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An Overview on Prevalence of Vitamin B_{12} Deficiency and Approaches for its Management

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Abstract: The prevalence of B_{12} deficiency is now common in most of the developing countries, especially in part of Southeast Asia and Europe. The deficiency of vitamin B_{12} leads haematological disorders and neuropsychiatric manifestations. The deficiency could be due to insufficient food intake, or malabsorption syndromes. With the emerging technologies and advancement in food manufacturing practices, various researches have been conducted in past to combat vitamin B_{12} deficiency among individuals. This review will discuss the prevalence of vitamin B_{12} insufficiency at extremely vulnerable ages, its causes and bioavailability. Additionally, numerous components of therapy procedures like supplementation, food fortification, fermentation and encapsulation are also highlighted with previous researches.

Keywords: Encapsulation, Food fermentation, Prevalence, Supplementation, Vitamin B_{12}.

I. Introduction

Vitamin B_{12}, a water-soluble vitamin, also known as cobalamine, due to the presence of metal cobalt. It acts as a cofactor for various anabolic and catabolic reactions, DNA synthesis, methionine formation, and methylation processes. Herbivores animals rely on microorganisms for Vitamin B_{12}, and fermented foods are considered to be the cheapest and easily available food sources.

Clinical deficiencies are rarely visible however; subclinical deficiency can be seen as delayed cognitive development, in children. Cobalamine deficiency can happen to any age group but more prevalent in women of reproductive age, infancy, and children or in the elderly, where the demands are higher than normal. Inadequate intake, malabsorption syndrome, or reduced bioavailability of cobalamine, may lead to early symptoms like pernicious anemia, altered motor and neurological behavior, and skin manifestations. The appearance of one or more symptoms in combination may suspect the deficiency; however, confirmation must be made via a hematological laboratory test for cobalamine. Increased levels of methylmalonic acids, and homocysteine, abnormally decreased levels of B_{12} in blood, or transcobalamin –bound B_{12}, can be used as diagnostic criteria for B_{12} deficiency [1].

Early detection of B_{12} deficiency can be helpful in mitigating its adverse effect on human health Vitamin B_{12} deficiency in infants can easily be determined by a mother's serum B_{12}. Multivitamin deficiency during pregnancy not only causes low serum cobalamin in infants but also leads to inadequate liver stores at birth. The breastfed infant may develop hematological or neurological symptoms within 3 weeks, even though the mother doesn't show any deficiency symptoms. Adenosylcobalamin and methylcobalamin both are used as co-factor in methylation reactions and impairs myelin formation. In India, 52%-74% prevalence of vitamin B_{12} deficiency was noted among pregnant and lactating women that lead to the possibility of infancy or childhood subclinical deficiency [2]. Cobalamine is produced by grass fermentation in herbivore animals and when carnivorous eat these animals, they become a great source of B_{12}. In athletes, the body requires B_{12} for erythropoiesis and maintains oxygen level during strenuous exercises. It also plays a potential role in enhancing the transmission of neurological signals, improved immune function, and creatinine formation. As sportsmen and coaches are always keen on better performance, they commonly take vitamin B_{12} injections in an unjustified manner. The deficiency of B_{12} increases with any gastrointestinal disorders or athlete who is on a vegan diet. Although, B_{12} is immensely popular among sports persons, however, it's actual role during and after performance is limited [3].

II. Prevalence

In India, as a significant number of school going children are malnourished, vegetarian diets make them more susceptible for B_{12} deficiency. Sporadic dinner design, utilization of profoundly prepared calorie-dense fast foods can be a main micronutrient lack in urban youths.

Chakraborty *et al.* [1] investigated the serum B_{12}, concentration in 2403, school going adolescents by anthropometric and biochemical assessment tool to measure the prevalence of serum

B_{12} deficiency in adolescents and found B_{12} deficiency in total 32.4% study population, while urban population demonstrates lower percentage (30.1%) in comparison to rural adolescents (43.9%). Furthermore, males having higher (34.4%) prevalence of deficiency than female (31.0%). In addition to this, 32.8% rural adolescents were not able to match their dietary B_{12} with RDA, in comparison to urban adolescents (9.5%) (Table I). B_{12}, acts as cofactor for methionine synthesis and low serum B_{12}, may leads to hyper-homocystenimia and cardio vascular disease. WHR and BMI both are inversely related to serum B_{12} levels and showed the obese and overweight are at higher risk of deficiency. A positive association was found between dietary intake of B_{12} with serum B_{12} concentration for both urban and rural population [1].

Table I: Vitamin B_{12} Status of Total Study Population

Total Sample	*Number of Adolescents (n = 2403)*	*Low Serum B_{12} (%)*	*Serum B_{12} > 148 pmol/L (%)*
Rural adolescents	403	43.9	56.1
Urban adolescents	2000	30.1	69.9
Total Male population	1024	34.4	65.6
Total female population	1379	31.0	69.0
Adolescents with Normal weight	1675	28.1	71.9
Overweight	560	39.8	60.2
Obese	168	51.2	48.8

Source: [1]

Another same study was conducted by Gupta *et al*. [4], as the low levels of serum B_{12} for long duration in school going children result in their poor academic performance, impaired cognitive development and delayed onset of puberty. High prevalence of B_{12} deficiency was reported in various studies in India in plain region so Gupta *et al*. [4] investigated vitamin B_{12} deficiency among children between the age of 6-18 years in three district of Himachal Pradesh, India with anthropometric and biochemical analysis. The results showed, out of 215 children between the age group 6-17 years with 107 males and 108 females, higher serum B_{12} was reported in age group 6-11 years, than 12-18 years. Non vegetarians (72%) showed higher mean (331.3$\pm$ 129.9) serum B_{12} than vegetarians (27%) serum B_{12} levels (318.8$\pm$ 80.5). Based on SES, lower income group, have relatively lower B_{12} levels than middle and higher income group, due to lack of affordability of animal foods (Table II). Food frequency questionnaire showed high consumption of animal foods by subjects. This study found only 7.4% prevalence of B_{12} deficiency in hilly region, and that can be correlated with high consumption of animal foods like fish, eggs, or chicken at least once a week. The author assumption for low prevalence of vitamin deficiency could also be either due to majority of population were non vegetarians or lower WHO cutoffs (serum B_{12} < 150 pmol/L), utilization for study. Author also establishes a strong association between dietary B_{12} intake from animal foods and serum B_{12} levels, probably the reason for low serum B_{12} in LIGs in high altitude region of India [4].

Table II: Vitamin B_{12} from the Population in Hilly Region

Total Sample Parameters	*Number of Children (n = 215)*	*Mean Serum Vitamin B_{12}*
Age 6-11 years	71	384.6
Age 12-18 years	144	326.9
Male	107	335.1
Female	108	359.3
Upper income group	0	Nil
Middle income group	97	336.1
Lower income group	47	310.7
Vegetarian	40	318.8
Non vegetarian	104	331.3

Source: [4]

Considering the vitamin B_{12} during antenatal and post natal period, Mittal *et al.* [2] investigated serum B_{12} levels among exclusively breast fedinfants between age 1-6 months and their lactating mother and assess their correlation between the two. The study was conducted on 100 exclusively breast fed infants and found 57% prevalence of B_{12} deficiency. Surprisingly, two infants showed the levels less than 50 pg/ml Fig. 1. Infants from non vegetarian mothers relatively showed lower prevalence

rate (52.1%) in comparison to vegetarian mother (68.9%). Out of 100 lactating mothers 46% were found deficient in B_{12} and all were asymptomatic. With statistical analysis, a positive correlation was seen between vitamin B_{12} level of mothers and infants less than 3 months [2].

