NUTRACEUTICALS AS POTENTIAL ANTIOXIDANTS IN FERMENTED DAIRY PRODUCTS TO COMBAT PANDEMICS

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ABSTRACT: Nutraceuticals are gaining popularity due to their role in prevention and treatment of pandemic diseases. The stability of fermented foods using lactic acid bacteria (LAB) has been investigated by number of scientists. An isolated bacterial strain from homemade milk curd was used in the present investigation to prepare cow milk curd and soy milk curd and the nutrient contents of the curds were determined under different fermentation conditions to evaluate the nutraceutical potential of fermented milk products from animal and vegetable origins that made their importance in vivo. The B vitamins i.e., folic acid, riboflavin and thiamine contents were found to increase from 22 to 106.03 mg/g, 3.42 to 12.30 mg/g and 0.32 to 110.32 mg/g respectively, in cow milk curd and from 10 to 107 mg/g, 0.15 to 13.41 mg/g and 0.15 to 358.88 mg/g respectively, in soymilk curd. Structural changes in curd samples during fermentation were observed by SEM. Soy milk curd showed loosened microstructure. The degradation of proteins into peptides and amino acids were evaluated by SDS PAGE and amino acid analysis. Maximum production of amino acids i.e. cystine, histidine and asparagine were observed in both the cow and soymilk curd that can boost the immunity in our health. Therefore, such curd can be used as dietary antioxidant supplements for health benefit and to get rid of from pandemic diseases.

Keywords: *Streptococcus thermophilus, Lactobacillus spp.* Nutraceuticals, Milk curd, Soy curd, SEM, SDS PAGE, amino acid analysis, pandemics.

I. INTRODUCTION

Most probiotic food products in the market are milk based. These foods are well suited to promote the healthy image of probiotics. Consumers are familiar with the fact that fermented foods contain living microorganisms and if probiotics are used as starter organisms, this combines the positive images of fermentation and probiotic cultures [1]. A probiotic is a live microbial food supplement which beneficially affects the host animal by improving its intestinal microbial balance when consumed in appropriate quantities [2]. Other benefits include inhibition of bacterial pathogens, reduction of serum cholesterol levels, reduction of incidence of constipation, diarrhoea and bowel cancer, improvement of lactose tolerance, calcium absorption, vitamin synthesis and stimulation of immune system [3, 4].

Certain species of *Lactobacilli*, when included in the diets can have beneficial effects for humans. In order to provide a beneficial effect on the health of the host, it is necessary that the probiotic strain is able to survive in the gastrointestinal tract. To survive, the strain must be resistant to bile salts present in the lower intestine, gastric conditions (pH 1–4), enzymes present in the intestine and toxic metabolites produced during digestion [5, 6, 7, 8, 9, 10, 11]. It is therefore necessary to determine the number of *Lactobacilli* in faeces and, if possible, to identify the different species. Recently, a number of fermented dairy products have developed and marketed [12]. The utilization of lactose during fermentation can make the product easily digestible to lactose intolerant people.

Soy milk is consumed by people who can not consume cow milk due to lactose intolerance or allergy to milk proteins. Soymilk in association with probiotic bacteria can produce beverage having multiple functional properties. By the fermentation of soy milk with probiotic bacteria, the nutritional value of this product is extended and can be consumed by the people who do not consume milk [13].

The growth of lactic acid bacteria at different temperature and with or without addition of sugar have been investigated. Bacterial strains like *Lactobacillus casei*, *Bifidobacterium bifidum*, *Lactobacillus acidophilus* was found to grow better at 37 ºC than 43 ºC [14]. However, the growth of *L. acidophilus* was found to be worst. This may be due to the complex growth requirement of *Lactobacillus spp.* they require low oxygen tension, fermentable carbohydrates, proteins and their breakdown products, B complex vitamin, unsaturated fatty acids, minerals such as Mg, Mn and Fe for their growth [15]. Soy milk contains all the above nutrients except iron which may be the reason of poor growth [16]. Sugar beet pulp is a cheap source of iron and was used in this study to the extent of 2 % (w/w) without imparting any colour or flavour to the soy milk. The probiotic food with health claim should contain at least 10^6 cfu ml^{-1} of the bacteria because 10^8 – 10^9 of the viable cells is the minimum therapeutic dose per day. In order to improve the functional properties of fermented soy milk and cow milk by single and mixed culture of different lactic acid bacteria have been investigated. This also requires consumer acceptance of the product which is directly related to the textural characteristics of fermented food like soy milk and cow milk curd.

Textural characteristics of these products can be correlated well with their microstructures. Scanning electron microscopy (SEM) was used successfully by many investigators to reveal the microstructures of various semisolid gels like products such as yoghurt, soy curd, gelatin gel, bean curd and cheese [17, 18, 19]. Total solid content of cow milk was found to affect the textural characteristics of the product and so the consumer appeal. Lee and Rha (1978) [20] found that the physical and textural characteristics of the soy protein curds are related to their microstructure.

The aim of this work is to enhance the antioxidant properties of the fermented food products naturally i.e. in vivo with the use of culture of food grade LAB. So these studies are essential to develop a milk based fermented food from cow milk and soy milk using single or mixed culture of lactic acid bacteria to improve its nutritional value from the view point of vitamin content, bioavailability of protein and the problem of lactose intolerance. The present paper describes some of our observation on the above aspects.

