{7}	4.1×10^{7}	3.9×10^{7}	5.6×10^{7}	5.2×10^{7}	4.6×10^{7}	4.1×10^{7}
	Ave.	2.7×10^{7}	2.3×10^{7}	2.6×10^{7}	1.9×10^{7}	3.1×10^{7}	2.6×10^{7}	2.8×10^{7}	2.2×10^{7}

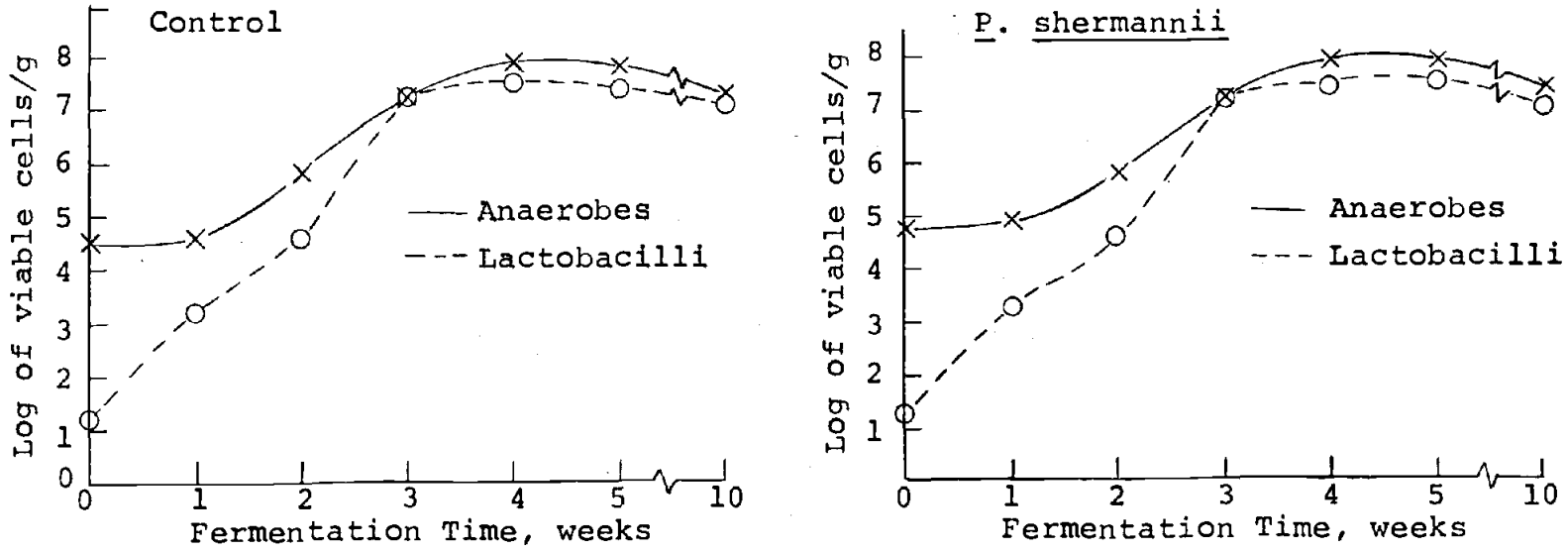

Figure 6. Total anaerobic microorganisms and lactobacilli per gram of control and kimchi inoculated with P. shermannii and fermented at 4°C.

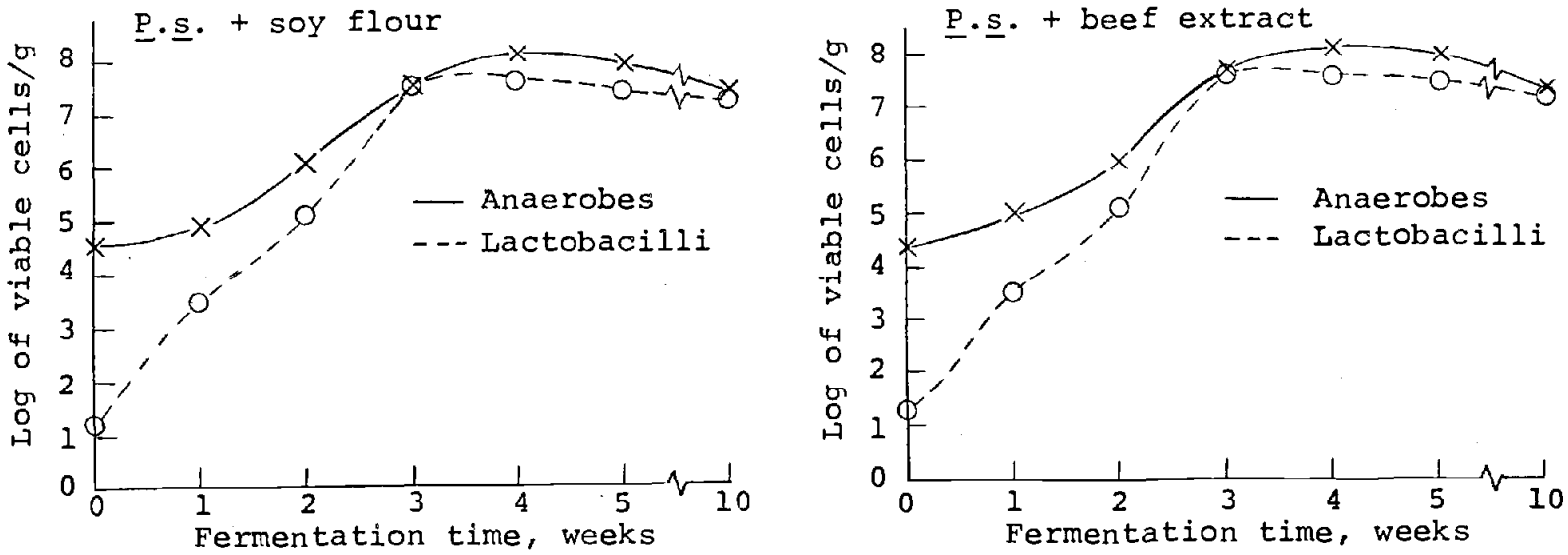

Figure 7. Total anaerobic microorganisms and lactobacilli per gram of kimchi inoculated with P. shermannii and added soy flour or beef extract, and fermented at 4°C.

bacteria from kimchi: Lactobacillus plantarum, L. brevis, Leuconostoc mesenteroides, and Pediococcus cerevisiae.