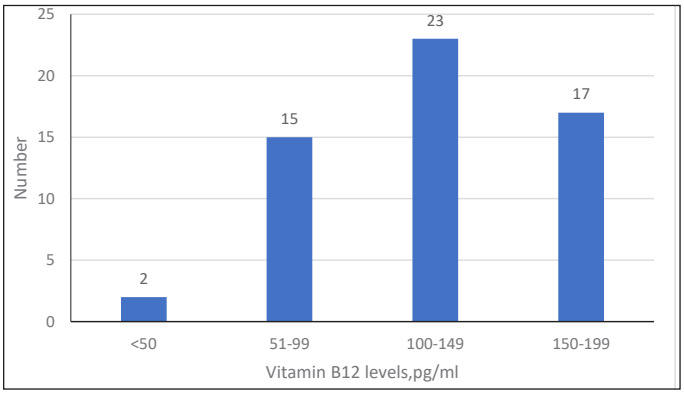


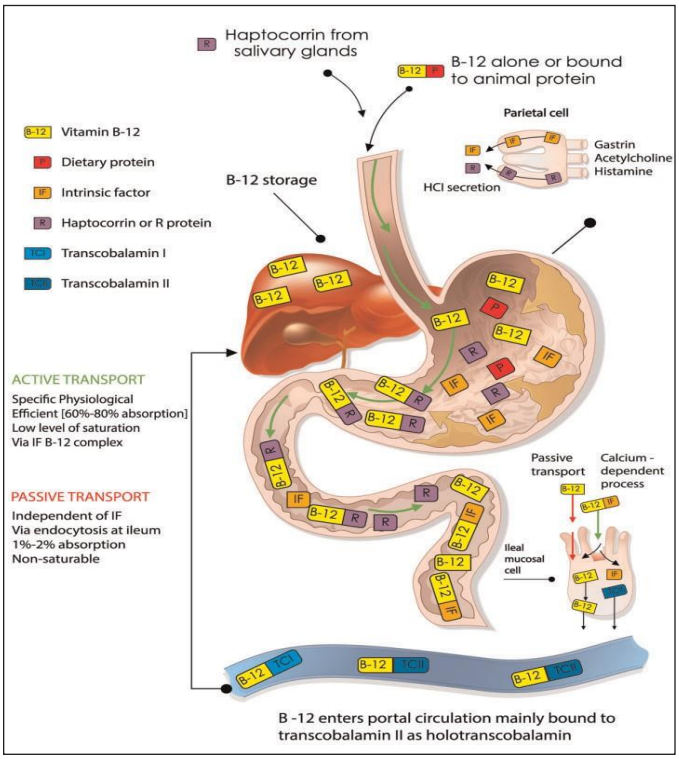
Fig. 1: Vitamin B_{12} Distribution in Deficient Infants

Another study from Warsaw, Poland by Krzywański *et al.* [3], on 1031 athlete sample, no one found to be deficient in B_{12}. An average B_{12} concentration was 739±13 pg/ml in the entire sample. Strength athletes showed below 300, 350 and 400 pg/ml B_{12} concentration while endurance athletes, the concentration of B_{12} was above 700 pg/ml. Those athletes, who were taking B_{12} injections, no difference were found in serum B_{12}. Furthermore, those athletes, who were taking injections, have found higher serum B_{12} levels. Due to the intake of sports supplements, no deficiency was found among athletes. However, a significant difference was found between endurance and strength class groups. Endurance athletes demonstrate higher B_{12} concentration than strength and that might be the result of high dose of B_{12} oral supplementation. A very weak positive correlation was established between B_{12} concentration in sports person and their haemoglobin and HCT levels. To conclude, it's never mistaken to say, athletes should maintain their B_{12} between 400-700 pg/ml and special attention must be given to vegetarian, vegan or sports person who have low B_{12} concentrations (200-400 pg/ml). Those athletes who possess insufficient B_{12}, the supplementation must be considered keeping in mind its effects on RBCs [3].

III. Bioavailability of Vitamin B_{12} in Humans

Vitamin B_{12} plays an essential role in neurological and RBCs formation. It actively participates in the formation of methionine from homocysteine, synthesis of creatinine, proteins, lipids, DNA, RNA, and neurotransmitters. Thus B_{12} is involved in various metabolic reactions, neurological reactions, and overall health like energy production and utilization, and prevention of congenital disorders. Biochemical test for B_{12} deficiency is characterized by low concentration serum B_{12} and holotranscobalamine in addition to increased total homocysteine and methylmalonic acid in urine and serum. The adults Recommended dietary allowances for vitamin B_{12} is 2.4 mcg/day and Eggs, lean meat, fish are considered to be rich sources of B_{12}. Normally, the bioavailabilities of vitamins are 50% in the human body. For vitamin B_{12}, the studies found 37%-25% from eggs, and 84%-54% from lean meat. The presence of protein in a meal increases the absorption of B_{12} and thus bioavailability by inducing gastrin secretion and Intrinsic Factor by stomach cells.

Prolonged and/or recurrent *Helicobacter pylori* infection, genetic disorders, or bacterial infection may alter intrinsic factors production and affect B_{12} absorption. Furthermore, any gastrointestinal irritability due to malabsorption syndromes like tropical sprue, gluten insensitivity, totals, or partial gastrectomy also adversely affects B_{12} absorption. Vitamin B_{12} absorption takes place in the distal ileum via active transport. B_{12} is bound with hepatocorrin released from salivary glands, in the stomach and makes a covering that protects it from acid degradation. Once it reaches the duodenum, the protein hepatocorrin partially degraded by the effect of pancreatic trypsin, and B_{12} get attached with IF, which is resistant to proteolysis. It enters then to distal ileum mucosal cells, by cubam-receptor-mediated endocytosis. After absorption of vitamin B_{12}, IF gets degraded in lysosomes and releases B_{12} free. Free B_{12} binds with transcobalamin II and transported in serum as HoloTC. It is then taken up by nearby tissues and then degraded in lysosomes, leaving it for converting as cofactor forms (Fig. 2) [5].



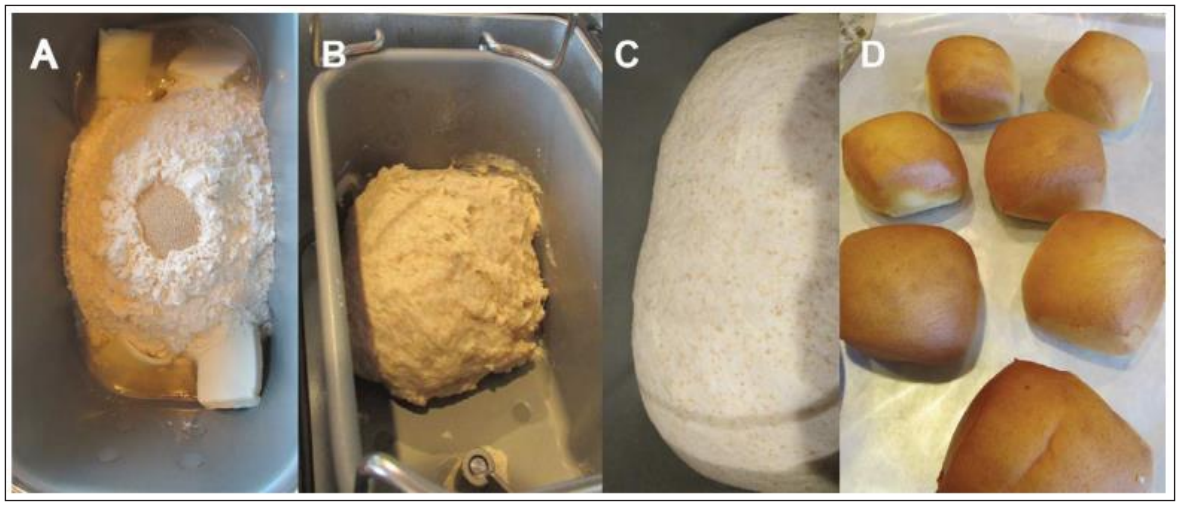
Source: [5]

Fig. 2: Process of B_{12} Absorption in Distal Ileum

Various researches have been conducted in past to assess the bioavailability of B_{12} in body. Professor Robert F. Schilling developed a multi-stage method, called Schilling's Test. It was used to detect B_{12} absorption and its efficiency, and diagnose malabsorption. In the first stage, the author used a radiolabel B_{12} usually ^{57}Co or ^{58}Co, followed by 1mg intramuscular unlabeled B_{12} dose, just after an hour, in the aim of to saturate blood transport of B_{12} and to ensure none of the radioactive B_{12} binds with any B_{12} depleted tissues. After 24 hours, urine collection was done to monitor absorption and excretion. If <10% oral dose was appeared in urine, it showed the malabsorption and stage 2 has been followed. Where, the author, suggest to administer B_{12} dose in combination with IF to diagnose pernicious anaemia. The result of the study showed, Normal cobalamin levels in urine sample were found upon administration of radiolabeled B_{12} in normal individuals. In case of absorption defect, the urine levels were found to be abnormal. The normal radioactive B_{12}, is 8-40% in 24 hours. In case of abnormal urine cobalamine, and on administration of intrinsic factor, urine cobalamin is improved that leads to deficiency of IF. And if Urine cobalmine is still abnormal, represents defective B_{12} absorption in distal ileum. Shilling test gives a quantitative estimate of B_{12} absorption. However due to high cost and exposure of radioactive substances, it is restricted in various countries [5].

