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ISOLATION AND APPLICATION OF FOLATE PRODUCING INDIGENOUS LACTIC CULTURES IN MILK BASED FOODS

Submitted in partial fulfilment of the degree of
Bachelor of Technology under the Supervision of

Dr. Gunjan Goel

By

RITURAJ SURYAVANSHI (101705)
AKANSHA BAJPAI (101713)

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,
WAKNAGHAT (DISTT. SOLAN)

CERTIFICATE

This is to certify that the project entitled "Isolation and Application of folate producing indigenous lactic cultures in milk based food" submitted by Akansha Bajpai (101713) and Rituraj Suryavanshi (101705) in partial fulfillment for the award of degree of B. Tech (dual degree) in Biotechnology of JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY has been carried out under my supervision.

This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Gunjan Goel
Assistant Professor (senior grade)
Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology
Waknaghat

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Rituraj Suryavanshi (101705)
Akansha Bajpai (101713)

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CHAPTER 1
INTRODUCTION

Folate is a natural water soluble vitamin B9 from class of Vitamin B. This vitamin is mainly present in two forms either natural or synthetic one. The natural occurring form is folate and the synthetic form is the folic acid. The naturally occurring form folate lacks stability in food storage and preparation but the synthetic folic acid is stable and is used for supplements and food fortification.

Folate forms an essential nutritional component (important B vitamin) in the human diet. It is essential for the normal functioning and development of the human body. Mammalian cells cannot synthesize folate *de novo*. Folic acid is itself not biologically active in the body. Human body needs folate to synthesize DNA, repair DNA, methylate DNA and is involve in many metabolic pathways such as purine & pyrimidine biosynthesis and amino acid inter conversions. Folates also possess antioxidant properties that protect the genome by inhibiting free radical attack of DNA in addition to their role in DNA repair and replication mechanisms. Foods that are naturally high in the folic acid include leafy vegetables spinach, broccoli, lettuce, okra and asparagus. Foods such as bananas, melons, lemons, beans, yeast, mushroom, orange juice, tomato juice also contain folate. It is also found in legumes such as dried beans and peas. Yeast and yeast extract have also been reported to contain considerable amount of folate.

Folate deficiency has been associated with the higher incidence of neural tube defects during the embryo development. Therefore, a higher intake is recommended for women before conception and during pregnancy. A low folate intake also leads to a number of health disorders such as Alzheimer, coronary heart diseases, osteoporosis, increased risk of breast and colorectal cancer, poor cognitive performance, hearing loss, etc. Due to the health benefits associated with the increased folate intakes many countries now have mandatory folate enrichment programs. As folic acid is more bioavailable than the natural folate, food fortification and supplementation with folic acid is concentrated upon. Regulations for mandatory fortification of wheat flour with folic acid are currently in place in 53 countries but in many cases these regulations have not been implemented. In 2006, the World Health Organization (WHO) and the

Food and Agricultural Organization (FAO) of the United Nations published guidelines that helped the countries to set the Target Fortification Level, the Minimum Fortification Level and the Maximum Fortification Level of folic acid to be used to fortify flour with folic acid. In the United States, mandatory fortification of enriched cereal grain products with folic acid was authorized in 1996 and fully implemented in 1998. The required fortification level is 154 µg/100 g flour [1]. In addition to the United States, 30 other countries now add folic acid to flour, including Canada (150 µg/100 g), Chile (220µg/100g wheat flour), Costa Rica (180 µg/100 g), Dominican Republic (180 µg/100 g), El Salvador (180µg/100g), Guatemala (180µg/100g), Honduras (180µg/100g), Indonesia (200µg/100g wheat flour), Mexico (200µg/100g wheat flour), Nicaragua (180 µg/100 g) and Panama (180 µg/100 g) [1].

Many researchers have been looking for novel methods to increase the concentrations of naturally occurring folate or its variants in the foods. Numerous researchers have reported that Lactic Acid Bacteria (LAB) have the ability to synthesize folate [2]. The proper selection and the use of folate producing microorganisms is an interesting strategy to increase natural folate levels in foods. Hence, folate fortification could be carried out by incorporating folate producing lactic culture in milk based product. On average, milk and dairy products provide 10% to 15% of the daily folate intake. A number of physical and nutritional growth factors like composition of the medium i.e. C & N sources, growth factors, inorganic salts and the conditions for growth such as temperature, pH, oxygen tension, and incubation period have been reported to affect the folate production by microorganisms (lactic cultures) [2]. The folate levels in fermented milks can possibly be increased through judicious selection of the microbial species and cultivation conditions. Such information can be valuable for selecting the folate producing strains to produce fermented products with elevated levels of folate.

Keeping in view the above necessasity of folates in our diet, this study aimed to *isolate lactic cultures from dairy products and screening them for folate production and thereafter incorporating these high folate producing cultures into milk based product.* Milk may act to increase efficiency of folate absorption by protecting dietary folates from uptake by bacteria in the gut, thus increasing absorption in the small intestine [3].

CHAPTER 2
REVIEW OF THE LITERATURE

2.1 Folate and its derivatives

The term "folate" includes both the naturally occurring polyglutamate form in foods and the synthetic folic acid used for food fortification and nutritional supplements. Folic acid or pteroylglutamic acid (PGA) is comprised of *p*-amino benzoic acid linked at one end to a pteridine ring and at the other end to L-glutamic acid.

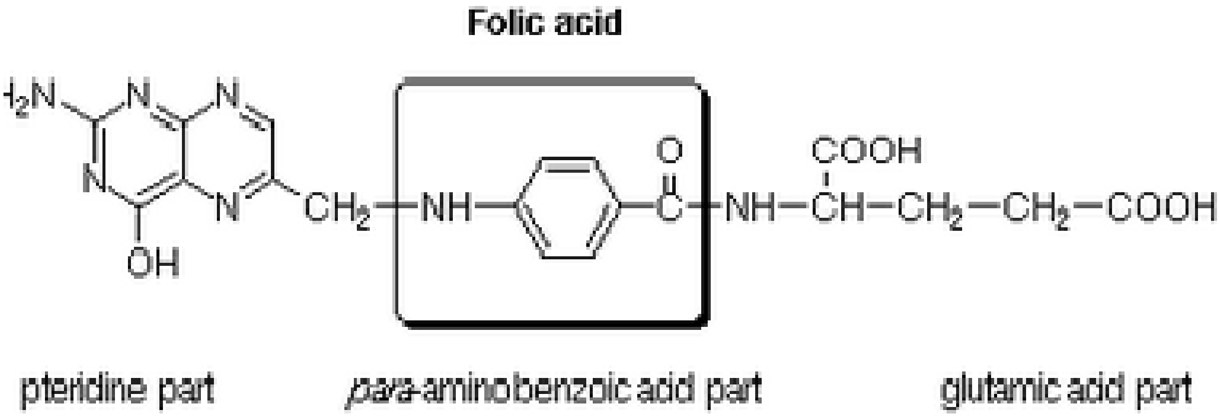

FIG 1:- Structure of folic acid

The naturally occurring forms of folate differ in the extent of the reduction state of the pteroyl group, the nature of the substituents on the pteridine ring and the number of glutamyl residues attached to the pteroyl group. The naturally occurring folates include 5-methyltetrahydrofolate (5-MTHF), 5-formyltetrahydrofolate (5-formyl-THF), 10-formyltetrahydrofolate(10-formyl-THF), 5,10-methylenetetrahydrofolate (5,10-methylene-THF), 5,10 methenyltetrahydrofolate (5,10-methenyl-THF), 5-formiminotetrahydrofolate (5-formimino-THF), 5,6,7,8-tetrahydrofolate (THF) and dihydrofolate (DHF). Most naturally occurring folates are pteroylpolyglutamates, containing two to seven glutamates joined in amide (peptide) linkages to the γ-carboxyl of glutamate. The principal intracellular folates are pteroylpentaglutamates, while the principal extracellular folates are pteroylmonoglutamates.

2.2 Bioavailability and metabolism of folate

Folate bioavailability refers to the proportion of ingested folate that is absorbed and becomes available for metabolic processes or storage. As discussed above folate is a naturally occurring form of the vitamin and is found in food, while folic acid is synthetically produced and is used in fortified foods and supplements.