II. MATERIALS AND METHODS

Microbial cultures

A strain of lactic acid bacteria was isolated from home made curd using plate and dilution technique and later was identified as *Streptococcus thermophilus* DG1 using DNA base composition by Bangalore Genei (India) (GenBank accession number BIS-08-072). Other lactic cultures i.e. *Lactobacillus plantarum* (MTCC 1325) and *Lactobacillus mesenteroides sub spp. mesenteroides* (MTCC 107) were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Preparation of curd from cow milk

Cow milk (Amul Dairy, Gujrat) and skim milk powder (SAGAR) was purchased from the local market at Jadavpur. 60 g of skim milk powder was mixed with 240 ml of cow milk, stirred well and heated to 40 ºC in a water bath. After cooling to about 37 ºC each of three 100 ml of this milk sample were inoculated aseptically with 2 % v/v of culture of *S. thermophilus* DG1 and 2 % v/v of a mixed culture (0.5:1.5 v/v) of *S. thermophilus* DG1 and *L. plantarum* previously grown in MRS broth with the following composition (g l^{-1}): Tryptone 10.0, Yeast extract 5.0, D-glucose 20.0, Tween- 80 1.0, Potassium dihydrogen phosphate 6.0, Ammonium citrate 2.0, Sodium acetate, hydrated 25.0, Glacial acetic acid 1.32 , Magnesium sulphate hydrated ($MgSO_4$, $7H_2O$) 0.575, Manganese sulfate, hydrated ($MnSO_4$, $4H_2O$) 0.14, Ferrous sulphate, hydrated ($FeSO_4$, $7H_2O$) 0.034. The pH was adjusted to 5.4 [21].

The inoculated milk samples were incubated at 37 ºC for 16 h to form curd. In this present study home made cow milk curd was used as control which was prepared by addition of 2 % v/v of a starter culture to the boiled and cooled milk at 37 ºC and allowed to incubate at this temperature for 16 h.

After the formation of curd, the curd samples were cut into pieces and stirred for about 10 min to drain off the whey portion through a fine cheese cloth and stored in a covered beaker at 4 ºC. The aluminium foil was cut into pieces and small amount of stored curd sample from different preparation was transferred into the petridish containing foil. Samples were then heated to 60 °C in a hot air oven for 2.5 h until the moisture in the semisolid curd gel was evaporated to dryness. Different samples covered in the petridish prepared in this way were kept in a desiccator (containing fused $CaCl_2$) to prevent for the moisture absorption.

Preparation of curd from soybean

Production of soy milk

The soybean were purchased from the local market and carefully selected to remove infected ones. The beans were then soaked in water for about 8 h, dehulled and again soaked in water overnight to absorb sufficient water (water/bean ratio 6:1). The soaked beans were grounded in a blender for 15 min and then boiled and cooled to destroy trypsin inhibitor and improve flavour, and then filtered through a fine cloth [22]. The produced soymilk was then heated to boiling and cooled to 37 °C and was used for the preparation of curd.

Preparation of soy milk curd

60 g of skim milk powder (SAGAR) was added to 240 ml of soymilk and mixed well and was homogenized. Each of 100 ml of soymilk and skim milk mix was inoculated with different ratios of lactic cultures (stated earlier) and also a mixed culture containing *S. thermophilus* DG1, *L. plantarum* and *L. mesenteroides sub spp. mesenteroides* in the ratio of 1:1:1 (v/v) along with 2 % (v/v) beet pulp and samples were incubated at 37 °C for 18 h to form soy curd.

After preparation of soymilk curd the samples were dried in a desiccator (method stated earlier).

Sample Preparation

1 g curd was taken in 25 ml distilled water and homogenized. Homogenized sample was used for determination of nutrient contents.

Determination of Riboflavin Concentration in Curd Samples

Concentration of Riboflavin in milk curd and soy curd prepared using different concentration of skim milk and different concentration of inoculum were determined by spectrofluorometric method of Kodicek et.al. [23]. 1 ml homogenized curd sample was used for this purpose.

Spectrophotometric Analysis of Folic Acid and Thiamine [24].

Folic acid and thiamine contents were measured as per procedures of USP VIII [24].

Microstructure examination by Scanning Electron Microscopy (SEM)

The dried samples in aluminium foil were fixed in 7 % glutaraldehyde for 4 h at 22 °C. Specimens were washed twice with deionised water, dehydrated successively in 20, 40, 60, 80, 95 % absolute alcohol respectively, defatted twice in chloroform and kept in absolute alcohol. At regular intervals fresh alcohol was added to the sample. The samples in the petridish were kept in hot air oven at 60 °C -70 °C for 1-2 h and then were placed in the desiccators. Each dried specimen was fractured to expose the internal structure, mounted in an aluminium stub coated with mica plate of 100 °A thickness. Microscopy was carried out on a JSM 6360, Tokyo, Japan; SEM at an accelerating voltage of 17 KV and pictures were taken on polarized film.

SDS-PAGE

The prepared curd samples were treated with NaOH solution of pH 12.0 for its complete dissolution. Aliquots (2 ml) were taken and mixed well with an equal volume of Tris buffer (pH 8-9) for stabilizing the protein containing ß-mercaptoethanol. After being centrifuged at 5000 rpm for 10 min, the supernatants were separated. SDS-PAGE analysis method as described by Geighton was used for the evaluation of protein degradation using gradient (10 % w/v) gels. The gel was 1.5 mm thick and consisted of a 2 cm stacking gel and a 10 cm running gel. 20 µg of protein was applied to sample slots. The period of electrophoresis was 3.5 h at 115 volt. After the end of electrophoresis, the matter from gel was eluted. Protein bands were stained with Coomassie Brilliant Blue in methanol/water/acetic acid (5:5:1, v/v), and then distained in the same solvent [25]. Standard protein markers were used. The molecular weights of different proteins (in k Da) were as follow: ovalbumin, 43.0; glutamic dehydrogenase, 55.0; bovine serum albumin, 66.2; phosphorylase b, 97.4; ß-galactosidase, 116.0; myosin, 200.0. The gel images were visualized and photographed using Photoshop version 7.0.

Analysis of amino acids of curd samples by Amino acid Analyser

Waters Pico·Tag amino acid analyser was used to determine the amino acids present in the curd samples.