Another method for assessing bioavailability of B_{12} was used by Garrod *et al.* [6]. In his study they used absorption test from crystalline fortified egg yolk. ^{14}C $B_{12,}$ was prepared from *Salmonella* aerobically and injected intramuscularly into hens to produce vitamin B_{12} enriched egg. The enriched eggs were cooked well and ingested by 10 human subjects. Eggs usually provide 1.4 to 2.6 mcg B_{12}. 24 hours post ingested and baseline urine, stool and blood sample were collected and analysed for B_{12} contents using AMC (Accelerator mass spectrometry) technique. The results showed the bioavailability of ^{14}C B_{12} was found between 13.2% to 57.7% with mean 30.2%. The total B_{12} retention and absorption are similar in most of the subjects. The limitation of the study was, the bioavailability was calculated from ^{14}C B_{12} on a very low dose (1.11 KBql) in comparison to previous studies. In addition to this, ^{57}Co, emits gama radiation which are more harmful than low energy beta radiation emitted by ^{14}C. Bioavailability from egg is found to be similar as in previous research [6].

In another study, Garrod *et al.* [7] prepared ^{14}C B_{12} fortified flour to assess B_{12} bioavailability after baking it into bread. ^{14}C B_{12} was extracted from *Salmonella enterica*, by incorporating ^{14}C-[C2] dimethylbenzimidazole (DMB) to get high activity ^{14}C B_{12}. ^{14}C B_{12} was incorporated at 2 mcg/100 gm flour and each prepared bread provides approximately total of 0.8 mcg B_{12} (Fig. 3). The aim of the study was to assess the vitamin B_{12} bioavailability from crystalline fortified B_{12} flour in elderly subjects. The results showed increased ^{14}C levels in plasma at 4 hours and it was at its peak after 7-9 hours of ingestion. 72 hrs after dose ingestion it reaches to equilibrium. In the first 48 hours of urine sample, ^{14}C label was appeared. However, it varied among the participants (mean 20.1%) (Table III). The researcher clearly illustrate that when B_{12} is added as fortificant in wheat flour, it retails its bioavailability by 50%. The RDA of B_{12} for an adult is 2.4 µg/day with assumption of 60% bioavailability of low dose vitamin, in crystalline form, and 50% from meat, fish and eggs. Furthermore, B_{12} fortification in the amount of 2 mcg/100 gm flour also supports the absorption same as meal and fish [7].



Source: [6]

Fig. 3: (A) ^{14}C B_{12} First Dissolved in Water, (B) Flour is Added along with Sugar, Salt and Yeast, (C) The Dough was Rose, (D) Baked in Bread Machine and Cut into Pieces

Table III: Assessment of Bioavailability in Different Elderly Subjects

Age	*Serum B_{12} pmol/L*	*Pepsinogen I (ng/mL)*	*nCi Dose*	*B-12 Dose (µg)*	*Total nCi Excreted (Urine/ Stool)*	*% Bioavailability*
62	302	76	31.03	0.79	16.47	46.9
61	251	112	31.48	0.80	14.92	52.6
69	263	149	31.70	0.81	11.50	63.7
66	592	109	31.59	0.81	22.62	28.4
78	475	90	31.64	0.81	16.02	49.4

Source: [6]

Greibe *et al.* [7] used cobasorb test for evaluation of B_{12} bioavailability. Cynocobalaminis a synthetic vitamin which is used in pills, while HO-Cbl (hydroxycobalamin) or coenzyme methylcobalamine or 5'hydroxycobalamin form of B_{12} is found in food. Colasorbtest involve administration of different dosage of cynocobalamine and hydroxycobalamin, and check the change in serum holotranscobalamin (holoTC). Here the researcher has taken 2 groups, group A with decreased cobolamin and Group B with normal serum cobalaminlevels. In this study, the dose of cobalamin administration was estimated for highest increase in holoTC. In addition to this, comparative study was conducted on holoTC by administration of both cynocobalamin (CN-Cbl) and hydroxycobalamin (HO-Cbl) and the difference between the holoTC spike among low and normal levels cobalamin. No significant difference was found in holoTC values with 1.5 µg and 3 µg CN-Cbl dosage. Likewise, no significant mean difference in holoTC was found on 1.5 mcg and 3 mcg HO-Cbl dosages. For 6 mcg HO-Cbl dosage, the holoTC is increased. For group B, the highest mean holoTC (32 pmol/L) was recorded in 6 mcg CN-Cbl administered subjects. The mean increase in holoTC was 2.8 times higher in 9 mcg CN-Cbl administered subjects in comparison to 9 mcg HO-Cbl administered subjects. In addition to this, while comparing colosorb test for group A and group B, no significant difference was observed between the same dose of CN-Cbl. A 2-3 times higher holoTC was reported in subjects with CN-Cbl administration in comparison to HO-Cbl. With the help of previous studies, it can be concluded that higher and efficient uptake of HO-Cbl, by nearby tissues than CN-Cbl. That leads to higher serum levels of holoTC [7].

IV. Vitamin B_{12} Supplementation

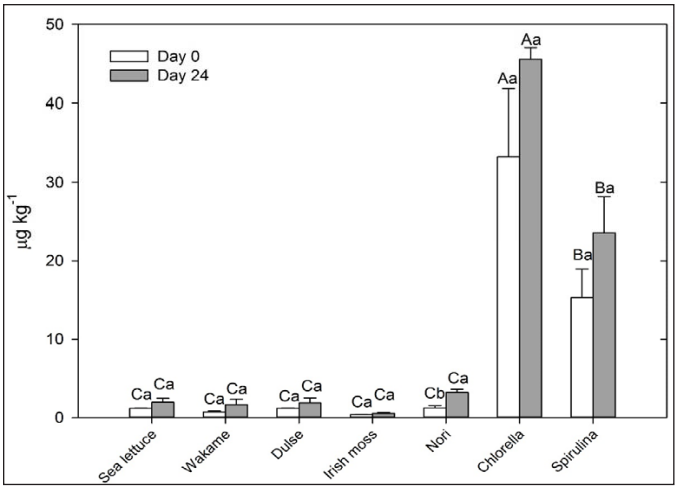
High prevalence of B_{12} deficiency in low socioeconomic group and in elderly makes the vitamin highly essential to be consumed through diet. Several methods have been used so far like enrichment, supplementation and fortification to overcome these micronutrient deficiencies. Formulation of a low cost nutrient dense food is often used as sound approach to reach a large community.