Folic acid is the core molecule pteroyl glutamic acid and is less reduced than natural folates. It only contains one glutamic residue. It is heat stable and synthetic. It is susceptible to the effects of oxidizing and reducing agents. It is water soluble therefore demands supplementation in the body more frequently. After ingestion, folic acid is converted into the same active form of the vitamin. Natural folate is a natural compound found in foods that have the same core molecule as folic acid. It has various levels of reduction of the pteridine ring, one carbon substitution and a number of glutamate residues. Folic acid is chemically more stable than natural forms of folate because it is less reduced and has just one glutamate residue. These two properties make folic acid more bioavailable when taken as a supplement or are in fortified foods.

The polyglutamate chain to which most of the natural folate is attached hampers the bioavailability of dietary folate and so this polyglutamate chain must be removed by the enzyme *α-glutamyl hydrolase* or *human conjugase* that are present in the brush border of the small intestine. [4] [5]. Then it is absorbed and transported as a monoglutamate into the portal vein. This critical step is not required for absorption of folic acid because it is already present in the monoglutamate form. It is transported to all the cells in the body through the blood circulatory system. The folate monoglutamates are in the 5 methyl-tetrahydrofolate (5-methyl-THF) form which passes readily by diffusion from blood into the all body cells. A large proportion of food folates are already in the 5-methyl-THF form whereas folic acid must be converted to this form.

The body cells cannot retain the 5- methyl-THF. Therefore, in the body cells through tha action of enzymes (that also require vitamin B12 to function) 5-methyl-THF is converted to the tetrahydrofolate (THF). Tetrahydrofolate (THF) can be retained in cells. It is the active substrate required for the synthesis of forms of the folate that are needed by the body (THF-polyglutamates). Thus after ingestion the folic acid / folate is first converted to dihydrofolate by the action of enzyme *dihydrofolate synthetase* and then to THF by the action of enzyme *dihydrofolate reductase* which is then used by the cells.

The absorption efficiency of natural folates is approximately half from that of the synthetic folic acid and thus relative bioavailability of dietary folates is estimated to be only 50% compared with synthetic folic acid but there are still controversies [7]. Folic acid when taken on an empty stomach is twice as available as food folate and folic acid when taken with food is 1.7 times as available as food folate. [6] **.** Despite the large amount of information available on folate bioavailability, knowledge of this important part of folate nutrition is still described as fragmentary [3].

Natural folates (polyglutamates) are consumed.

Absorption (occurs in the small intestine)

Folate polyglutamate

Folate conjugase

5-methyl –THF (folate monoglutamate)

Dihydro folate synthetase

Dihydrofolate

Dihydro folate reductase

Tetrahydrofolate

Used by the body cells.

FIG 2:- FOLATE METABOLISM IN THE HUMAN BODY

2.3 Folate deficiency and requirements

Folate is required in many metabolic pathways such as DNA and RNA biosynthesis and amino acid interconversions. Mammalian cells cannot synthesize folate therefore its exogenous supply is imperative to prevent nutritional deficiency. Though folate is present in a normal human diet but folate deficiencies still occur frequently both in developing and developed countries. Folate deficiency develops due to low intake of folate-containing foods, or as a result of severe alcoholism. It may also occur as a result of malabsorption, especially in case of diseases affecting either intestinal pH or the jejunal mucosa. For example, celiac disease, Malabsorption syndromes, includes Crohn's disease, tropical sprue and gluten sensitive enteropathy. In addition, the few drugs can also lead to folic acid depletion such as oral contraceptives, anticonvulsants, H-2 receptor antagonists, barbiturates, cholestyramine, anti-inflammatory drugs, methotrexate, aspirin, antacids and alcohol. Hence folate deficiency in humans is associated with several health problems. Some of them are discussed below.

2.4 Implications of folate deficiency on health

2.4.1 Anemia

Folates function as a carbon carrier in the formation of heme which is the iron containing non-protein portion of haemoglobin [7] therefore without enough folate the body cannot make or maintain new blood cells thus leading to anaemia. As per the study by Dugdale short-term folate deficiency produces characteristic effects on the RBC parameters. The mean cell volume of RBC increases and haemoglobin decreases. It causes Megaloblastic anemia. Megaloblastic anaemia is a type of anemia in which there are very large red blood cells. Also the inner contents of each cell are not completely developed. This malformation causes the bone marrow to produce fewer cells and sometimes the cells die earlier than their normal 120-day life expectancy. Instead of being round or disk-shaped, the red blood cells can be oval. It results from the inhibition of DNA synthesis during RBC production. And when DNA synthesis is impaired, the cell cycle cannot progress from the G2 growth phase to the mitosis (M) stage. It leads to the continuous cell growth without division. This anemia has a slow onset when compared to that of other anaemia's.

There are several factors which lead to the occurrence of megaloblastic anemia. It is typically caused by a poor diet lacking in adequate amounts of folic acid. Alcohol interferes with the absorption of folate, so people who are prone to drinking alcohol are at risk of folate deficiency anemia. Folate deficiency can also be seen in certain diseases of the lower digestive tract such as celiac disease or in people with cancer. Folate deficiency often occurs during pregnancy because the increased amounts of folate are needed by the fetus and also because it is absorbed more slowly from the mother's digestive tract. Vomiting from morning sickness, anorexia and poor dietary habits also contribute to folate deficiency during pregnancy .Thus pregnant women with inefficient dietary folate intake may have a risk of megaloblastic anemia.

The inability to absorb folic acid may also be inherited. Inherited congenital folate malabsorption is a genetic disorder in which infants cannot absorb folic acid in their intestines and it can lead to megaloblastic anemia. It requires early intensive treatment to prevent long-term problems such as intellectual disability.

The most common symptoms associated with folate deficiency anemia are –

1) Decreased appetite

2) Irritability

3) Lack of energy or tiring easily

4) Diarrhoea

5) Smooth & tender tongue

6) Abnormal paleness

Treatment usually involves taking an oral or injected dietary folic acid supplement for at least two to three months, eating foods high in folic acid and decreasing alcohol intake.

2.4.2 Pregnancy and neural tube defects

Pregnancy is associated with a marked acceleration in 1-carbon transfer reaction including those that are required for nucleotide synthesis and for cell division. It forms the basis for the substantial increase in folate requirements during pregnancy. Thus, as pregnancy doubles the need of dietary folates, recommendations in most countries are therefore set to 400 μg dietary folates/d. Deficient maternal folate status is associated with premature birth, low birth-weight and increased risk of neural tube defects (NTDs) in the offspring. [6]. NTDs result due to incomplete development of the central nervous system and its closely related surrounding structures during the early stages of pregnancy

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NTDs are the most common major malformation of the central nervous system. They arise at a very early stage of pregnancy normally between 21 to 28 days after conception (a time when most women are just beginning to suspect they are pregnant). At this stage, the cells are developing they form a tube-like structure known as the neural tube. And from the neural tube entire nervous system develops. The neural tube eventually becomes the brain and spinal cord. The development and closure of this neural tube is vital to the normal development of the baby. Failure of the proper closure of the neural tube results in a NTD. NTDs can involve the brain, spinal cord, meninges (covering membranes pia mater, arachnoid mater and dura mater), skull and spine.[8] In spina bifida the spinal cord at the lumbar vertebra is not covered with bone . In Anencephaly there is no brain. In encephalocele the brain tissue protrudes out of the skin from an abnormal opening in the skull.

Table 1: Different neural tube defects and the percentage of their occurrence

NTDs	**Accounting for the percentage of NTDs**
Spina bifida	51%
Anencephaly	40%
Encephalocoele	8%
Iniencephaly	1%

The major reason for the occurrence of the neural tube defects is that it occurs at an early stage in embryonic life. It occurs at a time when many women do not even realize that they are pregnant. Also most women do not consult their doctor at this very early stage in pregnancy. As increased intakes of folic acid have been proven to reduce the risk of NTDs, the pre-conception period is the crucial time for the folic acid consumption. Thus, promoting the normal closure of the neural tube and consumption of folic acid during pre-conception period is a significant challenge because in spite of the major advances in contraception in developed countries more than half of all the pregnancies are unplanned. Overwhelming evidence exists since the early 1990s that increased intakes of the vitamin folate in its synthetic form folic acid can prevent up to about 70% of these birth defects [9, 10]. Researchers in Sweden have found that when the grain supply has not been fortified with folic acid, folate deficiency was associated with a 50% increase risk of early miscarriage. Other data suggest that folic acid supplementation before conception may have the potential to reduce the frequency of Down's syndrome. Some mothers of the infants with Down's syndrome have abnormal metabolism of folate[11]. Folic acid is also important for lactating women. Due to the demands of breastfeeding on the mother's folic acid stores, the RDA (recommended daily allowance) for lactating women is increased.