III. RESULTS AND DISCUSSIONS

Vitamin Analysis

Curds were prepared from cow milk and soy milk using different concentrations of skim milk and inoculated with different concentrations of LAB. The riboflavin, thiamine and folic acid contents were determined. The maximum Riboflavin content was observed in both cow milk curd (12 µg/g) and soy milk curd (13.5 µg/g) using 1 % inoculum (Fig. 1, 4).Thiamine content was maximum in cow milk curd (110 µg/g) and soy milk curd (350 µg/g) prepared using 1 and 2.5 % of inoculum, respectively (Fig. 2, 5). Folic acid content was found to be maximum in both cow milk and soy milk curd (110 µg/g and 108 µg/g respectively) when 1 % (v/v) inoculum was used for their preparation (Fig. 3, 6). Fresh soybean milk contains almost negligible amount of folic acid but, after fermentation with LAB, the folic acid content increased to an appreciable level of 107 mg/g in soy milk curd.

Microstructure modification of curds during fermentation by different lactic acid bacterial strains with addition of skim milk were studied. The microstructure of both the milk curd and soy curd during fermentation stages was examined by SEM technique. Scanning electron micrographs obtained are shown in the figures (Fig. 7-12.). In Fig. 7, the texture was uniform. The average size of pores was smaller and appeared to be uniformly distributed. After addition of inoculum 2 % (v/v) *S. thermophilus* DG1, some of the pores became bigger but the rest of the pores in the surface were uniform in size (Fig. 8). Fig. 9 showed non uniform structure of the milk curd because of the addition of mixed lactic cultures. A very interesting observation was from Fig. 10 of soymilk curd where uniform pore size had appeared. Cracks were appeared in the few places on the curd surface. Addition of the Beet pulp (2 % v/v) into the mixture of soymilk- skim milk inoculated with the lactic cultures showed an interesting observation in Fig. 11. The pore size became bigger, possibly indicating that significant interaction of iron from beet pulp with the soy curd.

Proteolysis of curds during fermentation

It was well known that the degradation of proteins would lead to the formation of peptides and free amino acids. Such fragments could be monitored with many techniques. The degradation of proteins and formation of peptides (biogenic peptides) in curd samples were evaluated with SDS-PAGE. Earlier scientists have also studied primary proteolysis, secondary proteolysis and changes in hydrophilic (HI) and hydrophobic (HO) peptides during ripening of a goats' milk cheese (Murcia al Vino) manufactured with plant coagulant (PC) and calf rennet (AR) [26]. The distribution of protein bands for curd samples are shown in Fig. 12. It can be seen clearly that proteolysis of curd protein occurred during fermentation. Molecular weights of protein subunits range from 17 to 72 k Da in the ascending direction from bottom to top characterized by band lane 0 and lane 3. With the progress of fermentation cow milk and soy milk proteins in the curd samples were degraded by proteases. Most of the peptides in curd proteins had molecular weight less than 20 k Da, indicating that those proteins in soy curd and cow milk curd were degraded into small peptides. Casein components of curd samples were obtained as a broad band (Lanes 1 and 3) indicating that small peptides have been generated during separation which have been eluted from the protein gel and most of them have < 20 k Da of molecular weights where as whey proteins, serum albumins and other proteins were clearly visualized in the protein gel which indicated that their molecular weights were > 20 k Da and they have not been degraded before loading to the gel (Lane 0 and 2). At pH 12.0, possibly our casein protein forming the curd gel had been degraded extensively to form the polypeptides of smaller molecular weights. When this solution was put into the SDS gel at pH 7.0, the degraded proteins had been separated in the gel on application of electrical field which have been shown in Fig. 12.

Amino acid Analysis

From Table 1, values of chromatographed peak results, the percentage of amino acids (in terms of picomol) were calculated with respect to the reference values and it was shown that the amino acids viz. cystine, histidine and aspartic acid and asparagine were present in both types of curd samples and found to be increased significantly as shown in Table 1. The concentrations of amino acids (Table 1) vary with the different ratios of inoculum added to the curd. Interesting observation was that soy milk curd (inoculated with 1:1:1 v/v of *S. thermophilus* DG1, *L. plantarum* and *L. mesenteroides* in addition to 2 % beet pulp) had maximum amount of amino acid concentration compared to other probiotic curd samples. This is possibly due to the iron fortification with addition of beet pulp in the soymilk curd. Table 1 gives values of concentration of amino acids present in the curd sample.

IV. CONCLUSION

The incorporation of lactic acid bacteria (LAB) in milk during preparation of curd improved the nutritional value of both cow milk and soy milk curds provided the culture maintained its viability. The LAB produce B vitamins during fermentation of milk.

The present study demonstrated that during fermentation of curd (cow milk curd and soymilk curd) with the mixture of lactic cultures, the texture was modified and the nutritional characteristics were informed. There was significant difference in hardness in terms of consistency. Photographs of SEM showed that protein network was destroyed during fermentation with homogeneous and dense microstructure formed in curd samples. Results from SDS-PAGE analysis indicated that casein was degraded into small biogenic peptides (molecular weight < 20 k Da) during fermentation. Amino acid analysis also showed the production of increased amounts of amino acids in the curd samples leading to the improvement in nutritional and antioxidant quality of cow milk and soy milk by mixed culture fermentation.

V. FUTURE SCOPE

Nutraceuticals are essential for health benefit. Future study could show the production of natural drugs by using nutraceuticals that will be beneficial for combating pandemics by boosting our immunity. By forfitication of curd with plant materials may also enhance the immunity in vitro. Microbial fuel cell can be generated by using *lactobacilli* as immune booster.

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CONFLICT OF INTEREST

We have no conflict of interest with our research work to any other else.