Castillejo *et al.* [8] used fresh grapes and cucumber to prepare vitamin B_{12} and fucose supplemented smoothie. 9 edible algae, kombu, dulse, thongweed, Irish moss, wakame, spirulina, nori, chlorella, and sea lettuce were used for enrichment. 300 µm particle size grounded algae powder was used for smoothie preparation. Cucumber, grapes along with plant material sanitized with 75 mg/L NaClO for 2 min. and rinsed after rinsing with cold water. Using different algae, 9 different smoothies were prepared. The proportion of each vegetable and algae are given in Table IV. 80 gm of each smoothie was kept in a sterile squeeze polyvinyl chloride pouch for analysis on 0, 7, 14, 21 and 24 days. B_{12} was measured using the microbial kit, VitaFast, r–Biopharm; Berlin, Germany. The study aimed to determine bioactive components and quality check on algae supplemented smoothie. The results showed, initial B_{12} content in chlorella and spirulina smoothies were high (33.3 µg/kg and 15.3 µg/kg respectively) in comparison to other smoothies, which showed approximately the same i.e. 1 µg/kg fw, while Irish moss showed a little less i.e. 0.41 µg/kg fw. No changes in B_{12} content were reported throughout storage [9]. 70 gm and 160 gm of chlorella and spirulina smoothies would cover the recommended daily intake of vitamin B_{12}. In nori and chlorella supplemented smoothies, 60% B_{12} is comprised of in coenzyme form. The B_{12} content in the 250 gm portion of chlorella and spirulina smoothies was increased to 475 and 245% after storage for 24 days at 50C (Fig. 4). Fruits and vegetable supplementation with natural algae can be considered as a natural tool for B_{12} fortification. The highest B_{12} was observed in chlorella and spirulina smoothie. Despite the highest rise in B_{12} after storage, chlorella smoothie scored low in sensory quality and highest vitamin C degradation, in comparison to spirulina [8].

Table IV: Percentage of Each Ingredient in Smoothie

Vegetables	*Smoothie with Algae*	*Smoothie with No Algae (Control)*
White seedless grapes	56.5%	57.8%
Broccoli	15.5%	15.8%
Cucumber	25.8%	26.4%
Algae	2.2%	-

Source: [8]



Source: [8]

Fig. 4: B_{12} Content in Smoothies with Different Algae during the Day of Processing and After Day 24

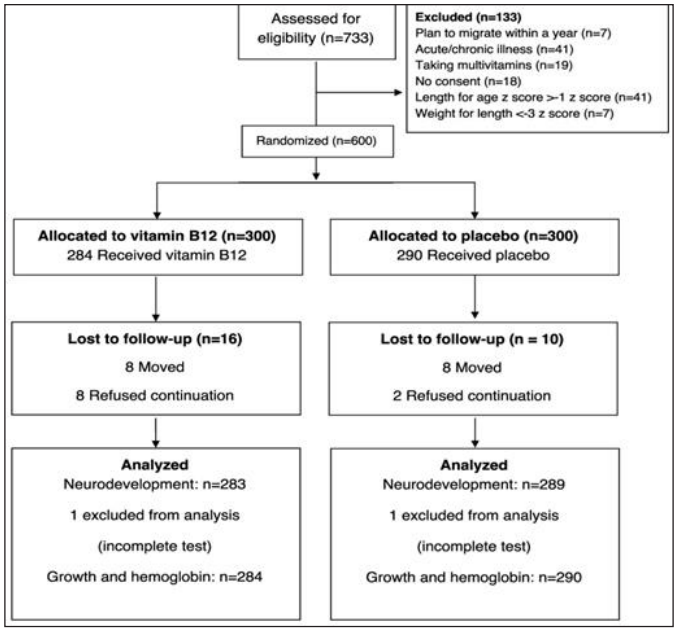
To combat B_{12} deficiency and to improve the cognitive development of toddlers, and to see the effect of meat, fortified foods, and local cereal on B_{12} and cognitive development in high poverty region, Sheng *et al.* [10], used a cluster randomized control trial study on 60 villages. These villages belong to the extremely low-income group. All villages were divided into 60 clusters randomly. 60 clusters were then divided into 3 groups with 20 clusters in each group. Infants at age 6 months, from every cluster, were randomized and given 50 gm/day pork called meat group (MG), another group received equivalent caloric B_{12}, Iron and Zinc fortified cereal supplement called fortified cereal group (FG), and one control group, received equi-caloric local cereals including rice flour, sugar and honey without B_{12}. The study was conducted for 1 year. All anthropometric assessment was done at age of 6 month and 18 months. Serum B_{12}, total homocysteine (tHcy) levels were tested at 18 months. Neurocognitive testing was done by Bayley Scales of Infant Development, 3rd Edition (BSID III). Out of 180 blood samples, (60 from each group) the mean infant's birth weight was 3075.7±380.2, 3039.6±362.1 and 3005.7±373.4 g in the non-vegetarian group, B_{12} fortified cereal group, and local non fortified cereal group respectively. Maternal education has been recorded at a median of 9 years. Since the significant difference was not found in birth weight and maternal education, among 3 groups, Weight for age Z score (WAZ), Length for age Z score (LAZ), Weight for length Z score (WLZ) and head circumference for Age (HcAZ) was almost similar at 18 months in all three groups. At age of 18 months, the median for B_{12} was 360.0 pg/ml. 62 (34.4%) children were found deficient (< 300 pg/ml). Out of 62 (34.4%) deficient children, 22 (36.7%) were from the non-vegetarian group, 3 (5%) from the fortified cereal group, and the highest 37 (62.8%) from the local nonfortified cereal group. The highest mean B_{12} (509.5 pg/ml) was shown by the fortified cereal group followed by (338.0 pg/ml) in the meat group and 241.0 pg/ml in the local cereal group. The median (IQR) for serum homocysteine was 8.2 µmol/L (6.9-10.2) in all toddlers. 21 children were found with homocysteine > 12 µmol/L while 14 toddlers found > 14 µmol/L homocysteine with B_{12} < 300 pg/ml (Table V). A significant difference in cognitive score was established among the 3 groups. The local cereal group showed a lower cognitive score in comparison to the meat group and fortified cereal group. Multiple linear regression analysis showed a positive correlation between serum B_{12} and cognitive and fine motor development. Animal foods are a good source of B_{12}. For vegetarian individuals, fortified foods should be encouraged to combat B_{12}. Fortified food and meat can also improve cognitive development [10].

Table V: Anthropometric and Biochemical Parameters of Subjects at 18 Months Age

Markers	*Total Subjects (n = 180)*	*Non Vegetarian Group (n = 60)*	*Fortified Group (n = 60)*	*Non Fortified Cereal Group (n = 60)*
Age in Months	17.9	17.9	18.0	17.9
Length for age Z score (LAZ)	−1.6	−1.5	−1.8	− 1.6
Weight for age Z score (WAZ)	−0.9	−0.9	−1.0	−0.8
Weight for length Z score (WLZ)	−0.2	−0.2	−0.2	−0.1
Head circumference for Age (HcAZ)	−0.3	−0.4	−0.3	−0.3
VitB_{12} (pg/mL)	360.0	338.0	509.5	241.0

Source: [10]

Strand *et al.* [11] opted for 600 children between the age group 6-11 months, intending to assess the impact of B_{12} supplementation on neurocognitive development, hemoglobin concentration, and on the growth of 6 months infants for one year The whole sample was divided into 2 subgroups. One group was given 2 mcg of Vitamin B_{12} daily and another group was kept as a placebo. Extra 15 vitamins and minerals were given as per RDA for both groups (Fig. 5). Neurodevelopment was measured by Barley Scales of Infant and Toddler Development 3rd Ed. (Barley-III). Weekly visits were carried out to ensure supplement intake.



Source: [11]

Fig. 5: Flowchart for Subject's Selection and Intervention

After 12 months of B_{12} supplementation, metabolic response, homocysteine, and MMA (methylmalonic acid) were measured. Out of 600 enrolments, 16 (2.7% from) B_{12} group and 10 (1.7%) children from the placebo group have lost the follow-up. Almost 94% sample had followed the routine and no significant effect of B_{12} intervention was found on mean growth and haemoglobin concentration among the two groups **(**Table VI). 12.5 cm mean growth was observed in both groups. 0.02 mean difference was found in haemoglobin in both groups. No effect on Neurocognitive development was observed after B_{12} supplementation; however, it improved the metabolic response and improves B_{12} status in toddlers [11] .