2.4.3 Cardiovascular disease

The homocysteine level in the blood is positively related to the risk of cardiovascular diseases. The results of more than 80 studies have shown that even moderately elevated levels of homocysteine in the blood increases the risk of occurrence of cardiovascular diseases [12].The Framingham Heart Study showed that the higher is the blood homocysteine level, the greater is the degree of narrowing of carotid arteries which increases the likelihood of strokes.

It has been seen that the diet rich in folate can reduce the homocysteine level. Folic acid aids in conversion of homocysteine to methionine. Out of the three vitamins that regulate the homocysteine levels, folic acid has been shown to have the greatest effect in lowering basal levels of homocysteine in the blood when there is no coexisting deficiency of vitamin B_{12} or vitamin B_6.Therefore, we may say that the Folate-rich diets may decrease the risk of cardiovascular disease. A study that followed 1,980

Finnish men for ten years found that those who consumed the most dietary folate had a 55% lower risk of an acute heart stroke when compared with those who consumed the least dietary folate [13].Contrary to these findings , it has been seen that folic acid had a negative effect on the occurrence of strokes. A preliminary meta-analysis of data from four of the on-going trials including about 14,000 subjects showed that the B vitamin supplementation had no significant effect on the risk of coronary heart disease or stroke [18]. Therefore, well-designed and controlled clinical trials are needed to prove the efficacy of folic acid in the prevention and treatment of cardiovascular disease.

2.4.5 Cancer

It is thought that folate has a significant role in the prevention of cancer. Epidemiologic evidence have shown an inverse associations between higher intakes of dietary folate and a reduced risk of cancer (of the colon, other parts of the gastrointestinal tract, pancreas). It has been found that Cancer arises from DNA damage due to excess of on-going DNA repair and the inappropriate expression of critical genes [14]. Folate helps in DNA & RNA synthesis and methylation thus folate intake may affect both DNA repair and gene expression. Several epidemiologic studies have suggested that the higher intakes of folate/folic acid may reduce the risk of this cancer .Folic acid from dietary sources resulted in a modest reduction in the risk of colon cancer. Folate deficiency leads to the misincorporation of uracil for thymidine during DNA synthesis and also to an increased frequency of chromosomal breaks .These aberrations are reported to be normalized when a person is on folic acid supplementation. The relation between folate and the cancer is still a matter of debate. More investigation and research is needed.

2.5 Recommended dietary allowance

In 1944, the nutrition advisory committee of the Indian research fund association (now the ICMR- Indian council of medical research) made some recommendations regarding the nutrient requirements, dietary allowances and balanced Indian habitual diets. It was set as the recommended daily allowance (RDA) of nutrients for Indians. It was based on the recommendations of the League of Nations health committee in 1935. The RDA for Indians was revised by the ICMR in 1958, 1968, 1978, 1989.

The ICMR expert group on nutrient requirement and safe dietary intake for Indians met from April 27th-29th, 2009 and then to carry out further revisions it met again on November 3rd 2009. The I.C.M.R. expert group recommended RDA for folate in terms of free folate. In an adult 180 μg of free folate was recommended. As a higher amount of folate is needed during pregnancy and lactation, medicinal supplementation of folate was recommended for the pregnant and lactating women. The table below shows the amount of folate (RDI) required by different age groups.

Table 2-The Amount of folate (RDI) required by different age groups.
(*Source* :- Bulletin of The Nutrition Foundation of India –Vol 31 2010, November 1)

	GROUPS	FOLIC ACID (mcg/d)
ADULTS	MAN	200
	WOMAN	250
	PREGNANT WOMAN	500
	LACTATING WOMAN	350
INFANTS	0-12 MONTHS	25
CHILDREN	1-6 YEARS	80-100
	7-9 YEARS	120-140
ADOLESCENTS	10-12 YEARS	120-140
	13-15 YEARS	150-250
	16- 18 YEARS	150-250

2.6 Folate deficiency in India

As published in the Indian journal of community medicine vol-33 , out of the one sixth of the global population residing in India, one third are suffering from vitamin and micronutrient deficiencies. It is estimated that forty per cent of the world's severely malnourished children under five live in India. At least half of the Indian infant deaths are related to malnutrition. Not only in terms of population but also economically micronutrient deficiency is costing a lot to the India. The loss due to micronutrient deficiency costs India 1 percent of its GDP. This amounts to a loss of Rs. 27,720 crore per annum in terms of productivity, illness, increased health care costs and death. Every day, more than 6,000 children below the age of five die in India. And more than half of these deaths are caused by malnutrition-mainly the lack of Vitamin A, iron, iodine, zinc and folic acid. Anemia prevalence among children under five years is 69% and among women it is over 55%. 200,000 babies are born every year with neural tube defects in India due to folic acid deficiency. For almost two years now all the states are depleted of iron and folic acid tablet stock for pregnant women acknowledging that anemia contributes to 20% of maternal deaths every year in India. 75% of selected urban population from India (Pune, Maharashtra) had hyper homocysteinemia and methylmalonic academia with cobalamin deficiency.

Every year 3- 4 lakh infants worldwide are born with spina bifida and anencephaly. The prevalence is approximately 1-5 per 1000 live births and the risk of recurrence is 2-3%.[15]More than 95% of cases are contributed by the first affected pregnancies. The distribution of neural tube defects shows considerable geographical, temporal and ethnic variation. In India the prevalence is 3.63/1000 live births, the highest cases are reported from the northern states, namely Punjab, Haryana, Rajasthan and Bihar. [16] In a study reported (from India) only 20% women had heard about folic acid but none of them knew that it should be taken before pregnancy or it prevents birth defects.[17] Folate unawareness does not only exists among general population but it also dominates among nurses, pharmacists and health professionals. A survey conducted among student pharmacists concluded that 94% knew folic acid supplements prevent birth defects, 74% knew supplementation should begin before pregnancy but only 55% knew the recommended levels or good folate sources (50%).[18] A telephone survey among obstetricians in Delhi, India reported that though all of them were aware of folic acid, only 63% knew that it prevents birth defects and 30% knew that it should be given before pregnancy.[19] None of them prescribed pre conceptional folic acid or folate rich diet through child-bearing age. It was more surprising that 80% of those surveyed were not aware of the preventive dose of folic acid.

2.7 Folate produced by food-grade microorganisms

The ability of *de novo* folate production is found in bacteria, green plants, fungi, and certain protozoa through the folate biosynthesis pathway. Numerous researchers have reported that LAB, such as the industrial starter bacteria *Lactococcus lactis, S.thermophilus*, and *Leuconostoc species* have the ability to synthesize folate [21]. Contrary to this many *Lactobacilli* species consume folate. The ability to produce folate can differ remarkably between different lactic cultures (2 to 214 μg/L folate). Lactic cultures such as *Lb. acidophilus, Leuconostoclactis, Bifidobacterium longum*and some strains of *Propionibacteria* can also produce large amounts of folate[21]. Majority of folate produced by *L. lactis* and *Leuconostoc* spp. is intracellular and *S. thermophilus* produces folate extracellularly. The major limitation of use of probiotic organisms for fortification of folate is the requirement of strict anaerobic conditions for folate production and possibilities of folate utilization by co-cultures when used as adjunct starter.

Among all these food-grade bacteria and dairy starters, *S. thermophilus* is the best folate producer that produces folate extracellularly in the milk during fermentation.

2.7.1 Streptococcus thermophilus: prolific folate producer

S. thermophilus is known to produce folic acid during growth in milk. *S. thermophilus* has a strain-specific ability of folate production [6]and has been reported to produce higher quantity of folate in comparison to other LAB. But great differences have been observed in the production ability of individual strains. It has been found that *S. thermophilus*is the dominant producer, elevates the folate levels in skim milk, whereas lactobacilli have been found to deplete the available folate in the skim milk. Holasova and others(2004) showed about more than 6-fold increase in the 5-MTHF content by *S. thermophilus*.