Propionibacteria were recovered from control kimchi and increased to a limited extent during the fermentation until 3 weeks (Table 11 and Figure 8). The number of propionibacteria present in the fresh control kimchi was 6.7 x 1 per g; at the 3 weeks of fermentation, it had increased to 5.0×10^2 per g. After this point, the propionibacteria decreased slightly over 10 weeks of fermentation. Propionibacterium shermannii, which was inoculated into kimchi, did not increase in numbers; however, it survived at the 5 weeks fermentation period. After 10 weeks, considerable numbers still remained. The survival pattern of the Propionibacterium was identical in all the inoculated samples. The average starting number in inoculated samples was 1.7×10^7 per g; the average number at 5 weeks, 7.4×10^5 per g; and the average number at 10 weeks, 5.1×10^4 per g. Since the Propionibacterium survived in the same pattern within all of the inoculated kimchi, the addition of soy flour or beef extract apparently aided the metabolic processes of the organism so that it produced more vitamin B_{12} rather than increasing the growth during the kimchi fermentation.

Identification of Propionibacteria

From the control kimchi, the following propionibacteria were identified: P. globosum, P. granulosum, P. avidum, P.

Table 11. Total counts of propionibacteria per gram of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

Fermentation Time	Replication	Propionibacteria			
		Control	Inoculated	Inoculated plus Soy Flour	Inoculated plus Beef Extract
0 day	1	3.0x1	$1.8x10^7$	$1.6x10^7$	$1.9x10^7$
	2	1.1x10	$1.7x10^7$	$1.8x10^7$	$1.9x10^7$
	3	6.0x1	$1.5x10^7$	$1.7x10^7$	$1.5x10^7$
	Ave.	6.7x1	$1.7x10^7$	$1.7x10^7$	$1.8x10^7$
1 week	1	$6.5x10^2$	$1.0x10^7$	$2.0x10^7$	$2.1x10^7$
	2	5.0x10	$1.3x10^7$	$2.0x10^7$	$2.2x10^7$
	3	1.2x10	$1.7x10^7$	$2.0x10^7$	$2.4x10^7$
	Ave.	$2.4x10^2$	$1.3x10^7$	$2.0x10^7$	$2.2x10^7$
2 weeks	1	$9.0x10^2$	$9.3x10^6$	$1.5x10^7$	$1.6x10^7$
	2	$6.6x10^2$	$7.9x10^6$	$1.1x10^7$	$1.2x10^7$
	3	1.0x10	$9.9x10^6$	$1.2x10^7$	$1.2x10^7$
	Ave.	$5.2x10^2$	$9.0x10^6$	$1.3x10^7$	$1.3x10^7$
3 weeks	1	$1.0x10^3$	$5.7x10^6$	$8.9x10^6$	$8.6x10^6$
	2	$4.7x10^2$	$3.7x10^6$	$7.5x10^6$	$7.9x10^6$
	3	1.9x10	3.5x106	8.2x106	8.3x106
	Ave.	$5.0x10^2$	$4.3x10^6$	$8.2x10^6$	$8.3x10^6$

Table 11 (continued)

Fermentation Time	Replication	Propionibacteria			
		Control	Inoculated	Inoculated plus Soy Flour	Inoculated plus Beef Extract
4 weeks	1	$9.9x10^2$	$2.6x10^6$	$4.7x10^6$	$4.2x10^6$
	2	$8.0x10$	$2.8x10^5$	$4.0x10^5$	$4.2x10^5$
	3	$1.0x10$	$2.3x10^6$	$2.6x10^6$	$3.2x10^6$
	Ave.	$3.6x10^2$	$1.7x10^6$	$2.6x10^6$	$2.6x10^6$
5 weeks	1	$5.6x10$	$4.9x10^5$	$9.8x10^5$	$1.3x10^6$
	2	$3.0x10$	$1.8x10^5$	$2.8x10^5$	$2.9x10^5$
	3	$2.0x10$	$6.4x10^5$	$1.1x10^6$	$1.4x10^6$
	Ave.	$3.5x10$	$4.4x10^5$	$7.9x10^5$	$1.0x10^6$
10 weeks	1	$2.0x1$	$1.9x10^4$	$2.8x10^4$	$2.6x10^4$
	2	$0 \quad x1$	$1.1x10^4$	$2.5x10^4$	$2.5x10^4$
	3	$1.3x10$	$4.2x10^4$	$6.3x10^4$	$2.2x10^5$
	Ave.	$5.0x1$	$2.4x10^4$	$3.9x10^4$	$9.0x10^4$

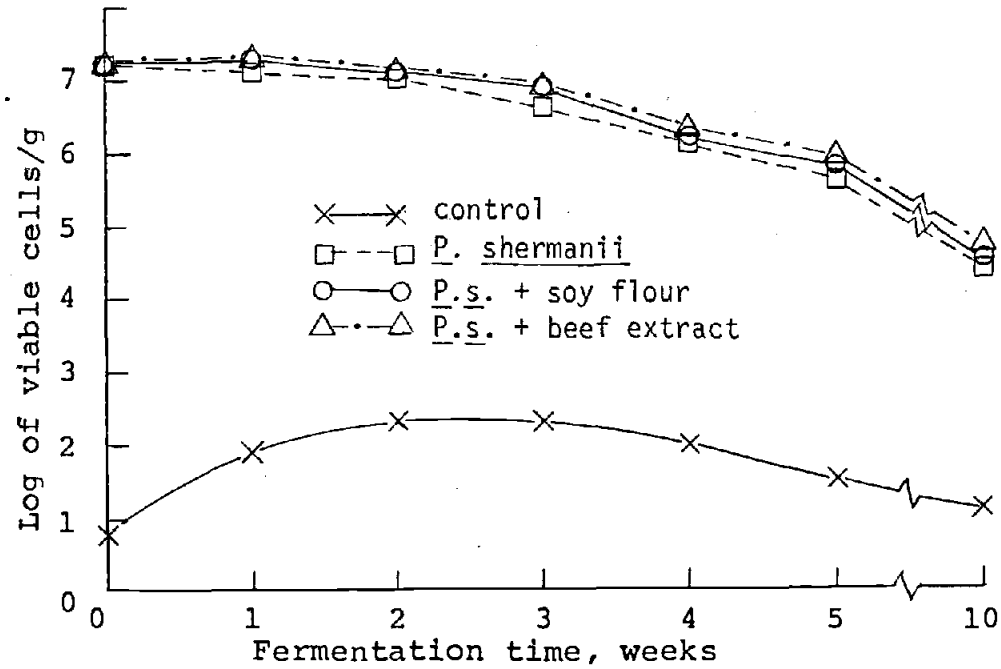

Figure 8. Total number of propionibacteria per gram of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

freudenreichii and P. acidopropionicia. Among the identified propionibacteria, only P. freudenreichii has been reported to be a vitamin B_{12}-producing organism. P. shermannii was not found in any of the control kimchi. Because of the predominance of P. shermannii in the inoculated kimchi samples, it was difficult to select typical colonies other than that from the sodium-lactate agar plates of those products.