Table VI: Effect of B_{12} Supplementation on Cognitive, Motor Development and on Growth and Haemoglobin After 1 Year

Bayley-III Subscales	*Vitamin B_{12} Group (n = 283) Mean ± SD*	*Placebo Group (n = 289) Mean ± SD*
Change in Raw Scores from Baseline Until End Study Cognitive	20.9 ± 4.3	20.7 ± 4.2
Language		
Expressive	14.5 ± 4.7	14.6 ± 5.0
Receptive	10.8 ± 4.0	11.4 ± 3.6
Motor		
Fine motor	14.00 ± 3.3	13.9 ± 2.9
Gross motor	21.5 ± 5.1	21.8 ± 4.7
Socio-emotional	29.6 ± 14.3	31.7 ± 12.0
Change from Baseline		
Length (cm)	12.5 ± 1.8	12.5 ± 1.8
Weight (kg)	2.1 ± 0.5	2.1 ± 0.6
Hemoglobin (g/dL)	1.0 ± 1.1	1.0 ± 1.2

Source: [11]

V. Microencapsulation: Improved Strategy for Vitamin B_{12} Supplementation

Food Fortification becomes necessary to prevent micronutrient deficiency. Several staple food items like yogurt, wheat flour, rice, salt have been used so far for fortification with essential nutrients. Although to prevent micronutrient deficiency, food fortification is low cost and straightforward method, but it does not protect damage of bioactive compounds under adverse conditions. In addition to this, sometimes the compounds added during process leaves unfavourable sensory changes in end food product. Vitamin B_{12}, also known as cynocobalamin, is water soluble vitamin and easily degradable when exposed to oxidizing agents, light, or acid medium. Prolonged heating can also destroy Vitamin B_{12}. As vitamin B_{12} can only be produced by microorganisms, the deficiency of this vitamin among vegan and vegetarian are highly prevalent. To overcome this problem, encapsulation technique has been adopted. Microencapsulation involves the entrapment of bioactive compounds under carrier agents and form microcapsules or microparticles. The ideal size of microparticles must be 5-300 micron. Microencapsulation provides better stability to bioactive compounds. Furthermore, it prevents degradation from light, heat does not leave odour or unfavourable changes after fortification [12].

The instability of B_{12} towards acids, oxidizing agents, light and heat makes the necessity of microencapsulation. As the microencapsulation of B_{12} was not studied yet and it can be a low cost and stable alternative for food fortification to combat B_{12} deficiency, Mazzocato *et al*. [12] formulated B_{12} containing micro particles by spray chilling method and studied its properties under different conditions. For encapsulation, the researcher used vitamin B_{12}, soy lecithin, and vegetable fat (melting point 48 °C) and produced solid lipid microcapsules (SLM). Total VI formulation of SLM was produced using different amount of B_{12}, soy lecithin and vegetable fat (premelted at

65 °C) (Table VII). Physicochemical and morphological analysis was done using various methods. The yield of atomization was found between 70-80%. Encapsulation yield and efficiency was obtained by Kirchner *et al.,* 2012 method, using HPLC equipped with 361 nm diode array detector. Approximate 76% vitamin B_{12} encapsulation yield was observed and 100% efficiency was obtained in encapsulation. No significant difference was found among 6 formulations in respect to soy lecithin concentration [12].

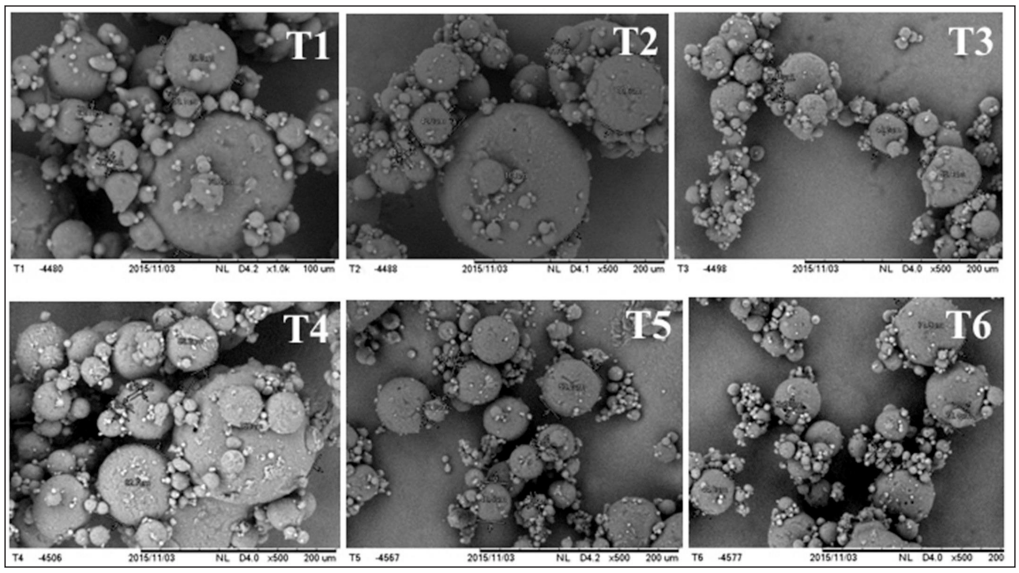
Table VII: Composition of Each SLM

	Formation of Different SLM		
	Vitamin B_{12} (%)	*Soy Lecithin (%)*	*Vegetable Fat (%)*
T1	0.10	0.00	99.90
T2	0.10	2.50	97.40
T3	0.10	5.00	94.90
T4	1.00	0.00	99.00
T5	1.00	2.50	96.50
T6	1.00	5.00	94.00

Morphology was studied by Scanning electron microscopy. Spherical shaped, microparticles were observed with heterogeneous size, defined surface, and undefined core (Fig. 6). The particle agglomeration was less in T_1 and T_4 formula than others as both have 0% soy lecithin, (refer Table I) soy lecithin acts as binder with lipid crystal. On contrary, agglomeration of particles was highest in T_3 and T_6 formulas due to highest (5%) soy lecithin concentration. The surface of particles was found smooth and continuous. This feature makes it more acceptable to use as fortificant in peanut butter, chocolates, and yogurt. SLM particle size plays as important role to maintain food texture and prevent rejection by consumers. SLM particles size was measured by laser diffraction. The medium diameter was found around 15 µm. The ideal microparticle size must be < 100 µm, which is an advancement of spray chilling (Table VIII) [12].

Table VIII: Physicochemical and Morphological Analysis Results of Encapsulated B_{12}

SLM Features	*Results*
Morphology	Heterogeneous, defined surface, smooth and continuous
Atomization yield	70%-80%
Encapsulation yield	76%
Encapsulation efficiency	100%
SLM particle size	15 µm



Source: [12]

T1 – Formula 1 with 0.1% vitamin-B_{12}, 0% soy lecithin and 99.9% vegetable fat;
T2 – Formula 2 with 0.1% vitamin-B_{12}, 2.5% soy lecithin and 97.4% vegetable fat;
T3 – Formula 3 with 0.1% vitamin-B_{12}, 5% soy lecithin and 94.9% vegetable fat;
T4 – Formula 4 with 1% vitamin-B_{12}, 0% soy lecithin and 99% vegetable fat;
T5 – Formula 5 with 1% vitamin-B_{12}, 2.5% soy lecithin and 96.5% vegetable fat;
T6 – Formula 6 with 1% vitamin-B_{12}, 5% soy lecithin and 94% vegetable fat.