2.8 Milk or milk based products as the food matrix

The U.S. Dept. of Agriculture (USDA) recommends the consumption of at least 3 servings of milk products as a part of a healthy daily diet. Taking into account this recommendation and also considering that a normal serving consists of 240 mL, currently available fermented milk products could contribute up to 23% of RDI for folate.

Due to the potential risks of fortification with folic acid, fermented milks containing elevated levels of natural form folates seem to be more rational for fortification purposes (Scott 1999). Fermented milks are considered as a potential matrix among dairy products for folate fortification because the folate binding proteins present in milk improves the folate stability and the bioavailability of 5- methyltetrahydrofolate. Therefore, proper strain selection and use of folate-producing microorganisms is an interesting strategy to increase the natural folate levels in foods.

Fermentation fortification, therefore, is a novel concept with a potential to increase folate content in food products by number of ways:

(1) Bacterial strain selection,

(2) Delivery engineering

(3) Metabolic engineering

2.9 Folate food fortification

The criteria for fortification strategies are:

- A high prevalence of inadequate folate intake
- Evidence of deficiency
- A high occurrence of deficiency-related disease such as NTD
- A widely available and highly consumed food vehicle that can be produced centrally.

The micronutrient malnutrition can be controlled by the consumption of a balanced diet, which is balanced in respect to all the six classes of nutrients. But this seems too arduous when seen in the global context. Every one in every country does not have access to all the vital nutrients. They all have varied dietary habits. Not everyone can retrieve the micro-nutrient rich food. Also natural sources loose half of the micronutrient content during its processing and cooking. About 75% of the folate in whole wheat is lost during milling.

Therefore, food fortification comes into existence. It accompanies the advantage of getting delivered to a larger population segment and does not require much changes in the dietary consumption patterns. In order to restore the micro- nutrient loss the food fortification has been used for over 80 years in the industrialized countries.

Regulations for mandatory fortification of wheat flour with folic acid are currently in place in 53 countries although in many cases these regulations have not been implemented. In 2006, the World Health Organization and the Food and Agricultural Organization (FAO) of the United Nations published guidelines which helped the countries to set the Target Fortification Level, the Minimum Fortification Level, the Maximum Fortification Level and the Legal Minimum Level of folic acid to be used to fortify flour with folic acid. In the United States, mandatory fortification of enriched cereal grain products with folic acid was authorized in 1996 and fully implemented in 1998. The required fortification level is 154 µg/100 g flour. [1]
In addition to the United States, some 30 countries now add folic acid to flour, including Canada (150 µg/100 g), Chile (220µg/100g wheat flour), Costa Rica (180 µg/100 g), Dominican Republic (180 µg/100 g), El Salvador (180µg/100g), Guatemala (180µg/100g), Honduras (180µg/100g), Indonesia (200µg/100g wheat flour), Mexico (200µg/100g wheat flour), Nicaragua (180 µg/100 g) and Panama (180 µg/100 g).

Table 3: Levels of folic acid fortification in countries with mandatory fortification programs.

Country	**Fortification level**	**Date of implementation**
United states	140 µg / 100 g	1998
Canada	150 µg/ 100 g	1998
Costa Rica	180 µg/ 100 g	1998
Chile	220 µg /100 g	2000
South Africa	150 µg / 100 g	2003

Cereal-pulse based Indian diets are qualitatively deficient in micronutrients particularly iron, calcium, vitamin A, riboflavin and folic acid. And also due to low intake of income-elastic protective foods such as pulses, vegetables particularly green leafy vegetables (GLV), fruits there is micronutrient deficiency in India. Fragmentary evidence suggests that Indians do tend to have high levels of homocysteine which can be treated with the consumption of folic acid. Sixteen various food fortification programs have been implemented by Indian government such as:-

National Nutritional Anemia Control Programme (NNACP)

In this programme supplements containing 100 mg of elemental iron + 500 µg folic acid are given to pregnant women for 100 days during pregnancy; 20 mg elemental iron and 100 µg folic acid are given daily to preschool children for 100 days in the year[22,23] . Recently adolescent girls have also been included as a part of the life cycle approach with same dose as pregnant women and weekly once administration throughout the year. Unfortunately despite of the scientific basis for the programme, iron- folic acid supplementation has failed to have an impact on the incidence or severity of anemia mainly due to lack of awareness regarding its importance and particularly due to poor outreach in a vast country like India.

Food fortification project (Rajasthan)

The Government of Rajasthan (GoR) has actively considered the fortification of multiple staple foods such as wheat flour, oil, milk and lentils. It has employed **Integrated Program Strategy.** It refers to “integrating” multiple food vehicles like wheat flour, oil, milk to be fortified with iron, folic acid, vitamin B12 and Vitamin A. Under the IPS, IIHMR has signed a Grant Agreement with the Global Alliance for Improved Nutrition (GAIN). The project is being carried forward with a multi-sectoral partnership with the Government of Rajasthan, the civil society and the private sector.

Micronutrient initiative (MI)

The Micronutrient Initiative (MI) supports projects to fortify cereal flours with vitamin and minerals containing folic acid and other essential micronutrients. It is developing and scaling-up an innovative intervention that aims at preventing anemia and NTDs in adolescent girls and women of child-bearing age. MI is also providing technical support to State Governments for the Weekly Iron Folic Acid supplementation (WIFS) program targeting adolescent girls and school children. MI has helped in training government employees involved in this program in Madhya Pradesh. Following a successful pilot project in selected districts of Chhattisgarh, MI is now a technical partner for the scale up of the program to all districts of the state. MI´s pilot project showed a reduction in anemia prevalence by about 14 percent and 7 percent amongst school going and out of school adolescent girls respectively. MI supports wheat flour fortification with iron and folic acid in India by hosting the flour fortification network (FFN).India flour fortification network under the guidance of flour fortification initiative (C.D.C AND U.S.A.) ensures wheat flour fortification with nutrients including folates in the states of West Bengal, Gujarat , Madhya Pradesh , Tamil Nadu, Kerala , Chandigarh, Rajasthan , Punjab , Delhi and Andhra Pradesh.

CHAPTER 3

MATERIALS AND METHODS

To achieve the objectives of this study, the study was divided into two parts, the first parts dealt with a survey about folic acid knowledge in the women in the surrounding areas of Waknaghat and Solan and in the second part of study the lab experiments were conducted to isolate a potential folate producing lactic culture.

3.1 The lactic cultures

Table 4: Different cultures and their MTCC number

STRAINS	**MTCC NO.**
Lactobacillus rhamnosus	5462
Lactobacillus plantarum	5422
Lactobacillus helveticus	5463
Lactobacillus casei	5462
Lactobacillus delbrueckii subsp. Bulgaricus	991
Lactobacillus rhamnosus GG	Procured from NDRI, Karnal
Streptococcus thermophilus	NCDC -218
AR 1 Curd	ISOLATED
AR3	ISOLATED
AR4	ISOLATED
AR 5	ISOLATED
AR 6	ISOLATED
AR1 Milk	ISOLATED
RA 3 Dosa mix	ISOLATED
RA 4	ISOLATED

2) Media and other chemicals

- MRS broth (HIMEDIA)

- MRS agar (HIMEDIA)
- S.T. agar (HIMEDIA)
- Folic Acid Media (HIMEDIA)
- Standard Folic Acid (SIGMA)
- 0.9% NaCl Solution
- Human Plasma
- Distilled Water

3.2 Survey on folic acid consumption

A questionnaire was prepared on different aspects of folic acid consumption, availability, role in the body metabolism. The survey includes mostly the women and the gynaecologists from Solan district.

- Surveyed 80 married women belonging to the area Solan, Waknaghat, Shoghi.
- Questionnaire for the Womens:
    - ➢ Their education , age , income
    - ➢ Where they live urban or rural
    - ➢ Number of children
    - ➢ Mode and duration of delivery
    - ➢ Feeding habits
    - ➢ What is folic acid?
    - ➢ Function of folic acid
    - ➢ Sources of folic acid
    - ➢ The duration and appropriate time for folic acid consumption
    - ➢ Name of any folic acid tablet

- Questionnaire to the doctors :

    - ➢ How many pregnant women come in a month?
    - ➢ How many cases of miscarriages, neo natal abnormalities and neural tube defects are reported in a year?
    - ➢ How many women take folic acid tablets pre pregnancy on the daily basis?