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Table legends

Table1. Concentration of amino acid in curd samples

Figure legends

Fig. 1. Riboflavin content in cow milk curd

Fig. 2. Thiamine content in cow milk curd

Fig. 3. Folic acid content in cow milk curd

Fig. 4. Riboflavin content in soy milk curd

Fig. 5.Thiamine content in soy milk curd

Fig. 6. Folic acid content in soy milk curd

Fig. 7. Milk curd inoculated with 2% (v/v) *S. thermophilus* DG1

Fig. 8. Milk curd inoculated with 0.5:1.5 (v/v) of *S. thermophilus & L. plantarum*

Fig. 9. Soy curd inoculated with 2% (v/v) *S. thermophilus* DG1

Fig.10. Soy curd inoculated with with 0.5:1.5 (v/v) of *S. thermophilus* DG1 *& L. acidophilus*

Fig. 11. Soy curd inoculated with with 1:1:1 (v/v) of *S. thermophilus* DG1, *L. plantarum* & *L. mesenteroides* in addition to 2 % beet pulp

Fig. 12. SDS-PAGE analysis of curd proteins. Lane M, protein markers with molecular weight ranged from 17 to 173 kDa; Lane 0, soy (whey) protein, Lane 1 soy curd protein, Lane 2, cow milk (whey) protein, and Lane 3, cow milk curd protein.

Table . 1Concentration of amino acid in curd samples

Amino acids	Concentration of amino acids present in curd samples (%)			
	Cow milk curd (prepared with 2% v/v *S.thermophilus*)	Cow milk curd (prepared with 0.5:1.5 v/v of *S.thermophilus* DG1 and *L.plantarum*	Soy milk curd (prepared with 0.5:1.5 v/v of *S.thermophilus* DG1 and *L.plantarum*)	Soy milk curd (prepared with 1:1:1 v/v of *S.thermophilus* DG1, *L.plantarum* and *L.mesenteroides* with 2 % beet pulp)

Asparagine	20.03±1.18	17.91±1.89	22.59±2.55	24.29±2.56
Serine	3.80±0.16	5.11±1.02	6.23±1.44	4.75±1.47
Glycine	0.14±0.03	1.52±0.16	1.19±0.25	1.78±1.36
Histidine	15.04±1.54	17.44±2.54	20.04±2.69	14.50±2.85
Arginine	1.33±0.25	5.35±1.22	4.03±0.25	4.13±2.11
Proline	4.71±0.55	0.16±0.01	2.25±0.48	0.001±0.000
Tyrosine	2.04±0.14	2.27±1.13	2.02±0.36	2.88±0.49
Valine	4.36±0.12	6.78±1.25	6.76±1.55	6.29±1.17
Methionine	1.05±0.02	0.92±0.04	0.53±0.02	1.20±0.15
Cystine	32.06±2.56	20.11±2.36	14.65±2.95	21.79±2.55
Isoleucine	3.16±0.45	13.53±1.55	1.78±0.25	1.82±0.54
Leucine	5.34±0.12	1.72±0.23	9.19±1.33	7.73±1.69
Phenylalanine	2.74±0.06	3.39±0.58	3.56±0.58	3.98±1.03
Lysine	4.15±0.11	3.87±1.08	5.22±1.42	4.90±1.27

Data presented are average values. ± indicates standard deviation (SD) of n = 3 experiments