Fig. 6: Scanned Electronic Micrograph of All 6 Solid Lipid Microparticles Loaded with B_{12}

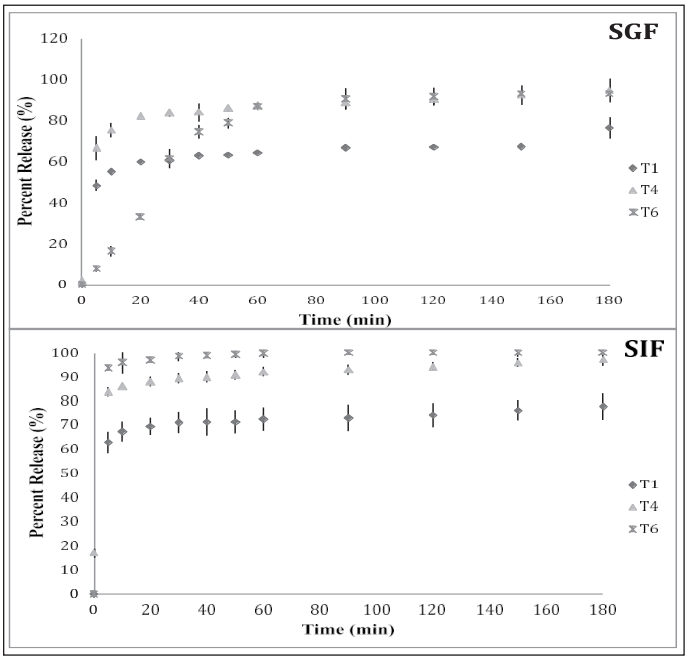
The stability of encapsulated and free B_{12} was measured under controlled conditions at 0, 30, 60, 90 and 120 days. Among all formulations, Formula 5 (T5) with 1% B_{12} and 2.5% soy lecithin showed highest (95%) stability at day 120. Free vitamin B_{12} with 98% purity was also used for storage at same time and highest (7%) degradation was seen just after 30 days (Table IX).

Table IX: Stability of B_{12} Enriched SLM After 120 Days at 25 °C

Formulation	*Storage Time (Days)*				
	0	*30*	*60*	*90*	*120*
T1	100.00 ± 0.00	98.82 ± 0.70	97.14 ± 4.20	93.69 ± 4.53	91.35±4.36
T2	100.00 ±0.00	98.08 ±3.90	96.44 ±3.29	94.40 ±4.29	93.42 ±4.38
T3	100.00 ±0.00	98.03 ±4.29	96.76 ±1.79	96.15 ±3.75	94.25 ±4.84
T4	100.00 ±0.00	99.06 ±1.07	97.09 ±1.45	96.93 ±1.91	91.15±2.63
T5	100.00 ±0.00	98.07 ±3.27	97.84 ±2.68	95.96 ±2.40	95.28 ±2.07
T6	100.00 ±0.00	97.97 ±1.72	96.25 ±2.74	93.89 ±4.41	92.46 ± 4.25
0.1% free from B_{12}	100.00 ±0.00	92.71 ±4.09	86.94 ±3.66	84.94±2,97	75.20 ±1.56
1% free from B_{12}	100.00 ±0.00	93.00 ±0.46	89.65 ±1.79,	85.72 ±1.10	76.56±1.77

Source: [12]

Vitamin B_{12} release profile test was performed on T1, T4 and T6 formulations using simulated gastric fluid (SGF) and Intestinal fluid (SIF). Highest release (55% and 75%) of B_{12} was seen in T1 and T4, respectively in first 10 min. During exposure to gastric juices while T6 showed only 8% release initially due to the presence of soy lecithin (5%) in T6 (Fig. 7). Soy lecithin acts as binding agent and protect bioactive compounds. As the absorption of B_{12} mainly occurs in intestine, the high amount of B_{12} was released in initial time [12].

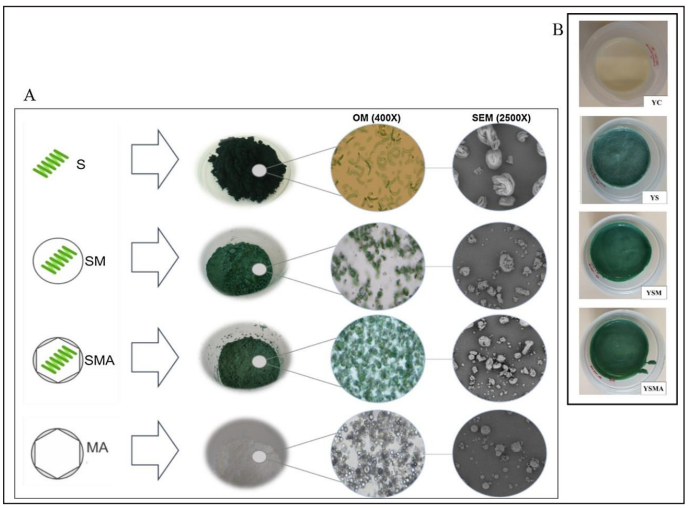


Source: [12]

Fig. 7: Percentage of Vitamin-B_{12} Released from Solid Lipid Microparticles

With increasing awareness about Nutrition, health and preventing diseases, lots of functional food has become popular among consumers. In this reference, *Spirulina platensis,* blue green microalgae are considered to be safe and natural source of bioactive compounds. Spirulina was used as fortificant in various food products; however the unpleasant odour and flavour make it unacceptable by consumers. Since, spirulina is nutritionally dense; da Silva *et al*. [13] used spray drying encapsulating technique to increase its stability and added to yogurt to improve nutritional value and bioactive compounds in yogurt. The researcher used spray drying method for encapsulation of spirulina and fortify yogurt to improve nutritional value and prevent diseases. Microencapsulation of spirulina was done using two encapsulating material: 1. Maltodextrin and 2. Maltodextrin cross linked with citric acid, naming SM and SMA respectively. Maltodextrin was used as wall material for encapsulation. When maltodextrin was mixed with citric acid, it makes cross linking due to esterification and increase thermal and mechanical resistance of encapsulating agent. For SM, maltodextrin/S. platensis was in ratio of 1:1. For SMA, maltodextrin/S. platensis was in same ratio i.e. 1:1 and 1.58 gm citric acid was also added additionally. Bioactive analysis was carried out for both free and encapsulated spirulina. Both free and encapsulated spirulina were added in yogurt and functional properties were validated. Da Silva *et al*. (2019) had performed proximate analysis in free spirulina. Protein was tested by Kjeldahl method (N × 6.25) and found 75.6 ± 0.3 g/100 g dw, carbohydrate was 17.5 ± 0.3 g/100 g dw, ash content by incineration at 550 °C and found 6.2 ± 0.1 g/100 g dw, lipid content was checked by Soxhlet extraction with petroleum ether and found 0.65 ± 0.04 g/100 g dw, and energy was calculated by Atwater system and found 378 ± 1 kcal/100 g dw. An anti-inflammatory agent called quinic acid (792 mg/100 g dw) was also found. After encapsulation, the

yield of SM was 66% and SMA (S. platensis encapsulated in MD crosslinked with citric acid) was 75%. Morphological analysis was done for both free spirulina and encapsulated (SM and SMA) spirulina by scanning electron microscopy (SEM). Fig. 8 (A), SMA microparticles are found more uniform than SM. MA (maltodextrin and citric acid, no spirulina) showed spherical structure while SM and SMA showed spiral structure but somewhere spherical showing covering of maltodextrin on spirulina. Free spirulina showed spiral structure. The most prominent structure was spherical showing encapsulation of bioactive compound. The encapsulated spirulina when added to yogurt, it showed more homogenous appearance and stability and controlled release was observed with SMA (spirulina with maltodextrin and citric acid). See Fig. 8 (B).