pH and Total Acidity

The pH of all samples decreased during the fermentation; control and inoculated kimchi with or without soy flour or beef extract showed no significant difference in values (Table 12 and Figure 9). The average pH value of the fresh, unfermented kimchi was 6.7; and that of kimchi at 5 weeks was 4.2. At 3 weeks of fermentation, the pH averaged 4.8. This was the optimum maturation time of the fermentation. The total acidity of all samples increased as the pH decreased during the fermentation (Table 12 and Figure 9). Again, no significant difference was observed between control and treated samples. The average value for total acidity of the fresh, unfermented kimchi was 0.05% expressed as lactic acid; that at 5 weeks of fermentation, 0.56%. At the optimum point, 3 weeks of fermentation, the average value for total acidity was 0.35%. During the 5 to 10 weeks of fermentation, the pH dropped slightly, from 4.2

Table 12. The pH and total acidity (TA) of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

Fermentation Time	Replication	Control		Inoculated		Inoculated plus Soy Flour		Inoculated plus Beef Extract	
		pH	TA %	pH	TA %	pH	TA %	pH	TA %
0 day	1	7.0	0.02	6.9	0.03	6.8	0.05	7.1	0.03
	2	6.6	0.06	6.7	0.05	6.7	0.05	6.7	0.05
	3	6.6	0.06	6.4	0.08	6.5	0.07	6.5	0.06
	Ave.	6.7	0.05	6.7	0.05	6.7	0.06	6.8	0.05
1 week	1	5.0	0.15	6.1	0.14	5.9	0.16	6.0	0.15
	2	6.0	0.17	6.0	0.18	6.1	0.17	6.1	0.17
	3	6.2	0.20	6.2	0.19	6.1	0.21	6.4	0.13
	Ave.	6.1	0.17	6.1	0.17	6.0	0.18	6.2	0.15
2 weeks	1	5.9	0.20	5.9	0.20	5.8	0.23	5.8	0.22
	2	5.8	0.28	5.8	0.29	5.9	0.25	5.9	0.25
	3	5.4	0.32	5.5	0.27	5.6	0.23	5.8	0.19
	Ave.	5.7	0.27	5.7	0.25	5.8	0.24	5.8	0.22
3 weeks	1	5.1	0.31	5.1	0.30	4.8	0.36	4.8	0.35
	2	4.8	0.35	4.8	0.34	4.9	0.30	4.8	0.35
	3	4.6	0.38	4.5	0.38	4.7	0.31	4.6	0.40
	Ave.	4.8	0.35	4.8	0.34	4.8	0.32	4.7	0.37

Table 12 (continued)

Fermentation Time	Replication	Control		Inoculated		Inoculated plus Soy Flour		Inoculated plus Beef Extract	
		pH	TA %	pH	TA %	pH	TA %	pH	TA %
4 weeks	1	4.4	0.40	4.5	0.39	4.2	0.47	4.3	0.46
	2	4.5	0.40	4.5	0.41	4.4	0.45	4.3	0.47
	3	4.2	0.44	4.3	0.43	4.2	0.45	4.2	0.48
	Ave.	4.4	0.41	4.4	0.41	4.3	0.46	4.3	0.47
5 weeks	1	4.3	0.47	4.2	0.49	4.2	0.53	4.2	0.54
	2	4.2	0.60	4.2	0.58	4.3	0.54	4.3	0.50
	3	4.1	0.60	4.1	0.64	4.2	0.59	4.3	0.59
	Ave.	4.2	0.56	4.2	0.57	4.2	0.55	4.3	0.54
10 weeks	1	4.2	0.65	4.2	0.65	4.1	0.69	4.1	0.67
	2	4.2	0.68	4.2	0.67	4.3	0.61	4.2	0.64
	3	4.1	0.71	4.1	0.69	4.1	0.72	4.1	0.73
	Ave.	4.2	0.68	4.2	0.67	4.2	0.67	4.1	0.68

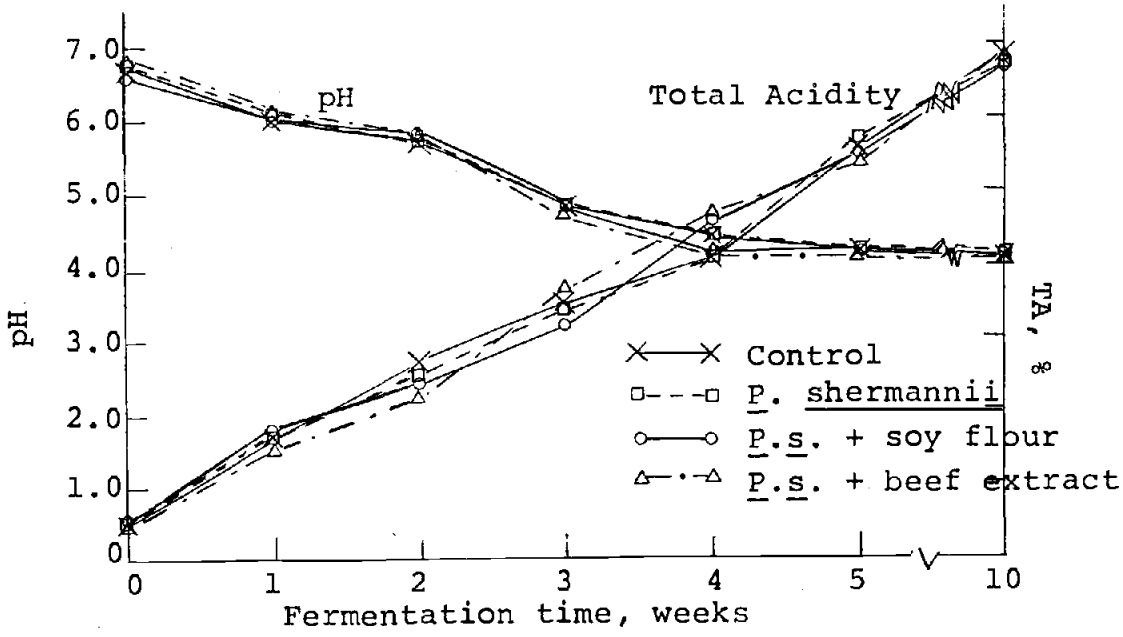

Figure 9. The pH and total acidity of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

to 4.1, but the total acidity increased from 0.56% to 0.68%.