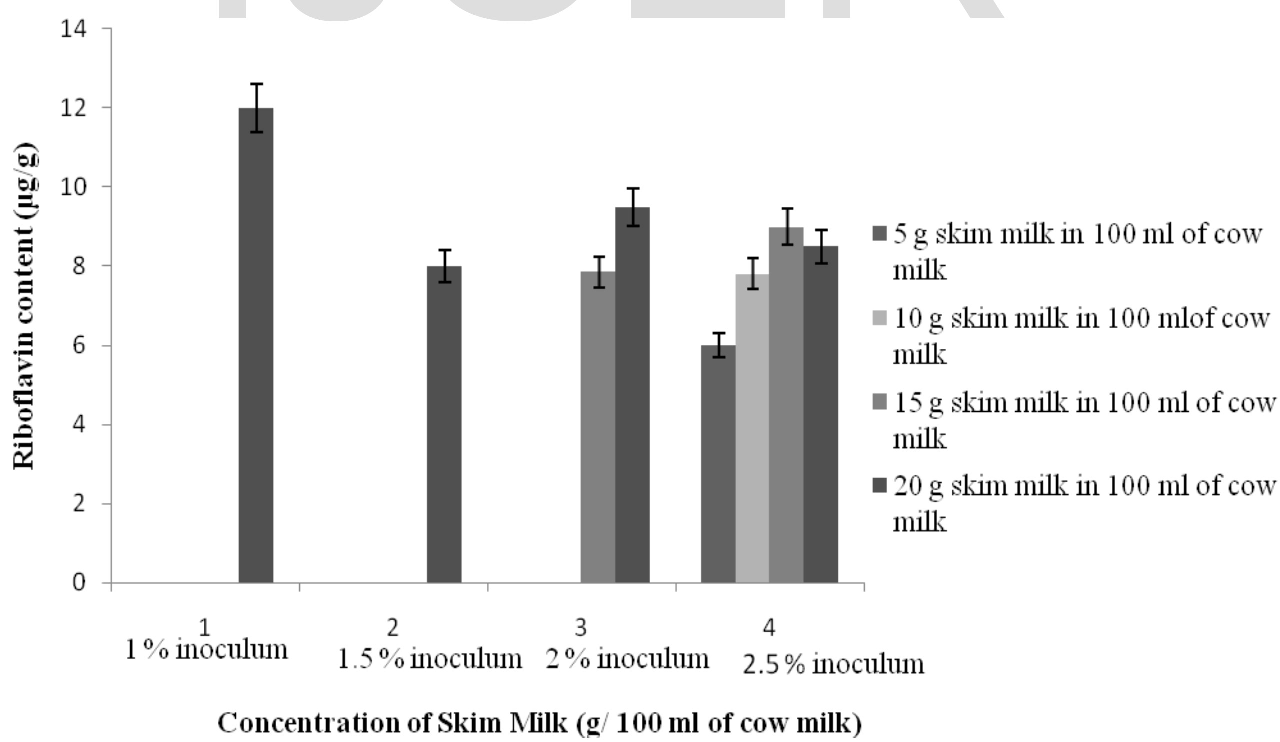

Fig 1. Riboflavin Content in Cow Milk Curd

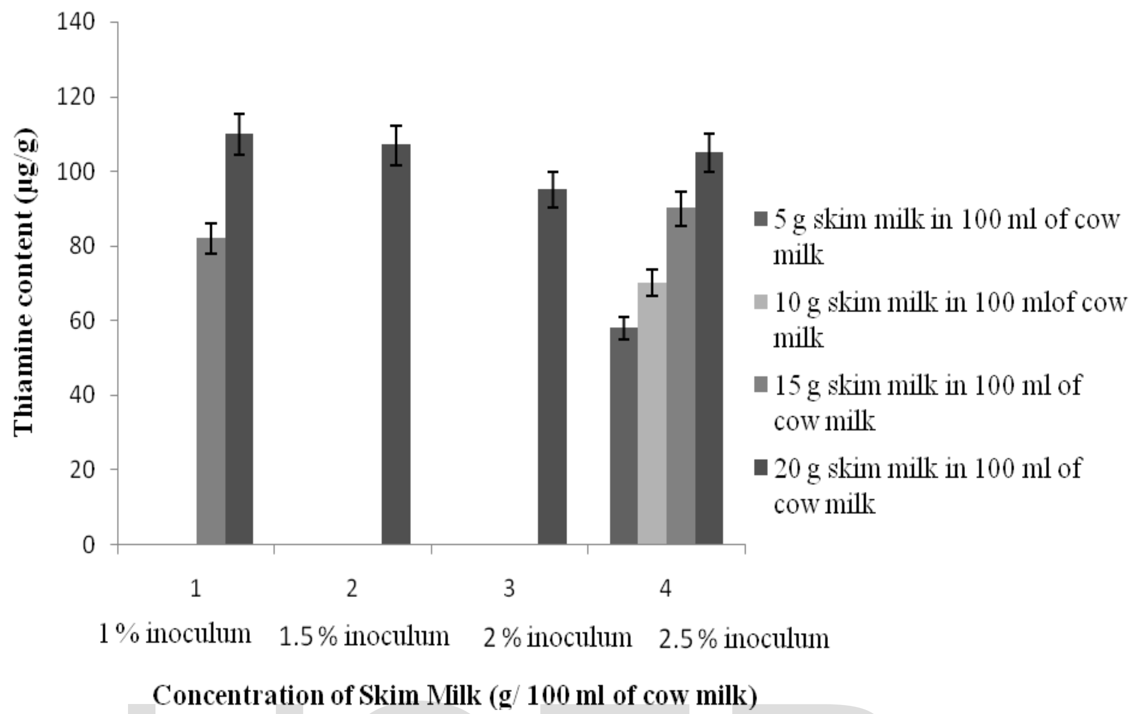

Fig 2. Thiamine Content in Cow Milk Curd

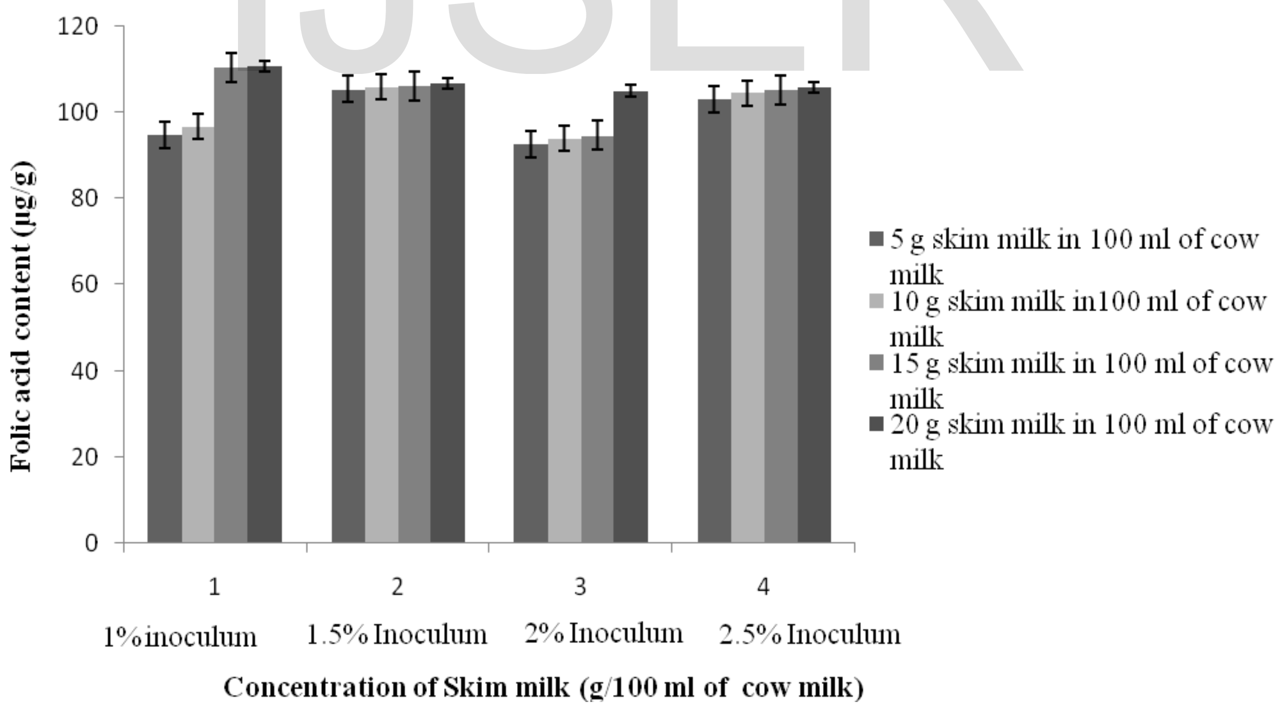

Fig 3. Folic Acid Content in Cow Milk Curd

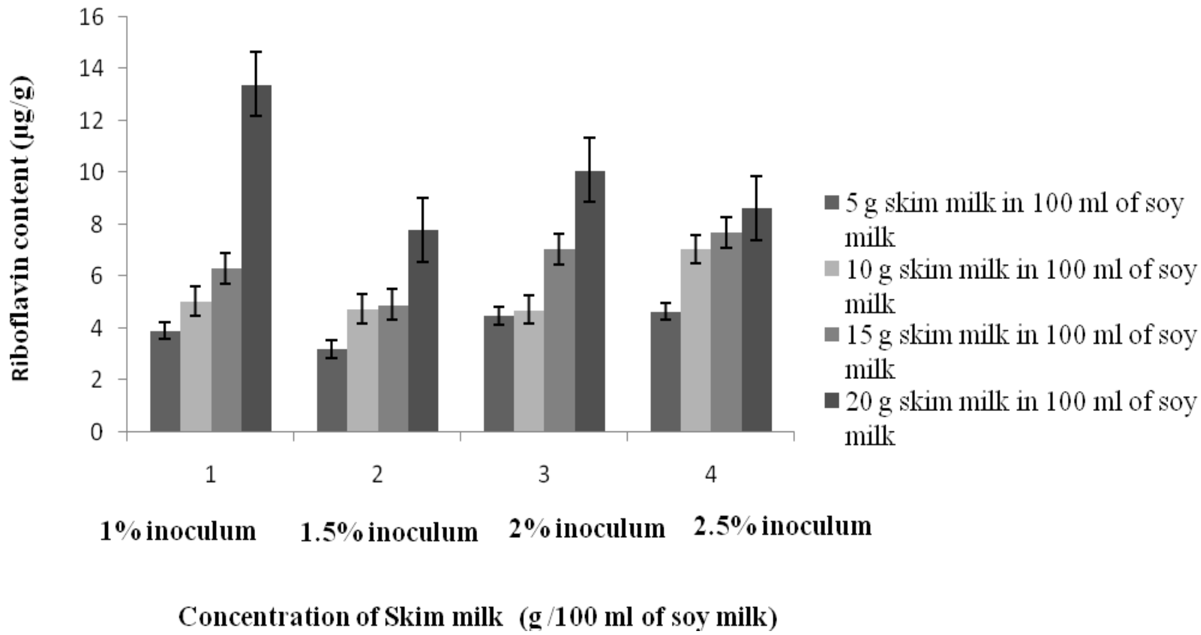