Source: [13]

Fig. 8: (A) Morphological Analysis of Free Spirulina (S), MA (without Spirulina), SM (Spirulina with Maltodextrin) and SMA (Spirulina with Maltodextrin and Citric Acid); (B) Visual Appearance of Yogurt After Adding Microparticles

Keršienė *et al.* [14], focused on the nutritional needs for elderly people. To meet the nutritional requirements, elderly requires more nutritionally dense food to prevent neurogenerative disorders. Micronutrients like, Vitamin B_{12}, Iron, Zinc, iodine, vitamin C, plays an important role for disease free ageing. Food fortification with naturally occurring vitamin and antioxidants may have some limitations in terms of stability and unfavourable changes during processing. To protect bioactive compounds essential for elderly people and providing them in low cost, double emulsifying technique for encapsulation was adapted. The aim was to provide more stable and controlled release that makes it more acceptable for elderly population. The researcher used double emulsion technique for encapsulation of multiple bioactive compounds. The double emulsion (DE), water in oil in water ($w_1/o/w_2$) provides a unique morphology. A two-step double emulsification method was used. Water soluble vitamin like C, vitamin B_6 and B_{12} and anthocynin -rich black chokeberry pomace extract (BCPE) was added in inner water phase and fat soluble vitamin A and D_3 was added in oil phase. For water soluble emulsifier, milk protein concentrate and for lipophilic emulsifier Polyglycerol Polyricinoleate (PGPR) was used. For further analysis, and competitive analysis, two types of double emulsion was formulated: Empty Double Emulsion (without vitamin and BCPE) and loaded double emulsion (with all vitamin and BCPE in oil and water phase). BCPE characterization was done by Folin–Ciocalteu's reagent and found approximate 82.7 ± 0.1 mg g−1 polyphenols. The double emulsion was found to be complex and easily destroyable during formation and storage. The stability study was done by creaming stability and droplet size change during storage for 30 days. The creaming stability was found to be very stable for both loaded and empty double emulsion (DE) (Table X) [14].

Table X: Creaming Stability and Droplet Size of Empty and Loaded DE

Sample	*Storage Time (in Days)*				
	1	*3*	*7*	*14*	*30*
Creaming Stability, %					
Empty DE	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	99.2 ± 0.03
Loaded DE	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	96.2 ± 0.05
Droplet Size (d43), µm					
Empty DE	61.65 ± 0.94	65.80 ± 2.31	62.10 ± 1.94	64.11 ± 2.16	-
Loaded DE	37.67 ± 0.79	31.02 ± 1.03	31.34 ± 1.03	32.53 ± 0.59	-

Source: [14]

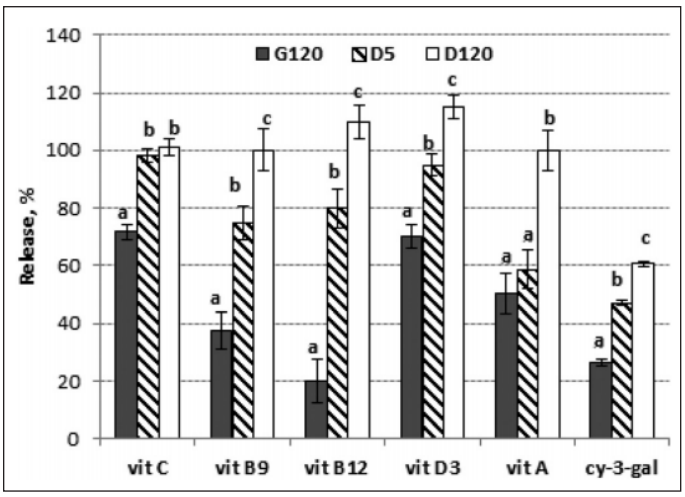
After 30 days storage, the loaded DE stability was found at 96.0%, while for empty, it was observed 99.2%. Droplet size in terms of volume–weighted mean diameter (d_{43}) for empty DE was 61.65 ± 0.93 µm, while after loading with bioactive compounds the diameter size was reduced to 37.67 ± 0.77 µm. Initial encapsulation efficiency (EE) for BCPE was found highest (96.55). Encapsulation stability was also high for BCPE after 14 and 30 days storage (95.25 ± 0.14 and 96.00 ± 0.41). Water soluble vitamins like Vitamin C, B_9, and B_{12} stability was found lower than fat soluble vitamin A and D_3. Among all water soluble vitamins initial EE of B_{12} was found highest (84.00) than other 2 vitamins. Highest degradation was seen in B_9 after storage for 14 and 30 days at 4 °C. Fat soluble vitamin A and D_3 were significantly protected throughout storage when incorporated in oil phase (Table XI) [14].

Table XI: Encapsulation Efficiency and Stability of Loaded Double Emulsion

Item	*Encapsulation Efficiency %*	*Encapsulation Stability, % After 14 Days Storage*	*Encapsulation Stability, % After 30 Days Storage*
BCPE (in terms of cy-3-galactoside)	96.55 ± 0.05	96.00 ± 0.14	96.00 ± 0.40
Vitamin C	76.63 ± 0.79	75.62 ± 0.84	74.00 ± 0.81
Vitamin B_9	75.00 ± 0.88	50.00 ± 0.85	45.00 ± 0.87
Vitamin B_{12}	84.00 ± 2.12	83.60 ± 1.98	78.00 ± 2.08
Vitamin A	99.37 ± 5.93	93.75 ± 6.19	90.00 ± 7.32
Vitamin D_3	98.52 ± 10.43	69.57 ± 12.30	78.26 ± 11.90

Source: [14]

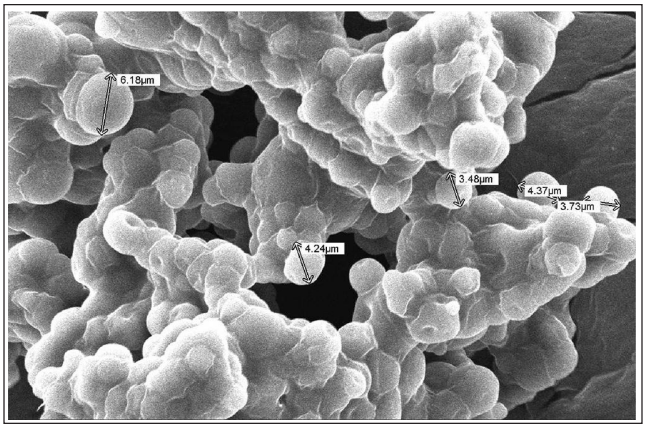
In vitro, study for release kinetics of vitamin was carried out. Gastric digestion study was carried out for 120 min. and afterwards, DE was exposed to duodenal fluids for initial 5 min. and then further 120 min. Vitamin C release was highest (71.75%) at end of gastric phase, and after adding duodenal fluid, almost 98% vitamin C was released within 5 min. In comparison to vitamin C, Vitamin B_{12}, and D_3, showed more controlled release in gastric phase, and 100% release was found in duodenal phase at 120 min. Vitamin D_3 and Vitamin A release in gastric phase was around 70% and 50% respectively and complete release at the end of the duodenal phase. Vitamin B_{12} showed only 4.4% release in gastric phase (Fig. 9). The double emulsion technique used to encapsulate multiple vitamins and antioxidants, demonstrated a good encapsulated efficiency and stability. Slow release of bioactive compounds, during in vitro experiments also makes it an essential technique to provide micronutrients for elderly [14].



Source: [14]

Fig. 9: In Vitro Digestions of Vitamins and Anthocyanins, at Different Duration, Gastric at 120 Min, Duodenal at 5 Min and Duodenal at 120 Min