Since there was no significant difference in the pH and total acidity between control and treated samples, these factors did not appear related to vitamin B_{12} production or destruction. Vitamin B_{12} should not be unstable at the pH of kimchi, since the pH was above 4.0 even after 10 weeks of fermentation.

Sensory Evaluation

Results of the triangle test showed no significant difference (5%) in flavor between the control and kimchi inoculated with P. shermannii and with added soy flour or beef extract (Table 13). Correct judgments from 26

Table 13. Results of sensory evaluation for control and inoculated kimchi with added soy flour or beef extract and fermented at 4°C.

	Fermentation time weeks	Total judgments	Numbers correctly identified
Reference[b] vs. P.s. plus soy flour	1	26	11[a]
	3	26	9[a]
Reference[b] vs. P.s. plus beef extract	1	26	2[a]
	3	26	9[a]

[a]Not significant at the 5% level.

[b]Reference was control kimchi which had not been inoculated with P. shermannii or had soy flour or beef extract.

assessments were not significantly different at the 5% level. The descriptive terms used by the panel members to identify differences were: salty, sour, tasty, crispy, or bitter.

Although the panel was too small to give an assessment of acceptability, they described all of the samples, including the control kimchi, as good kimchi.

Additional Studies, Preliminary

Preservation by Canning

To see if kimchi can be stored to preserve vitamin B_{12} at the maximum level, control kimchi and inoculated kimchi with added soy flour were canned after 1 week of fermentation. In the control kimchi, the vitamin B_{12} level was 54 ng/100 g just after canning, and decreased to 35 ng/100 g after 4 weeks storage at room temperature (Table 14). The vitamin activity of the fresh control at 1 week of fermentation at 4°C (59 ng/100 g) was markedly reduced after 4 more weeks of fermentation (to 15 ng/100 g). In the inoculated kimchi with added soy flour, the vitamin B_{12} level of 186 ng/100 g in fresh kimchi at 1 week of fermentation again sharply decreased to 92 ng/100 g by the end of 5 weeks of fermentation. The vitamin level in kimchi canned at 1 week (180 ng/100 g) decreased, but less sharply, to 129 ng/100 g after 4 weeks storage of the jars at room temperature. Although a slight amount of the vitamin activity was

Table 14. Vitamin B_{12} and ascorbic acid in fresh and canned kimchi.[a]

Sample	Control		Inoculated plus Soy Flour	
	Fresh	Canned	Fresh	Canned
Vitamin B_{12}	ng/100 g	ng/100 g	ng/100 g	ng/100 g
1 week:				
With salted shrimp	59	54	186	180
Without salted shrimp	17	16	82	81
5 weeks:				
With salted shrimp	15	35[b]	92	129[b]
Ascorbic acid	mg/100 g	mg/100 g	mg/100 g	mg/100 g
1 week:				
With salted shrimp	12.0	9.0	13.4	8.9
Without salted shrimp	14.4	8.8	14.3	9.3
5 weeks:				
With salted shrimp	8.7	7.7[b]	8.6	7.5[b]

[a]The data are averages of 2 replications.
[b]Canning was done at 1 week of fermentation and jars were stored at room temperature for 4 weeks to compare with unprocessed product fermented for 5 weeks.

destroyed by heat processing, the greater loss occurred during the storage of the jars at room temperature. The change in ascorbic acid content was also determined in canned kimchi. The ascorbic acid content was significantly decreased both in control and treated samples by the canning process (Table 14). Storage of canned products at room temperature for 4 weeks further decreased the ascorbic acid content: in the control, ascorbic acid decreased from 9.0 mg/100 g to 7.7 mg/100 g, and in treated kimchi, from 8.9 mg/100 g to 7.5 mg/100 g.

Assuming that most microorganisms and oxidases in kimchi were inactivated by canning, it is difficult to postulate the mechanism by which the vitamin B_{12} activity was reduced in the canned products.

Despite the disappearance, within one month, of free oxygen present at the time of sealing, a continuous loss of ascorbic acid in canned food throughout the storage life was reported by Feaster et al. (1949) and in canned orange juice by Nagy and Smoot (1977). Anaerobic destruction of ascorbic acid in citrate-phosphate buffer at pH 3-4 was observed by Huelin (1953). The destruction was accelerated by fructose, fructose-6-phosphate, and fructose-1,6-diphosphate, the effect increasing with lower pH.

Commercial Kimchi

Three brands of commercial kimchi (sold refrigerated in glass jars) were collected from markets in Corvallis and vitamin B_{12} and ascorbic acid determined (Table 15). As

Table 15. Vitamin B_{12} and ascorbic acid in commercial kimchi.

Brand	pH	Vitamin B_{12} ng/100 g	Ascorbic Acid mg/100 g
1	4.4	2	9.9
2	4.0	10	9.4
3	4.2	10	9.3

expected, pH was low, below that of the optimum fermentation point, and the level of vitamin B_{12} was also low. None of the commercial kimchi contained salted shrimp or any fish. Ascorbic acid levels were comparable to those of the samples with similar pH studied in this research.

Vegetarian Kimchi

Kimchi samples were made without salted shrimp and vitamin B_{12} and ascorbic acid determined (Table 14). Vitamin B_{12} levels in those samples were much lower than that of kimchi samples with salted shrimp, showing the effect of shrimp on the metabolic processes of P. shermannii and other microorganisms that produce vitamin B_{12}. Ascorbic acid content was not significantly different with or without salted shrimp.

Applications and Further Research

Since Korean people are familiar with soybean flavor, using it in kimchi fermentation would not be strange. Beef is sometimes added to kimchi to add flavor although it is not a usual practice. To provide a practical procedure for the inoculation of P. shermannii, cultures of the organism could be dried and packaged so that homemakers can easily buy and use them. For commercial use, a mixed culture preparation for kimchi fermentation has been on the market since 1977 and the Propionibacterium could also be mixed with it (Ro, personal communication).

It is recommended to test the propionibacteria identified from kimchi for the production of vitamin B_{12} during the fermentation. These cultures might be used for selected mutation or selection experiments. Exploration to stimulate the growth of inoculated P. shermannii also might be worthwhile for further increase in the vitamin production.

Although kimchi has been a long tradition in Korea, the scientific basic studies of nutritional significance have not been made extensively. Folic acid, for example, has not been studied in kimchi. Study of folic acid along with vitamin B_{12} would be highly recommended.

Preservation of kimchi at optimum fermentation has been long studied yet no desirable method has been developed. Research for the development of commercial products is also a continuous effort.

Fermented products of soybean and of fish also are consumed in Korea in considerable amounts throughout the year. Intensive studies of vitamin B_{12} in these products are needed.