- How many pregnant women take folic acid and in what amount and for how much time?
- Do patients know about the use of folic acid?
- Is folic acid intake equally important for all age groups?
- Is folic acid tablet equally important for both the genders?
- Is folic acid deficiency associated with the life style diseases?
- Is folic acid deficiency really a matter of concern for our country?
- Do you think food fortification with folic acid would be beneficial for our country?

3.2.2 Isolation of lactic cultures from different sources

- The bacterial lactic cultures were isolated from different sources such as milk, curd, dosa mix, juice in MRS agar as well as on ST agar
- The single colonies were sub cultured on the same agar to obtain a pure culture
- The Grams stain was performed to check the morphology and Gram's reaction of the isolates

3.2.3 Folic acid assay curve by microbiological method.

For finding the standard folic acid assay curve referred to the protocol given by Iyer and Tomar, 2009. The medium used for this assay was "folic acid assay medium" given in the Himedia Lab Manual. The following steps were followed:-

- The Folic acid assay medium was prepared as per manufacturer's instructions medium.
- Standard Folic acid was added at a concentration (0- 10 ng/ml of broth.)
- The overnight grown culture in MRS broth was centrifuged and added to folic acid assay medium with an OD of 0.50.
- The tubes were incubated at 37C for 16 h.
- After incubation, OD was taken at 600nm.

3.2.4 Screening of Lactic cultures for folic acid production by using microbiological assay

The standard available cultures and the isolated cultures were grown in the folic acid medium lacking folic acid as a supplement. After incubation at 37C for 16h, their absorbance was measured at 600 nm. The following steps were followed:-

- The cultures were inoculated in MRS broth.
- The tubes were incubated at 37°C for 24 hours.
- After incubation, the broth was centrifuged at 7000 g for 15 minutes at 4°C.
- The supernatant was removed and the pellet was washed twice with 0.9% saline.
- The absorbance of the pellet in normal saline was taken at 600 nm.
- The absorbance of the pellet was optimized to 0.5 nm**.**
- The 50 µl of pellet was inoculated in folic acid assay medium test tubes.
- The tubes were incubated at 37°C for 16 hours.
- After incubation, OD was taken at 600nm.

3.2.5 Extraction of folic acid from lactic cultures

The folic acid was extracted from the lactic cultures grown in folic acid assay medium as per the following method:

- The cultures were grown in folic acid medium and were incubated for 18 hours.
- A volume of 5 ml of cultivation broth was used.
- It was centrifuged at 7000g for 15 minutes at 4◦c.
- The cells were washed with 0.1M sodium acetate buffer (pH 4.8) and ascorbic acid.
- The cells were resuspended within the same buffer.
- The samples were then incubated at 100◦c in water bath for 5 minutes to release the folate from the cells.
- The samples were then centrifuged at 7000g for 15 minutes at 4◦c.

- The samples were then deconjugated with human plasma.
- After deconjugation, the samples were incubated at 37◦c for 4 hours.

3.2.6 Estimation of folic acid by HPLC

The folic acid production in lactic cultures was determined by HPLC as per the method prescribed by Iyer and Tomar 2009. The following procedure was adopted:

- HPLC specifications: C18 column with UV detector was used.
- The stock solution was prepared by dissolving 1.005 mg/ml of standard folic acid in 0.1M phosphate buffer (pH 6.5)
- It was further diluted in the range of 1,10,100,500,1000 µg/ml for determination of the retention time (RT) and relationship between folic acid concentration and peak area.
- The standard folic acid and the samples were loaded after passing through 0.22 µM disc filter.
- Column and UV detector cell temperature was 40°C.
- Column gradient and elution were achieved with the mobile phase consisting of acetate buffer (pH 2.8) and acetonitrile.
- The acetate buffer and acetonitrile were filtered through 0.45µM nylon filter and were degassed by sonication before use.
- Detection was carried out in the UV – region at 290 nm at a flow rate of 1 ml/min using 10% acetonitrile and 90% buffer aqueous phase in the beginning, changing to 24% acetonitrile and 76% buffer aqueous phase after 12 minutes.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Survey

Table 5 : Discussion on folic acid with the public

Urban	35	Rural	45
Had children	65	Did not had children	15
Consumed fruits on daily basis	70	Do not consume fruits on daily basis	10
Consume rice, grain chapati, pulse/ vegetables, milk/curd	All	Do not consume rice, grain chapati, pulse/ vegetables, milk/curd	Nil
Aware of folic acid	20	Unaware of folic acid No schooling Till 5th grade Till 10th grade Till 12th grade Graduate/ post graduate/phd/post doc	60 15 5 20 15 25
Knew that folic acid is found in vegetables and fruits	18	Did not knew that folic acid is found in vegetables and fruits	62
Knew that folic acid should be consumed before conception	2	Did not knew that folic acid should be consumed before conception	78

Discussion with the doctors

- Apart from the pregnant women , whom you prescribe folic acid tablets on daiy basis?
  - Anemia patients
  - Dihydrofolate deficient patients
  - Chemotherapy
  - Cancer patients

- Primary infertility
- Recurrent abortion

- Is folic acid deficiency associated with any life style diseases ? Yes
  - Dietary deficiency
  - Dislipidemia
  - Cardiac
  - Hyperlipedemia
- According to you , what is the importance of folate in our diet?
  - Prevention of neural tube defects
  - Brain development
  - Iron deficiency
  - First three months
  - Anemia
  - Tissue metabolism