Fig 4. Riboflavin Content in Soy Milk Curd

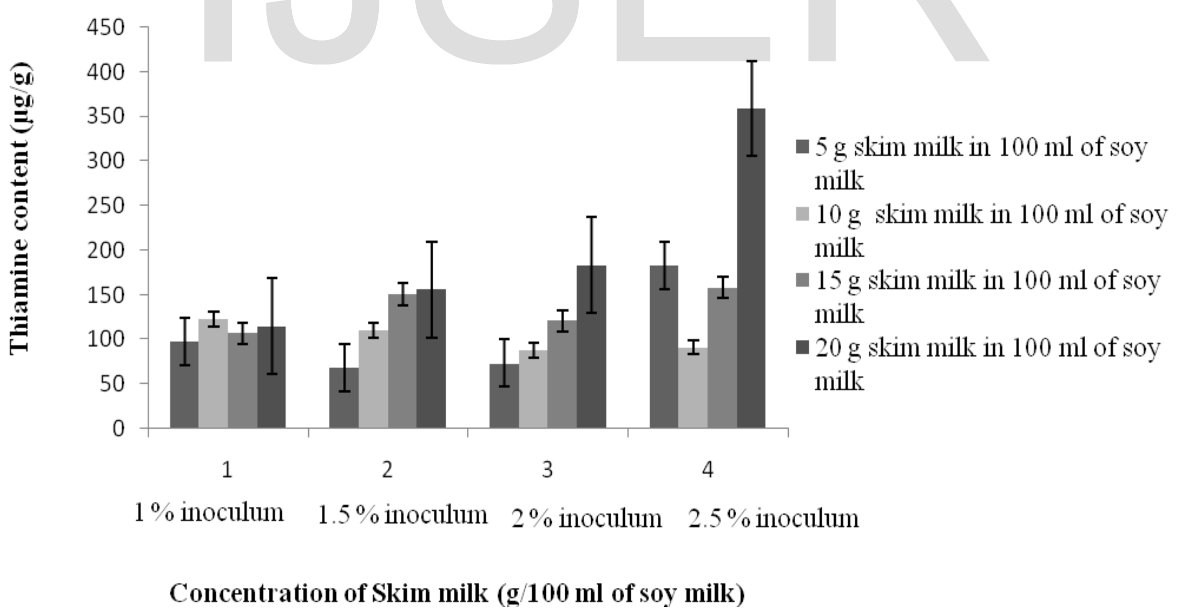

Fig 5. Thiamine Content in Soy Milk Curd

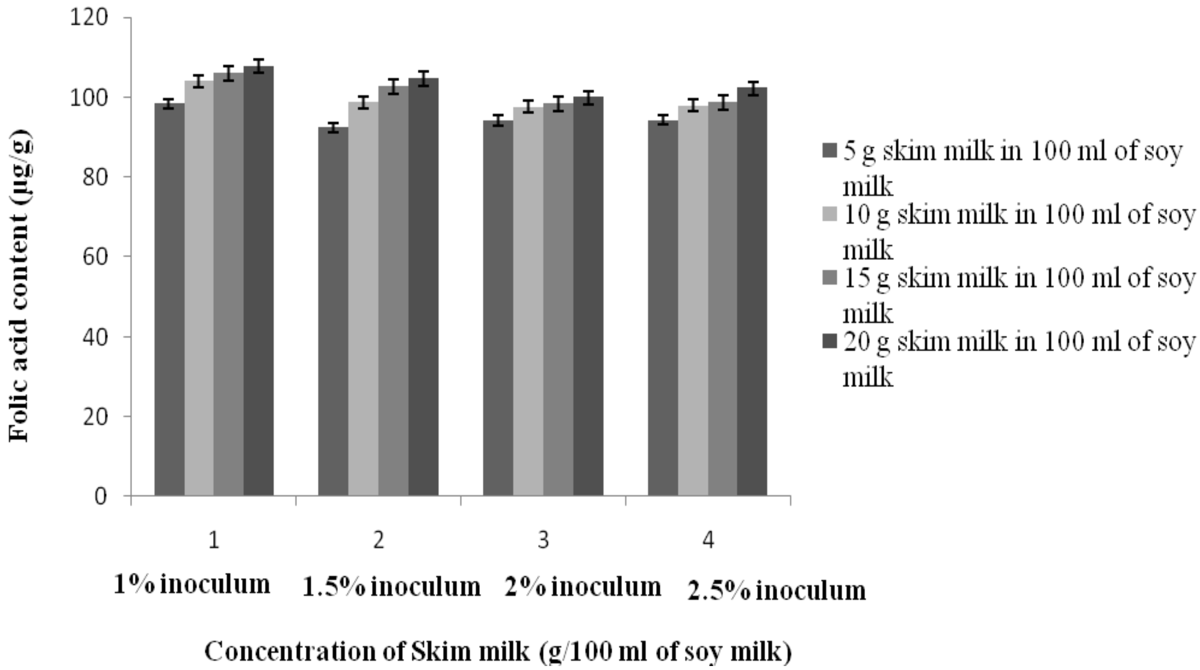

Fig 6. Folic Acid Content in Soy Milk Curd

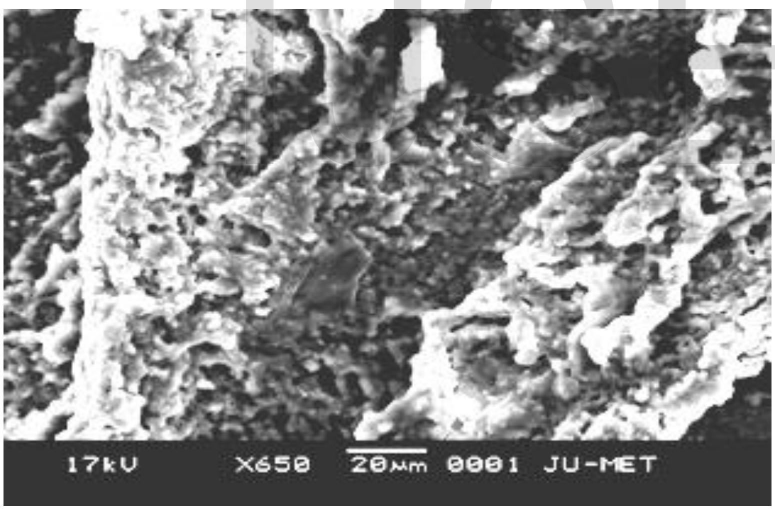