SUMMARY

To increase the production of vitamin B_{12} during kimchi fermentation, Propionibacterium shermannii (ATCC 13673) was inoculated on the basis of 1×10^6 cells per g of product at the beginning of fermentation. The kimchi samples were fermented at 4°C; optimum fermentation was reached at the end of 3 weeks of fermentation with a pH of 4.5-4.8.

The levels of vitamin B_{12} were determined by a microbiological assay method with Lactobacillus leichmannii (ATCC 7830) as the test organism. The assay procedures followed the Official Method of Analysis of A.O.A.C., 1975, 12th edition. Preliminary studies showed that a cyanide-buffer solution of pH 6.0 extracted vitamin B_{12} satisfactorily in kimchi samples and avoided the inhibitory effect on the growth of the test organism shown by the standard buffer. This buffer replaced the metabisulfite-buffer specified in the Official Method. Pseudovitamin B_{12} and deoxyribosides which are not cobalamins but resemble them in structure and act as growth stimulating factors for the test organism were determined after hydrolysis at pH 12.0 for 30 minutes at 121°C. This figure was used to correct the total vitamin B_{12} activity.

Maximum levels of vitamin B_{12} in all samples were produced by 1 week of fermentation. Control kimchi samples which were not inoculated had a maximum vitamin B_{12} content

of 47 ng/100 g. Kimchi inoculated with the Propionibacterium had an increased vitamin level, 102 ng/100 g. The addition of soy flour (0.5%) or beef extract (0.05%) to the inoculated kimchi increased the vitamin production to 197 and 203 ng/100 g, respectively, more than four times that of the control kimchi. After 1 week, the vitamin activity continuously decreased to the end of 5 weeks of fermentation. No further decrease was observed by 10 weeks. After 5 weeks of fermentation, the vitamin level was 12 ng/100 g in the control kimchi, 40 ng/100 g in the inoculated kimchi, 88 ng/100 g in the inoculated kimchi with added soy flour, and 95 ng/100 g in the inoculated sample with added beef extract. Cooking of the kimchi for 13 min after 10 weeks of fermentation lowered the vitamin activity by an average of 16%.

Statistical analysis showed that the F values for the effects of fermentation time and inoculation with the Propionibacterium were significant at the 1% level. The addition of soy flour or beef extract also had a significant effect (1% level) but there was no significant difference between the two protein additives (5% level).

Vitamin B_{12} activity was retained better in the 1 week product by home canning of the kimchi than by continued fermentation. Canned control kimchi had 54% of the original B_{12} after 4 weeks storage of the canned product at room temperature; inoculated canned kimchi with added soy flour

had 72%. The fresh, unprocessed control kimchi exhibited 25% of the peak vitamin activity after 4 weeks of continuous fermentation and the inoculated kimchi with added soy flour, 49%.

Vitamin B_{12} levels in kimchi samples made without salted shrimp were much lower than that of kimchi with added salted shrimp.

Addition of cobalt (1 ppm as $Co(NO_3)_2 \cdot 6H_2O$) did not increase the vitamin B_{12} production during the kimchi fermentation.

The ascorbic acid content did not show any significant difference between the control and inoculated kimchi with or without soy flour or beef extract during kimchi fermentation. The fresh, unfermented kimchi contained an average of 16.8 mg/100 g ascorbic acid. The content of ascorbic acid decreased continuously during the fermentation and after 5 weeks of fermentation was 7.7 mg/100 g. After 5 weeks, no further decrease was noticed at 10 weeks. The correlation coefficient, r, for the ascorbic acid levels and the fermentation time to 5 weeks was significant at the 1% level and a regression equation, $Y = 1.207 \text{ mg} - 0.069 X$, was derived: Y as the ascorbic acid level in log mg/100 g and X as the fermentation time in weeks. A 12-33% destruction of ascorbic acid by the heat process during home canning was observed. A further loss of 15% of ascorbic acid was seen in the canned product stored at room temperature for 4 weeks.

The total number of anaerobic microorganisms and lactobacilli also were not significantly different between the control and inoculated kimchi with or without soy flour or beef extract. The fresh, unfermented kimchi had average counts of anaerobic microorganisms of 5.9×10^4 per g. After 4 weeks of fermentation, the numbers increased to 2.8×10^8 per g and after this point decreased slightly to 2.8×10^6 per g at 10 weeks. Lactobacilli were involved actively in the kimchi fermentation. At 3 weeks of fermentation, the total anaerobic microorganisms were almost all lactobacilli; the total numbers of anaerobic microorganisms (2.7×10^8 per g) were almost the same as the total numbers of lactobacilli (2.3×10^8 per g).

Propionibacteria were isolated from the control kimchi which had not been inoculated. They multiplied to a limited extent during the fermentation. In the fresh, unfermented control kimchi, the propionibacteria counts were 6.7×1 per g and increased to 5.0×10^2 per g after 3 weeks of fermentation. After this, they decreased in numbers continuously to the end of 10 weeks of fermentation. Propionibacterium shermannii did not increase in numbers after inoculation into kimchi. Survival of the inoculum was not significantly different during the fermentation of inoculated kimchi with or without added soy flour or beef extract. The inoculated number of 1.7×10^7 per g as recovered from the kimchi juice had not decreased in 1 week

(1.9×10^7 per g) but decreased slightly to 7.4×10^5 by 5 weeks. After 10 weeks, there were 5.1×10^4 per g. The addition of soy flour (0.5%) or beef extract (0.05%) did not increase the number of Propionibacterium; however, vitamin B_{12} production doubled. Thus the metabolic processes of the organism rather than multiplication appear to be responsible. P. shermannii was not found in the control kimchi but other Propionibacteria were identified: P. globosum, P. granulosum, P. avidum, P. freudenreichii and P. acidopropionicia.

Changes in pH and total acidity were not significantly different between the control and inoculated kimchi with or without soy flour or beef extract. The kimchi samples at the optimum fermentation period of 3 weeks had a pH of 4.5-4.8 and total acidity of 0.30-0.40% as lactic acid. After 10 weeks of fermentation, the pH (4.1-4.3) was still slightly higher than 4.0, the lower limit of vitamin B_{12} stability, and total acidity was 0.61-0.73%.

The 9 Korean panel members found no significant difference (at the 5% level) in flavor between the control and inoculated kimchi with added soy flour or beef extract by triangular tests.

BIBLIOGRAPHY

Afroz, M., B. Bhothinard, J.R. Etzkorn, S. Horenstein and J.D. McGarry, 1975. Vitamin C and B_{12}. Journal of American Medical Association 232:246.

Amerine, M.A., R.M. Pangborn and E.B. Roessler, 1965. Principles of Sensory Evaluation of Food. Food Science and Technology, A series of monographs, Academic Press, New York and London.