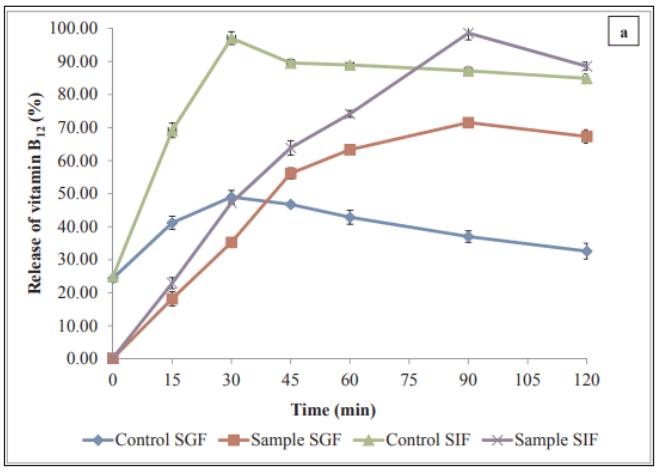
With increasing incidence of B_{12} and D_3 deficiency diseases globally, author Bajaj *et al.* [15] used vitamin B_{12} and D_3 for encapsulation by spray drying method. Various combinations for vitamins encapsulation have been done in past but the vitamin B_{12} and D_3 was never done before. Since the animal foods are good sources of these vitamins, and a large population is vegetarian globally, a stable fortification becomes necessity. Here, the author's objective is the co encapsulation of these two vitamins with most stable combination of wall material. Since vitamin B_{12} is water soluble vitamin and D_3 is a fat soluble vitamin, co-encapsulation was done by using gum acacia, Hi-Cap® 100, maltodextrin in an experimental design to optimize the best combination for wall material. Rice bran oil was chosen as medium for vitamin D_3 solution. Rice bran oil possesses excellent oxidative stability, contains oleic acid, linolenic acid and linoleic acid, and showed highest solubility after ground nut oil. Physicochemical, morphological study was carried out. To test the release profile for both free and encapsulated vitamin B_{12} and D_3, in vitro and in vivo study was done. Gum acacia, Hi-Cap® 100, maltodextrin in different ratios were used for encapsulation. 16 different experimental designs are formulated and undergone physicochemical analysis for Process encapsulation efficiency (PEE), Encapsulation efficiency (EE), Total encapsulation efficiency (TEE). Gum acacia represented highest PEE (85.00%), EE (93.43%), and TEE (79.41%) for rice bran oil, providing good emulsion stability. Considering the particle size of microparticles, maltodextrin by spray drying produces smaller size particle than GA, and HC. Small size particle emulsions are more stable and possess good entrapment efficiency. From all the experimental design, a best wall material combination for encapsulation of B_{12} and D_3 was found to be gum acacia: Hi-Cap® 100: maltodextrin = 38:60:2. This combination exhibits a good encapsulation efficiency and retention of bioactive compounds after storage. The scan electron microscope (SEM) images represents, smooth surface, spherical shape, without cracking in microparticles. The size was found between 3 and 7 μm (Fig. 10) [15].



Source: [15]

Fig. 10: SEM of Microcapsule Containing Vitamin B_{12} and D_3

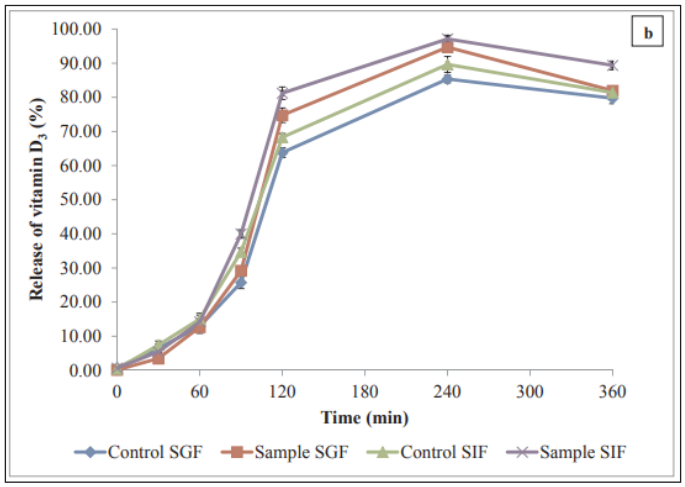
In vitro B_{12} and D_3 release rate was also tested in the presence of stimulated gastric fluid (SGF) at pH 1.2 and stimulated intestinal fluid (SIF) at pH 6.8. B12 release was lower in stomach conditions, while intestinal pH was more favourable for B_{12} release and absorption Fig. 11(A). Furthermore, at 30 min. the highest release was found from free (Control) B_{12} and at 90 min. from encapsulated B_{12}. The bioavailability of control B_{12} was 49% in SGF and 97% in SIF. On the other hand, encapsulated bioavailability was 72% and 99% in SGF and SIF respectively [15].



Source: [15]

Fig. 11(A): In Vitro Release Profile of Both Sample (Control) and Co Capsulated B_{12}

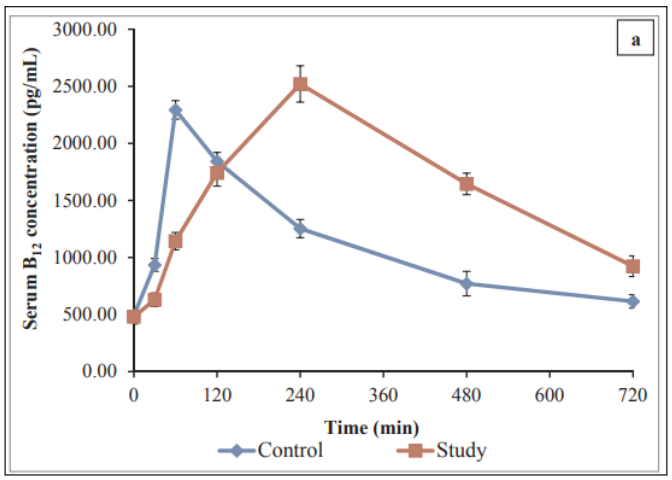
For Vitamin D_3 also higher concentration release was seen in SIF than SGF. The maximum vitamin D_3 release was at 120 min. for both control (Free) and sample (encapsulated) D_3, resulting that gum acacia and modified starch emulsion stability is independent of pH of the medium Fig. 11(B) [15].



Source: [15]

Fig. 11(B): In Vitro Release Profile of Both Sample (Control) and Co Capsulated D_3

During in Vivo study, a single dose of vitamin B_{12} (100 µg/ kg body weight) and vitamin D_3 (250 µg/ kg body weight) was given with gavage tube. The control group of animals were given a standard dose of both vitamin B_{12} and D_3 while experimental group of animals were given microcapsules (1.18 g) after dissolving in water. Maximum release was recorded at 240 min for vitamin B_{12} in experimental group, whereas, for control group maximum Vitamin B_{12} release was studied at 90 min. The in vivo study of Vitamin D_3 release findings were not in favour of in vitro release study Fig. 12(A), 12(B). During in vivo study, a quick release was seen in 8 hours from encapsulated D_3 than control group (24 hour). Gum acacia, Hi-Cap® 100, and maltodextrin in combination of 38:60:2 ratio has been found best as wall material. This combination showed best physic functional properties like, storage, thermal stability, Total encapsulation stability, entrapment efficiency, and process efficiency. During in vivo study, the maximum release of B_{12} was found around at 4 hours and better absorption whereas, encapsulated vitamin D_3 showed quick release with absorption at 8 hours. These encapsulation techniques provide enormous application because of its hydrophilic in nature [15].



Source: [15]

Fig. 12(A): In Vivo Study for Encapsulation Effect of B_{12} and D_3 Release

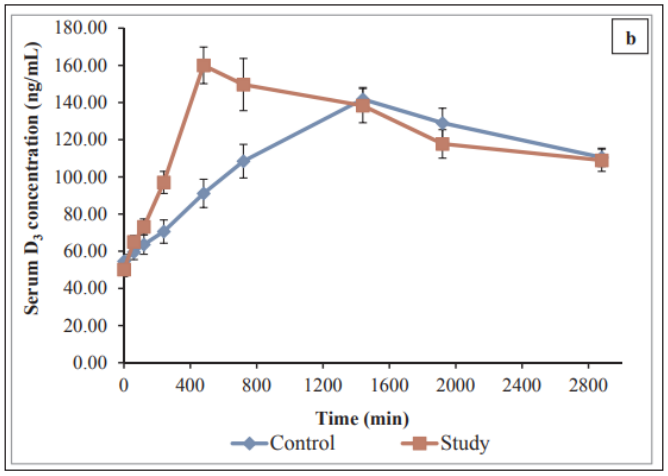


Fig. 12(B)

VI. Conclusion

In most of the countries, the treatment of vitamin B_{12} deficiency is based on either oral administration of cobalamin or intramuscular administration of cyanocobalamin or its derivatives. Now, with emerging technologies in food industries, innovations in natural vitamin B_{12} fortified food products can formulate to combat the deficiency and also to meet daily's requirement.

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