Fig. 7. Cow milk curd prepared with 2% (v/v) *S. thermophilus* DG1. Photograph shows uniform texture of the curd sample.

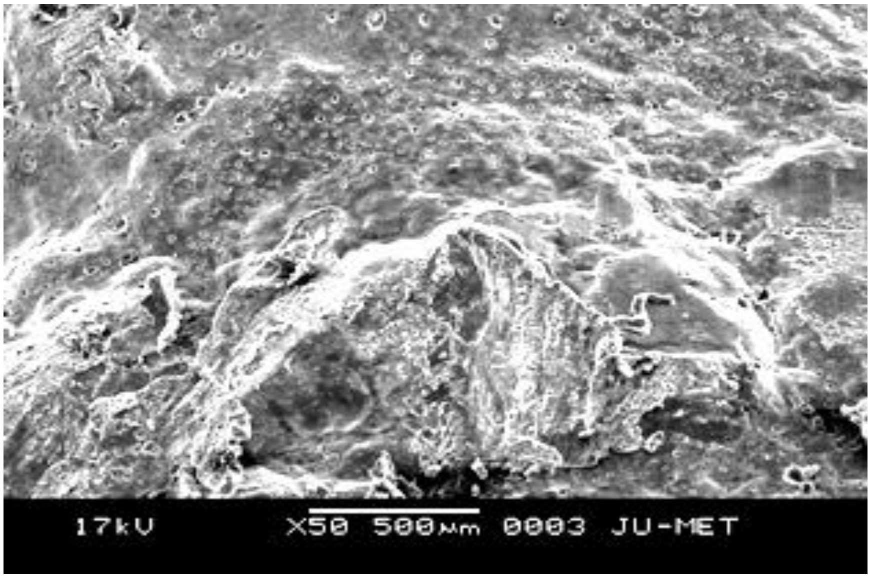

Fig. 8. Cow milk curd prepared with 0.5:1.5 (v/v) of *S. thermophilus* DG1 *& L. plantarum.* Photograph shows non uniform texture of the curd sample.