Antonelli, A., 1962. Vitamin B_{12} production by mycobacteria. Rend. 1st Super. Sanita., Rome, 25:417-422 (from Nutrition Abstract Review 33:No. 2460, 1963).

Bambha, P.P., P.A. Shankara, V.K.N. Nambudripad and H. Laxminarayana, 1973. Changes in vitamin B-complex content of milk during the production of dahi (fermented milk). Indian Journal of Animal Science 43: 210-215.

Banerjee, D.K. and J.B. Chatterjea, 1963. Vitamin B_{12} content of some articles of Indian diets and effect of cooking on it. British Journal of Nutrition 17:385-389.

Bartilucci, A. and N.E. Foss, 1954. Cyanocobalamin (vitamin B_{12}): A study of the stability of cyanocobalamin and ascorbic acid in liquid formulations. Journal of American Pharmaceutical Association 43:159-162.

Boxer, G.E. and J.C. Rickards, 1950. Chemical determination of vitamin B_{12}. 1. Determination of 5,6-dimethylbenzimidazole by colorimetric and fluorometric methods. Archives of Biochemistry and Biophysics 29:75-84.

Choe, C.E., S.K. Lee and Y.S. Chung, 1963. Studies on the vitamin B_{12} contents in fermented soybean inoculated with Bacillus megaterium. The Army Research and Testing Laboratory 2:22-23 (Seoul, Korea).

Coates, M.E., J.E. Ford, G.F. Harrison, S.K. Kon, and J.W.G. Porter, 1953. Vitamin B_{12}-like compounds. 1. Vitamin B_{12} activity for chicks and for different microorganisms of gut contents and faeces. British Journal of Nutrition 7:319-326.

Elliker, P.R., A.W. Anderson and G. Hannesson, 1956. An agar culture medium for lactic acid streptococci and lactobacilli. Journal of Dairy Science 39:1611-1621.

Ellis, B., V. Petrow and G.F. Snook, 1949a. The isolation of the crystalline anti-pernicious anemia factor from liver. Journal of Pharmacy and Pharmacology 1:60-61.

_____, 1949b. The chemistry of anti-pernicious anemia factor. Part 1. The liberation of phosphorus as phosphate from vitamin B_{12} by acid hydrolysis. Journal of Pharmacy and Pharmacology 1:287-291.

Fantes, K.H., D.M. Ireland and N. Green, 1950. A colorimetric assay method for vitamin B_{12}. Biochemical Journal 46:XXiV.

FAO-WHO Requirement of ascorbic acid, vitamin B_{12}, folate and iron, 1970. WHO Technical Report Series No. 452. WHO, Geneva, Switzerland.

Farquharson, J. and J.F. Adams, 1976. The forms of vitamin B_{12} in foods. British Journal of Nutrition 36:127-136.

Feaster, J.F., M.D. Tompkins and W.E. Pearce, 1949. Effect of storage on vitamins and quality in stored canned foods. Food Research 14:25-39.

Ford, J.E., 1953. The microbiological assay of vitamin B_{12}. The specificity of the requirement of Ochromonas malhamensis for cyanocobalamin. British Journal of Nutrition 7:299-306.

_____, 1957. Factors influencing the destruction by heat of vitamin B_{12} in milk. Journal of Dairy Research (London) 24:360-365.

Ford, J.E., E.S. Holdsworth, S.K. Kon and J.W.G. Porter, 1953. Occurrence of the various vitamin B_{12} active compounds. Nature 171(1):150-151.

Freedman, L., M. Blitz, D.B. Sabine and E. Eigen, 1960. Stable vitamin B_{12} solutions. U.S. Patent 2,939,821.

Gajcy, H., 1973. Biosynthesis of vitamin B_{12} by mesophilic and thermophilic strains of Bacillus megaterium. ACTA Microbiologica Polonica Ser. B. 5(22):No. 2-3, 119-127.

Gakenheimer, W.C. and B.A. Feller, 1949. A note on preliminary observation of the incompatibility of vitamin B_{12} and ascorbic acid. Journal of American Pharmaceutical Association 38:660.

Garibaldi, J.A., K. Ijichi, N.S. Snell and J.C. Lewis, 1953. Bacillus megaterium for biosynthesis of cobalamin. Industrial and Engineering Chemistry 45:838-846.

Goodwin, T.W., 1963. Vitamin B_{12} and related compounds (cobamides). In: The Biosynthesis of Vitamins and Related Compounds, p. 167-187, Academic Press, New York and London.

Gray, L.F. and L.J. Daniel, 1959. Studies on vitamin B_{12} in turnip greens. Journal of Nutrition 67:623-634.

Gregory, M.E., 1954. The microbiological assay of vitamin B12 in the milk of different animal species. British Journal of Nutrition 8:340-347.

Hall, H.H., R.G. Benedict, C.F. Wiesen, C.E. Smith and R.W. Jackson, 1953. Studies on vitamin B_{12} production with Streptomyces olivaceus. Applied Microbiology 1:124-129.

Hartley, F., P. Stross and R.E. Stuckey, 1950. Some pharmaceutical aspects of vitamin B_{12}. Journal of Pharmacy and Pharmacology 2:648-659.

Haw, K., K. H. Chang and J.Y. Yu, 1968. Nutrition report of Korea for 11th Pacific Science Congress. Korean Journal of Nutrition 1:191.

Hendlin, D. and M.L. Ruger, 1950. The effect of cobalt on the microbial synthesis of LLD-active substances. Science 3:541-542.

Herbert, V. and E. Jacob, 1974. Destruction of vitamin B_{12} by ascorbic acid. Journal of American Medical Association 230:241-242.

Hines, J.D., 1975. Ascorbic acid and vitamin B_{12} deficiency. Journal of American Medical Association 234:24.

Hodge, H. M., C.T. Hanson and R.J. Allgeies, 1952. Animal protein factor supplement produced by direct bacterial fermentation. Industrial and Engineering Chemistry 44:132-135.

Hodgkin, D., J. Pickworth, J. Robertson, K. Trueblood, R. Prosen and J. White, 1955. Structure of vitamin B_{12}. Nature 176:325-328.

Hoffmann, C.E., E.L.R. Stokstad, A.L. Franklin and T.H. Jukes, 1948. Response of Lactobacillus leichmannii 313 to the antipernicious anemia factor. Journal of Biological Chemistry 176:1465-1466.

Hoffmann, C.E., E.L.R. Stokstad, B.L. Hutchings, A.C. Dornbush, and T.H. Jukes, 1949. The microbiological assay of vitamin B12 with Lactobacillus leichmannii. Journal of Biological Chemistry 181:635-644.