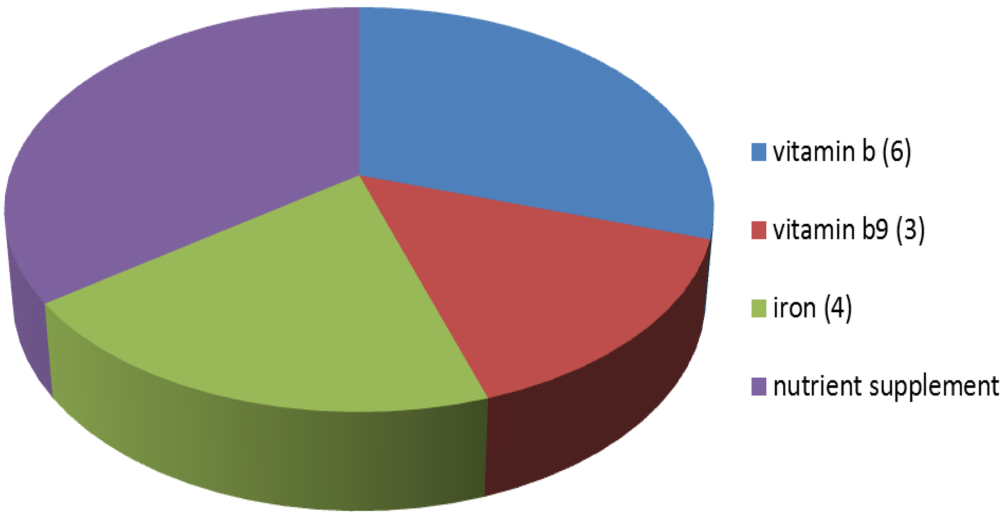


FIG 3 :- Percentage of different responses about the knowledge of folic acid

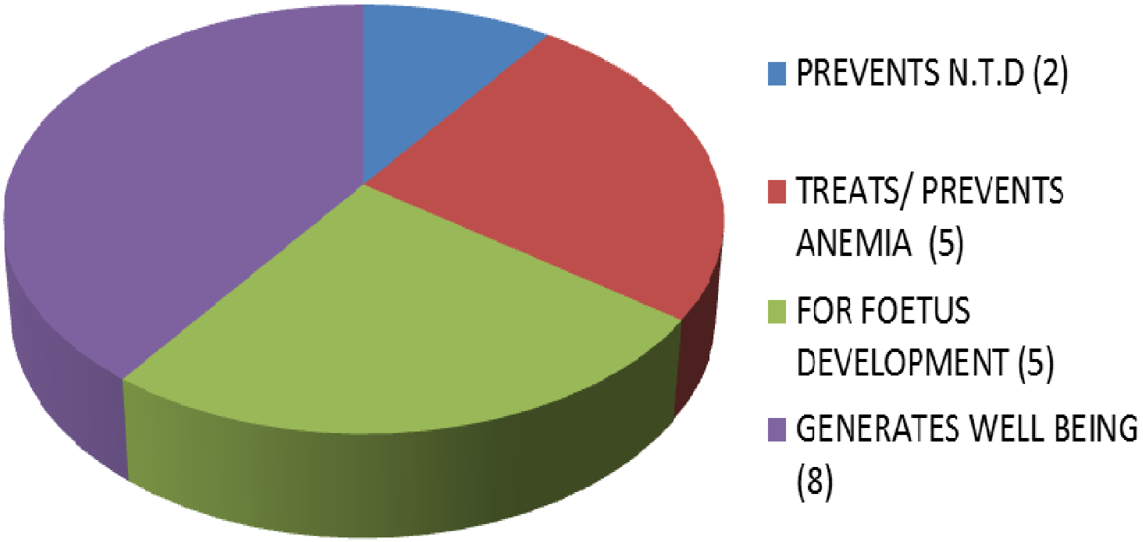


FIG 4 : Percentage of different responses about the function of folic acid

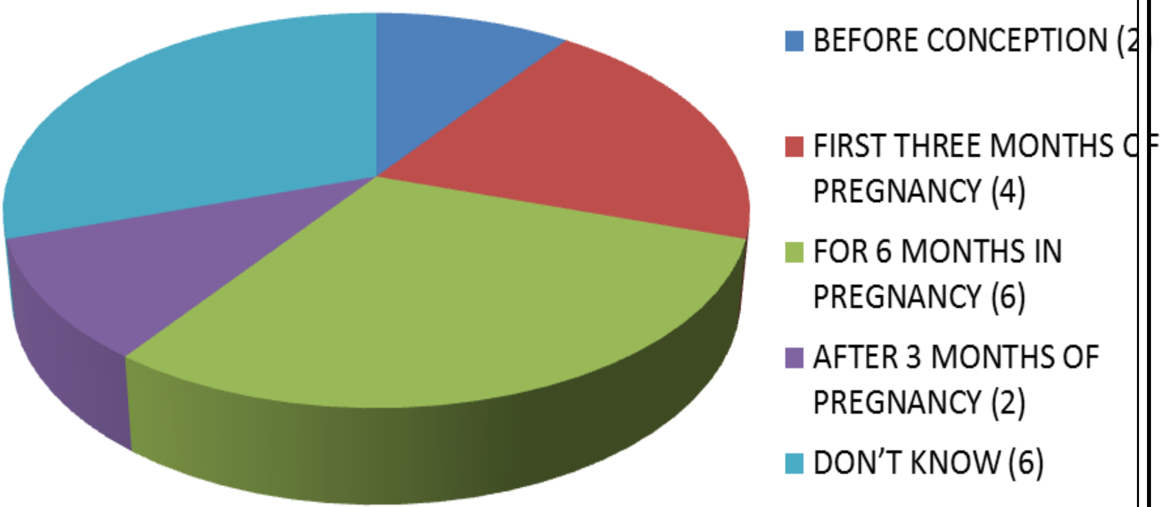


FIG 5: Percentage of different responses about duration and time of folic acid consumption

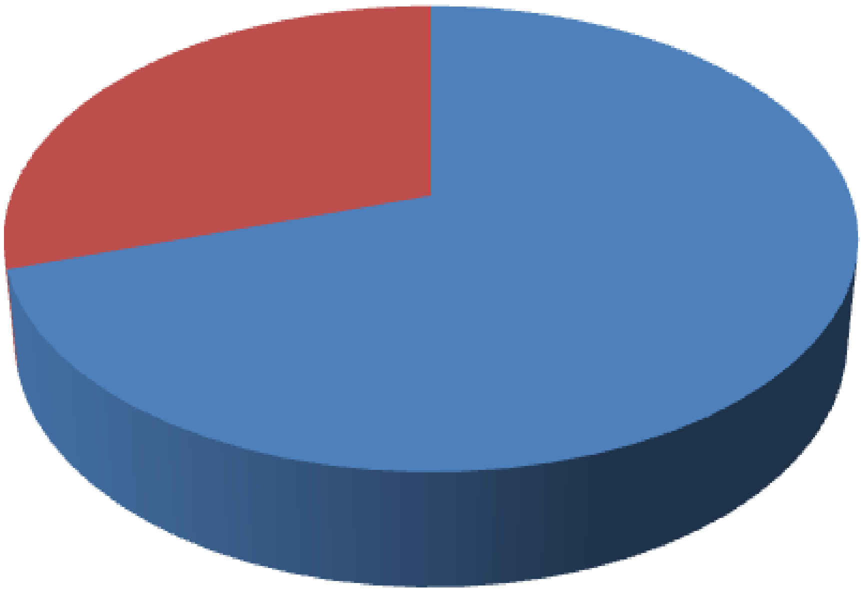


FIG 6: How many women take folic acid pre pregnancy ?

None – 70%

Take pills – 30

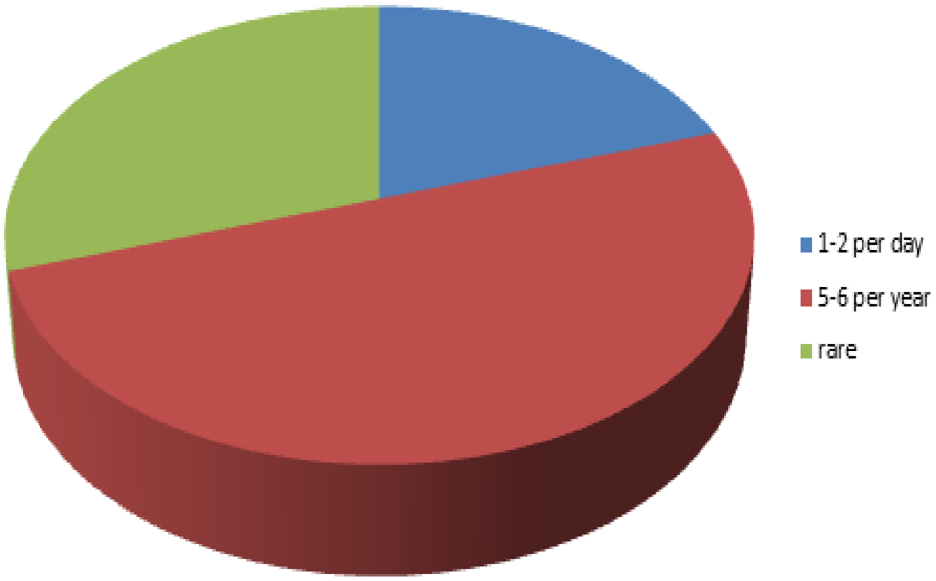


FIG 7 : How many cases of diseases associated with folic acid deficiency are reported ?

Photographs of the surveyor and the survee :-

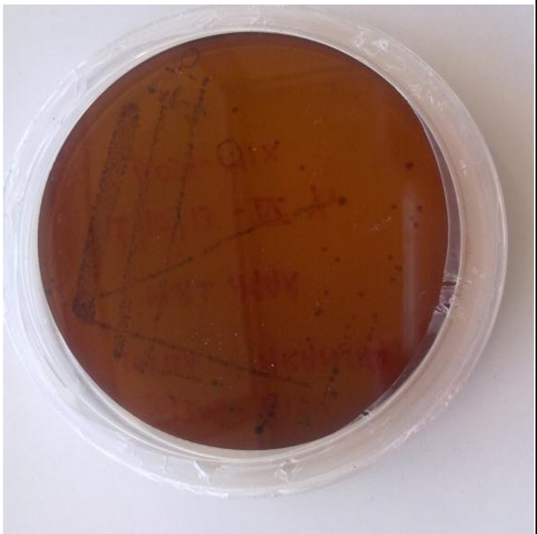
Photograph 4.1 :-

Discussion:- We surveyed around 80 female individuals from the Solan district. We found that most of them had healthy eating habits and had a normal delivery of the baby. But, majority of the women had no knowledge about folic acid. We were amazed that the majority of the educated women did not know the use of folic acid and that it should be consumed before conception. Thus, we can say that mass awareness about the folic acid is poor.