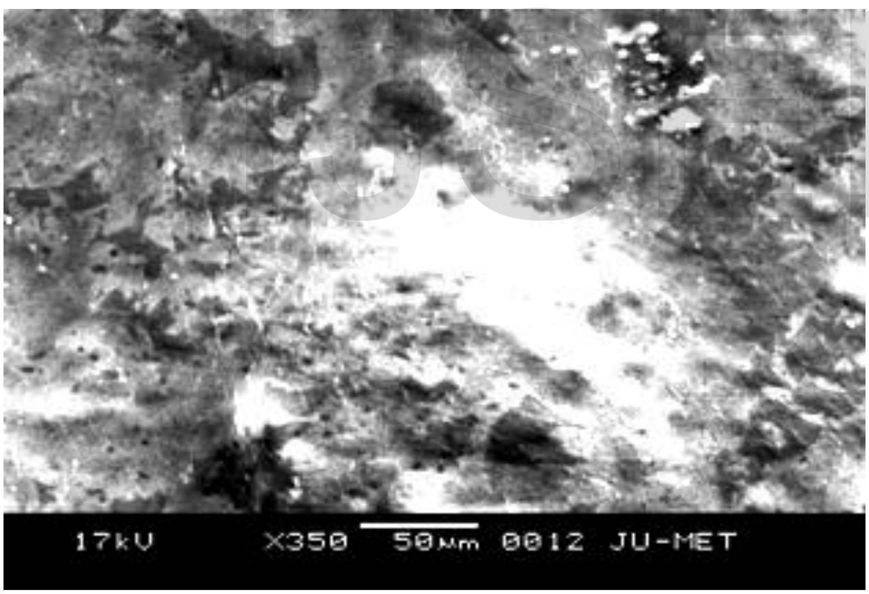

Fig. 9. Soy curd prepared with 2 % (v/v) *S. thermophilus* DG1. Photograph shows non uniform texture of the curd sample.

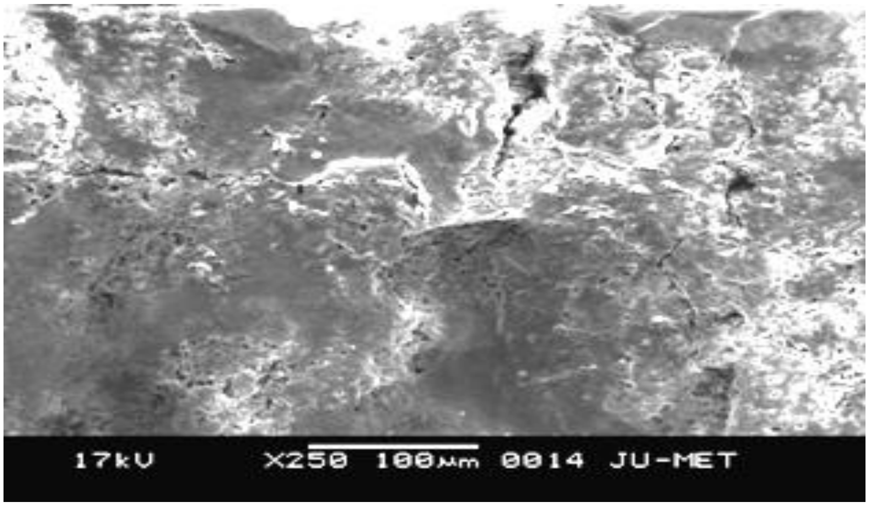

Fig. 10. Soy curd prepared with 0.5:1.5 (v/v) of *S. thermophilus* DG1 *& L. plantarum* showing uniform pore size.

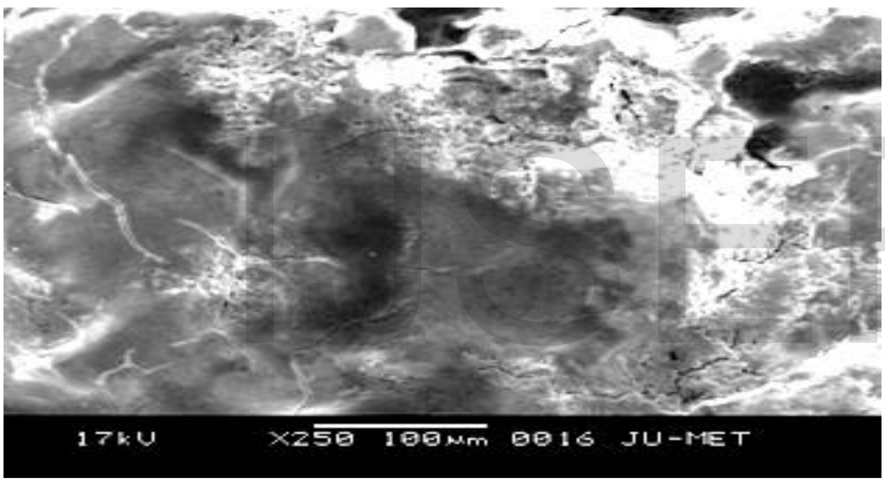

Fig. 11. Soy curd prepared with 1:1:1 (v/v) of *S. thermophilus* DG1, *L. plantarum* & *L. mesenteroides* in addition to 2 % beet pulp showing appearance of few cracks in the texture.

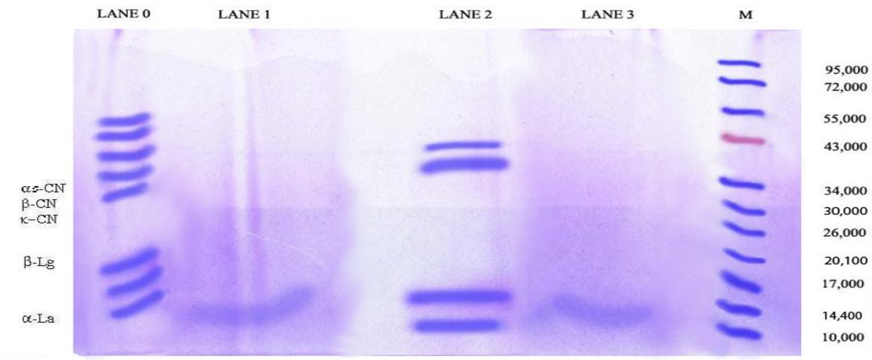

Fig. 12. SDS-PAGE analysis of curd proteins. Lane M, protein markers with standard molecular weight ranged from 10 to 95 kDa; Lane 0, soy (whey) protein, Lane 1 soy curd protein, Lane 2, cow milk (whey) protein, and Lane 3, cow milk curd protein. The SDS-electrophoretic profile of the milk proteins (lane 0) contains five major bands identified as α s–casein, ß-casein, α –La, 71 kDa-band, and three minor bands identified as β-Lg, κ-casein.