Holt, I.G., 1977. The Shorter Bergey's Manual of Determinative Bacteriology. 8th edition, p. 233-247, The Williams and Wilkins Co., Baltimore, Maryland.

Huelin, F.E., 1953. Studies on the anaerobic decomposition of ascorbic acid. Food Research 18:633-639.

Hutchins, H.H., P.J. Cravioto and T.J. Macek, 1956. A comparison of the stability of cyanocobalamin and its analogs in ascorbate solution. J. Am. Pharm. Assoc. 45:806-808.

Hutner, S.H., L. Provasoli, E.L.R. Stokstad, C.E. Hoffmann, M. Belt, A.L. Franklin and T.H. Jukes, 1949. Assay of anti-pernicious anemia factor with Euglena. Proceedings Society for Experimental Biology and Medicine 70: 118-120.

Jather, V.S., A.B. Inamdar-Deshmukh, D.V. Rege and R.S. Satoskar, 1975. Vitamin B12 and vegetarianism in India. ACTA Haematol. (BASEL) 53(2):90-97. (from Abstr. in Biological Abstract 60:No. 3766, 1975).

Karlin, R., 1961a. Increase in vitamin B12 content of fermented milks, possibility of fortification by adding Propionibacterium shermannii. Ann. Nutr. Paris 15: 247-256. (from Dairy Science Abstract 24:No. 2369).

_____. 1961b. The fortification of kefir with vitamin B12 by the addition of Propionibacterium shermannii. Soc. Biol., Paris 155:1309-1313. (from Dairy Science Abstract 24:No. 1475).

Kavanagh, F., 1963. Vitamins--Vitamin B12 and Congeners. In: Analytical Microbiology p. 527-549. F. Kavanagh ed., Academic Press, New York and London.

Ke, S.Y., Y.S. Chung and K.H. Lee, 1963. Studies on fermented food product by Bacillus megaterium group 1. Nutrition evaluation of fermented soybean by Bacillus megaterium. Korean Journal of Microbiology 1:26-29.

Kim, H.S. and Y.S. Chung, 1962. Identification of the aerobic bacteria isolated from kimchi and laver. Journal of Korean Agricultural Chemical Society 3:19-24.

Kim, H.S., K.C. Whang and K.H. Lee, 1960. Vitamin B_{12} biosynthesis by Pseudomonas species isolated from kimchi and lavers. Bulletin of the Scientific Research Institute, Korea, 5:65-67.

Kliewer, M. and H.J. Evans, 1962. B_{12} coenzyme content of nodules from legumes, alder and of Rhizobium melioti. Nature 194:108-109.

Kon, S.K., 1955. Other factors related to vitamin B_{12}. In: The Biochemistry of Vitamin B_{12}. Biochemical Society Symposia 13:17-35.

Kruglova, L.A., 1963. Enriching dairy products with vitamin B_{12} by the use of propionic acid bacteria. Izv. Timirjazev. Sel'skohoz. Akad. 4:208-213. (from Nutrition Abstract Review 33:No. 2470).

Kwon, T.W., 1972. Kimchi. A paper presented at section 11. Traditional foods of international symposium on conversion and manufacture of foodstuffs by microorganisms, Dec. 5-9, 1971, Kyoto, Japan.

Lee, I.J., S.I. Kim and K. Haw, 1958. Biochemical studies on Korean fermented foods, IX. Variation of vitamin B_{12} during the kimchi fermentation period. Report of National Chemistry Laboratories, Korea, 7:18-21.

Lee, T.Y., J.S. Kim, D.H. Chung and H.S. Kim, 1960. Studies on the composition of kimchi, 2. Variations of vitamins during kimchi fermentation. Bulletin of Scientific Research Institute, Korea, 5:43-50.

Lee, K.Y., S. Bang, M.H. Kim and E.S. Lee, 1968. A study on the food intake and nutritional status of elementary school children and their family in urban and rural Korea. A research report, Yonsei University, Korea.

Lehninger, A.L., 1970. Oxidation of odd-carbon fatty acids. In: Biochemistry p. 428-432, Worth Publishers, Inc., New York.

Leviton, A., 1956a. Process for the microbiological synthesis of vitamin B_{12} active substances. U.S. Patent 2,753,289.

_____, 1956b. Process for the preparation of concentrates of vitamin B_{12} active substances. U.S. Patent 2,764,521.

Leviton, A. and R.E. Hargrove, 1952. Microbiological synthesis of vitamin B_{12} by propionic acid bacteria. Industrial and Engineering Chemistry 44:2651-2655.

Lichtenstein, H., A. Beloian and H. Reynolds, 1959. Comparative vitamin B_{12} assay of foods of animal origin by Lactobacillus leichmannii and Ochromonas malhamensis. Journal of Agricultural and Food Chemistry 7:771-774.

Lichtenstein, H., A. Beloian and E.W. Murphy, 1961. Vitamin B_{12}-Microbiological assay methods and distribution in selected foods. Home Economics Research Report No. 13, United States Department of Agriculture, Washington, D.C.

Liem, I.T.H., K.H. Steinkraus and T.C. Cronk, 1977. Production of vitamin B_{12} in tempeh, a fermented soybean food. Applied and Environmental Microbiology 34:773-776.

Lillie, R.J., H.R. Bird, J.R. Sizemore, W.L. Kellogg and C.A. Denton, 1954. Assay of feedstuffs and concentrates for vitamin B_{12} potency. Poultry Science 33: 686-691.

Loy, Jr., H.W., J.F. Haggerty and O.L. Kline, 1952. Stability of vitamin B_{12b}. Journal of Association of Official Agricultural Chemists 35:169-174.

Making Pickles and Relishes at Home, 1970. Home and Garden Bulletin No. 92, U.S. Department of Agriculture, Washington, D.C.

Maugh, 11, T.H., 1973. Vitamin B_{12}: After 25 years, the first synthesis. Science 179:266-267.

Mukherjee, S.L. and S.P. Sen, 1959. Stability of vitamin B_{12}: Part 11. Protection by an iron salt against destruction by aneurine and nicotinamide. Journal of Pharmacy and Pharmacology 11:26-31.

Nagy, S. and J.M. Smoot, 1977. Temperature and storage effects on percent retension and percent U.S. Recommended Dietary Allowance of vitamin C in canned single-strength orange juice. Agricultural and Food Chemistry 25:135-138.

Newmark, H.L., 1958. Stable vitamin B_{12}-containing solutions. U.S. Patent 2,823,167.

Newmark, H.L., J. Scheiner, M. Marcus and M. Prabhundesai, 1976. Stability of vitamin B_{12} in the presence of ascorbic acid. American Journal of Clinical Nutrition 29:645-649.