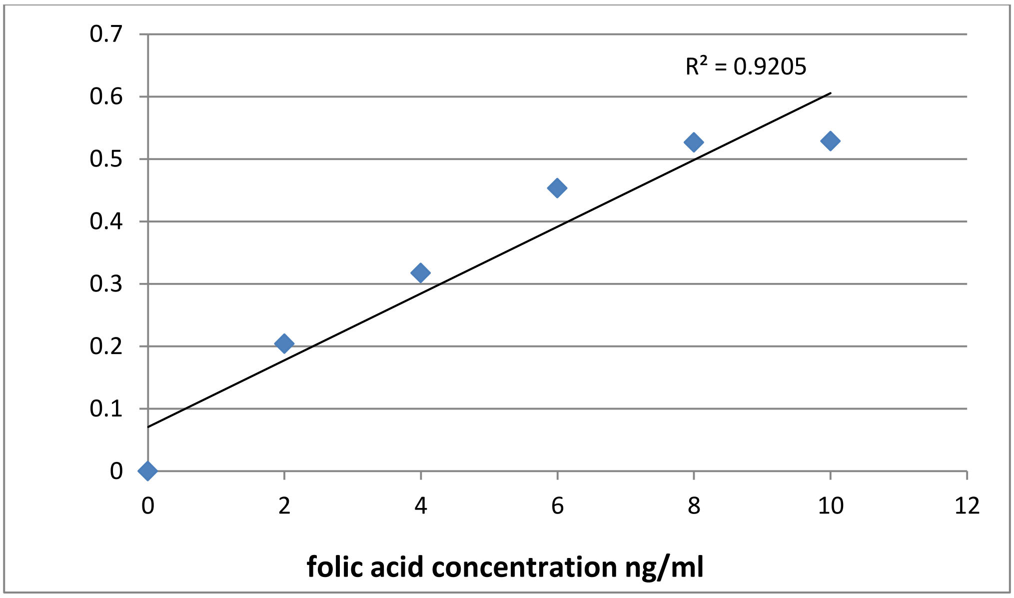
The doctors respondent that folic acid is an important vitamin. They prescribe it to pregnant women, anemic patients and cancer patients. They said that it is important for both the genders. According to them folic acid food fortification is a revolutionary concept and it should be brought into action provided it does not changes the colour and appearance of the fortified food.

4.2 Isolated bacterial cultures from different sources

	
4.2.1 :- Different curd colonies	4.2.3 :- Isolated MILK colonies on ST-AGAR

	
4.2.4 :- Isolated bacterial colonies from MILK on MRS AGAR.	4.2.5 :- Isolated bacterial colonies from dosa mix on MRS AGAR.

04.3 STANDARD CURVE OBTAINED BY MICROBIOLOGICAL ASSAY



FOLIC ACID STANDARD CURVE

4.4 **Estimation of folic acid content in the isolated and available cultures by microbiological assay**

FIG 4.4.1:-

NAME OF THE CULTURE	ABSORBANCE @600 n.m.	F.A. CONCENTRATION (µ.g./ l)
Lactobacillus rhamnosus MTCC -5462	0.4261	08.21
Lactobacillus plantarum MTCC -5422	1.6471	80.61
Lactobacillus helveticus MTCC -5463	0.3021	06.26
Lactobacillus casei MTCC -5462	0.0066	0
Lactobacillus delbrueckii subsp. bulgaricus MTCC -991	0.4410	09.11
Lactobacillus rhamnosus GG	1.5473	48.49
Streptococcus thermophilus NCDC -218	0.6585	11.58
AR1 Curd	0.2976	52
AR3 Curd	0.6877	12.206
AR4 Curd	0.8922	16.556
AR5 Curd	1.461	11.704
AR6 Curd	0.5864	2.001
AR1 Milk	1.4858	9.186
AR3 Dosa mix	0.8999	6.72
AR4 Dosa mix	0.7947	10.382

- Photographs clicked while performing the microbial assay

PHOTOGRAPH 4.4.1 :- **Bacterial Cell pellet**

PHOTOGRAPH 4.4.2:- Bacterial pellet washed and resuspended in normal saline.

Discussion:-

In the present study the standard curve for folic acid by the MA was found to have a R2 value of 0·9205 indicating a perfect linear relationship From the available cultures, L*actobacillus plantarum* (F.A.:80.61µg/l) , *rhamnosus GG* (F.A.:48.49 µg/l) showed the highest amount of folic acid content.AR6 curd (F.A.: 2.001 µg/l) showed lowest amount of folic acid content. These four samples were further analyzed for folic acid production by HPLC.

4.5 Extraction

Discussion:-We extracted intracellular as well as extracellular folic acid produced by cells, by resuspending the cell pellet in acetic buffer followed by addition of human plasma.

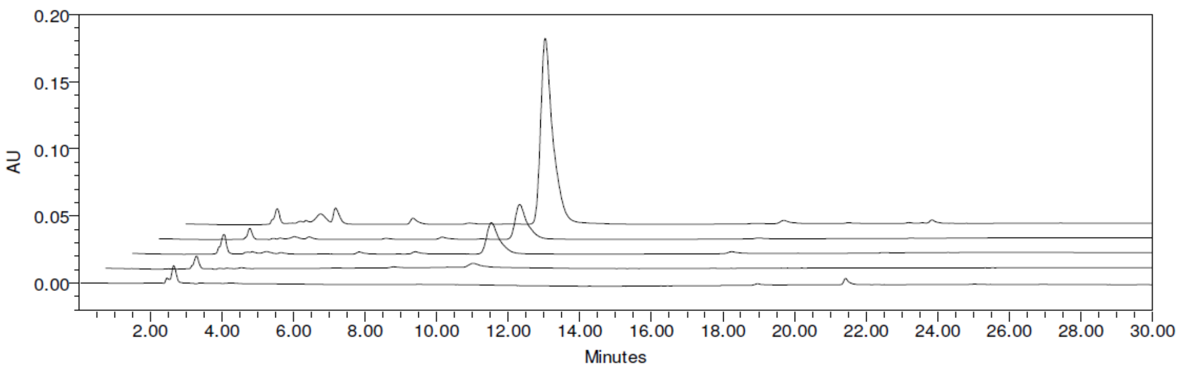
4.6) HPLC

4.6.1 Filtration of the chemicals for HPLC

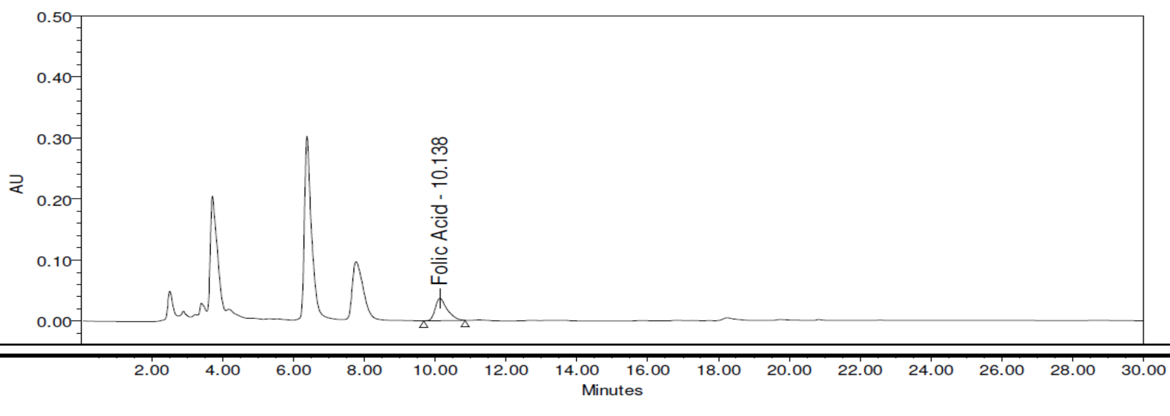
4.6.2 Filtration of cultures for HPLC

Snapshots of the HPLC results :-

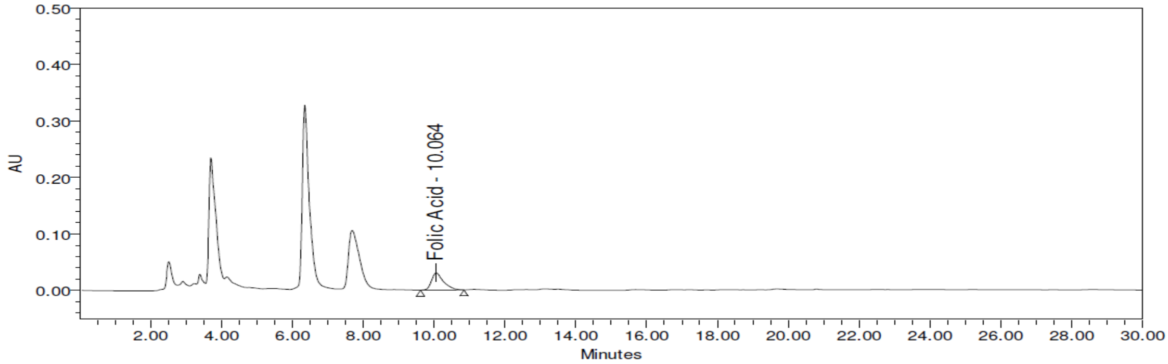
Snapshot 1:- Standard



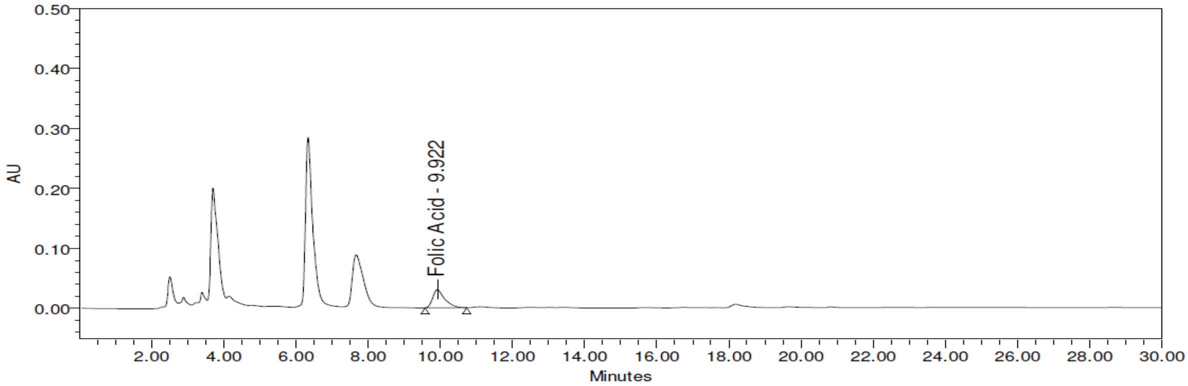
Snapshot 2:- ***Lactobacillus plantarum***



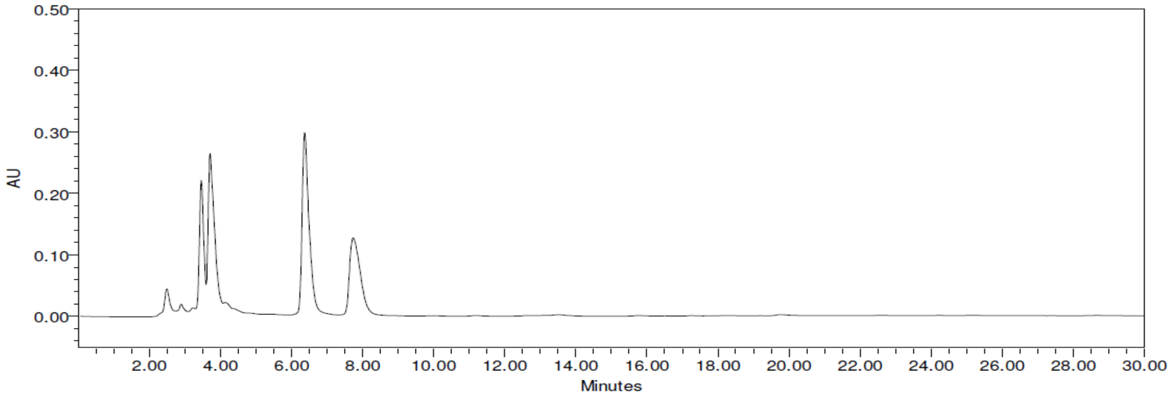
Snapshot 3: ***Lactobacillus rhamnosus GG***



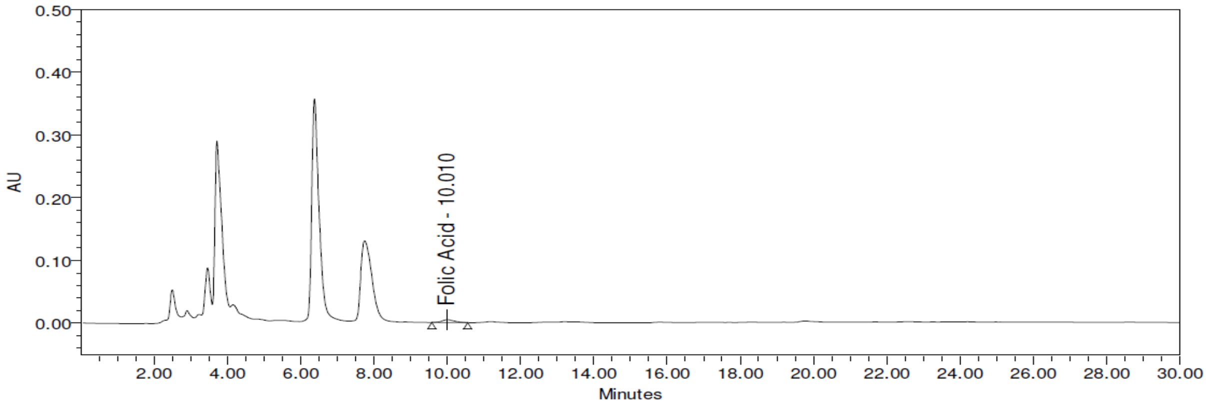
Snapshot 4*: AR1 Milk*



Snapshot 5 :- ***Lactobacillus casei***



Snapshot 6 :- AR6 Curd



Sample name	Retention time	Concentration (µg /ml)
Standard 1	**10.277**	**0.1**
Standard 2	**10.277**	**1**
Standard 3	**10.043**	**10**
Standard 4	**10.088**	**50**
Standard 5	**10.058**	**100**
Lactobacilus plantarum	**10.138**	**31.7**
Lactobacilus rhamnosus GG	**10.064**	**26.6**
AR1 Milk	**9.922**	**26.2**
AR6 Curd	**10.01**	**5.89**
Lactobacillus casei	**10.277**	**nil**

Discussion:- The HPLC of five standard samples and fifteen lactic cultures was performed. The highest folic acid producer was *Lactobacillus plantarum* followed by *Lactobacillus rhamnosus* GG , AR1 Milk. The lowest folic acid producer was AR6 Curd. No folic acid production was observed in *Lactobacillus casei.*

The absolute values from HPLC were lower than that of MA, probably because of the limitation of the UV detector to identify other folate derivatives. Therefore this suggests that HPLC with UV detector measures folic acid alone while MA measures total folate and threfore the difference represents the amount of folate derivatives except folic acid.

CHAPTER 5
CONCLUSION

Folic acid food fortification is very important in today's scenario. It will aid in reducing number of diseases such as neural tube defects, megaloblastic anaemia , cardiovascular diseases, miscarriages and other related diseases. In several studies it was found that pregnant women or anemic patients had folic acid deficient diet. The public awareness about folic acid and its use is less known. According to the survey conducted in the solan district majority of the population was not acquainted with the name and function of folic acid. The in vitro laboratory tests like microbiological assay and HPLC have shown *Lactobacillus plantarum* to be the highest producer of the folic acid. *Lactobacillus casei* was found to be the non producer of the same. Further , it is aimed to incorporate this bacteria into any suitable food matrix.. To conclude, folic acid fortification is the essence of the healthy rural India. It is a cost effective and sustainable approach to address micronutrient malnutrition.

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