Official Methods of Analysis of the Association of Official Analytical Chemists, 12th ed., 1975, Washington, D.C.

Orr, M.L., 1969. Pantothenic Acid, Vitamin B_6, and Vitamin B_{12} in Foods. Home Economics Research Report No. 36, p. 3, United States Department of Agriculture, Washington, D.C.

Ott, W.H., E. L. Rickes and T.R. Wood, 1948. Activity of crystalline vitamin B_{12} for chick growth. Journal of Biological Chemistry 174:1047-1048.

Pagano, J.F. and G. Greenspan, 1954. U.S. Patent 2,695,864.

Perlman, D., 1959. Microbial synthesis of cobamides. In: Advances in Applied Microbiology 1:87-122, Academic Press, New York and London.

Rickes, E.L., N.G. Blink, F.R. Koniuszy, T.R. Wood and K. Folkers, 1948a. Crystalline B_{12}. Science 107:396-398.

_____, 1948b. Comparative data of vitamin B_{12} from liver and from a new source, Streptomyces griseus. Science 108:634.

Robbins, W.J., A. Hervey and M.E. Stebbins, 1950. Studies on Euglena and vitamin B_{12}. Science 112:455.

Roessler, E.B., J. Warren and J.F. Guymon, 1948. Significance in triangular taste tests. Food Research 13: 503-505.

Rogosa, M., J.A. Mitchell and R.F. Wiseman, 1951. A selective medium for the isolation and enumeration of oral and fecal lactobacilli. Journal of Bacteriology 62: 132-133.

Rosenblum, C. and D.T. Woodbury, 1952. The determination of the stability of vitamin B_{12} in multivitamin mixture by radioactive indicator method. Journal of American Pharmaceutical Association, Scientific ed. 41:368-371.

Rosenthal, H.L., 1968. Vitamin B_{12}-estimation in foods and food supplements. In: The Vitamins: Chemistry, Physiology, Pathology, Assay, 2nd ed., vol. 11:145-170. Sebrell, W.H., Jr. and Harris, R.S. ed., Academic Press, New York and London.

Shenoy, K.G. and G.B. Ramasarma, 1954. Extraction procedure and determination of the vitamin B_{12} content of some animal livers. Archives of Biochemistry and Biophysics 51:371-378.

_____, 1955. Iron as a stabilizer of vitamin B_{12} activity in liver extracts and nature of so-called alkali-stable factor. Archives of Biochemistry and Biophysics 55:293-295.

Shorb, M.S., 1947. Unidentified essential growth factors for Lactobacillus lactis found in refined liver extracts and in certain natural materials. Journal of Bacteriology 53:669.

Skeggs, H.R., 1963. Lactobacillus leichmannii assay for vitamin B_{12}. In: Analytical Microbiology, F. Kavanagh ed., Academic Press, New York and London.

Skeggs, H.R., J.W. Huff, L.D. Wright and D.K. Bosshardt, 1948. The use of Lactobacilli leichmannii in the microbiological assay of the "animal protein factor." Journal of Biological Chemistry 176:1459-1460.

Skinner, J., 1976. Effect of ascorbic acid supplementation on vitamin B_{12} in plasma of elderly women. M.S. Thesis, Oregon State University.

Smith, E.L., 1948. Purification of anti-pernicious anaemia factors from liver. Nature 161:638-639.

_____, 1965a. Chemistry of vitamin B_{12}. In: Vitamin B_{12}, Ch. 4, p. 30-53. E.L. Smith, ed., John Wiley and Sons, Inc., New York.

_____, 1965b. Assay of vitamin B_{12}, analogues, and coenzyme B_{12}. In: Vitamin B_{12}, Ch. 11, p. 103-114. E.L. Smith, ed., John Wiley and Sons, Inc., New York.

Smith, E.L., J.L. Martin, R.J. Gregory and W.H.C. Shaw, 1962. Standardization of hydroxocobalamin. Analyst 87:183-186.

Speedie, J.D. and G.W. Hull, 1960. Cobalamin-producing fermentation process. U.S. Patent 2,951,017.

Strohecker, R., and H.M. Henning, 1965. Vitamin B_{12}: Cyanocobalamin and aquocobalamin. In: Vitamin Assay-Tested Methods, p. 151-170, Verlag Cheme. GMBH. Weinheim/Bergstr.

Sudarsky, J.M. and R.A. Fisher, 1957. Improvement in production of vitamin B_{12} products by Propionibacterium freudenreichii. U.S. Patent 2,816,856.

Trenner, N., R. Buhs, F. Bacher and W. Gakenheimer, 1950. A note concerning the incompatibility of vitamin B_{12} and ascorbic acid. Journal of American Pharmaceutical Association 39:361.

Toraya, T., B. Yongsmith, A. Tanaka and S. Fukui, 1975. Vitamin B_{12} production by a methanol-utilizing bacterium. Applied Microbiology 30:477-479.

Ungley, C.C., 1955. The chemotherapeutic action of vitamin B_{12}. Vitamins and Hormones 13:137-211.

Vedamuthu E.R. and G.W. Reinbold, 1967. The use of candle oats jar incubation for the enumeration, characterization and taxonomic study of propionibacteria. Milchwissenschaft 22:428-431.

Volcani, B.E., J.I. Toohey and H.A. Barker, 1961. Detection of cobamide coenzymes in microorganisms by the ionophoretic bioautographic method. Archives of Biochemistry and Biophysics 92:381-391.

Wagner, A.F. and K. Folkers, 1964. Cyanocobalamin and the cobamide coenzymes. In: Vitamins and Coenzymes, Ch. X, p. 194-243, Interscience Publishers, John Wiley and Sons, New York.

Williams, W.L., A.V. Stiffey and T.H. Jukes, 1956. Microbiological and chick assay of vitamin B_{12} activity in feed supplements and other natural products. Journal of Agricultural and Food Chemistry 4:364-367.

Wokes, F., J. Badenoch and H.M. Sinclair, 1955. Human dietary deficiency of vitamin B_{12}. American Journal of Clinical Nutrition 3:375-382.

Wood, T.R., M. Hill and D. Hendlin, 1952. Production of vitamin B_{12}. U.S. Patent 2,595,499.

Wuest, H.M. and D. Perlman, 1968. Industrial preparation and production. In: The Vitamins: Chemistry, Physiology, Pathology, Assay. 2nd ed., Vol. 11:139-144, Sebrell, W.H., Jr. and R.S. Harris, ed., Academic Press, New York and London.

Yun, D.J., 1968. On physical standard of Korean children. Korean Journal of Nutrition 1:121.