FORMULATION OF SYNBIOTIC FERMENTED SOYMILK PRODUCT

Project report submitted in partial fulfillment of

Degree of Bachelor of Technology

in

BIOTECHNOLOGY

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2017

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Certificate of Originality

This is to certify that the work titled "**Formulation of synbiotic fermented soymilk product**" submitted by **Shagun Choudhary** and **Manisha Singh** in partial fulfillment of the requirements for the award of degree of Bachelors of Technology in Biotechnology, of Jaypee University of Information Technology, Solan, has been carried out under the supervision of **Dr. Gunjan Goel**. 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.

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Acknowledgement

All praise belong the almighty lord to whom we thank for the strength, courage and perseverance bestowed upon us to undertake the course of the study. We feel privileged to express our deep sense of gratitude to our revered mentor, **DR.GUNJAN GOEL**, Associate professor, biotechnology, for valuable guidance, suggestions, support, constant encouragement, cooperation and providing requisite facilities to carry our project which otherwise would have remained incomplete. We gratefully acknowledge with deep gratitude, the cooperation and help given by **Ms**. **Kavita Sharma, Mr. Sampan Attri, Mr. Deepak Sharma** and all members of Jaypee University in helping with our project. We feel indebted to **Dr. Rajinder Chauhan**, Dean and Head, Department of Biotechnology and Bioinformatics, Jaypee University Of Information and Technology for his support and guidance . We would like to thank our institution and members of technical and non-technical staff of the department without whom this project will be a distant reality.

Shagun Choudhary

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Abstract

Soy milk, an aqueous abstract of soybean, and its fermented product contain great biological properties and are found to be a good source of bioactive peptides, isoflavones and can be utilized as a health enhancing ingredient in functional foods. A collection of sixteen strains of indigenous Lactic acid bacteria isolated from different fermented foods were evaluated for their acidification rate. The lactic cultures were evaluated for acidity using Horral Elliker's test. The pH of the fermented milk dropped from 6.8 to 6.19 with increase in acidity from 0.2 to 0.4 after incubation for 4 hrs. Therefore, from the sixteen strains, only six strains were observed as active starters which have low fermentation time and were used for further studies. A 100 ml of soymilk was fermented by 200µl different lactic cultures and was incubated overnight for fermentation at 37ºC. After fermentation, pH and titratable acidity of fermented milk was determined. For the maximum proliferation of LAB, two prebiotics (fructooligosaccharide and inulin) were screened at a concentation of (0-1.5%) in soymilk. The effect of prebiotic was evaluated by viable plate assay on MRS agar after 24 h of fermentation of soymilk. Both the prebiotics (FOS and inulin) had similar affect on the LAB count with an increase of log cfu from 6.00 to 7.89 at 1.5%.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BSA	Bovine serum albumin
CFU	Colony Forming Unit
DNS	Dinitrosalicylic acid
FC	Folin Ciocalteu
FOS	Fructooligosaccharide
GRAS	Generally Regarded As Safe
GI	Gastro intestinal
HDL	High density lipoproteins
HPLC	High Performance Liquid Chromatography
LDL	Low Density Lipoproteins
LAB	Lactic Acid Bacteria
ml	Millilitre
M	Molar
mg	Milligram
mM	Millimolar
N	Normal
nm	Nanometer
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
Rpm	Revolution per Minute
ssp.	Subspecies
sp.	Species
µl	Microlitre

1.Introduction

Soybean: Soybeans, one in all the most rich plant sources of dietary protein, contain thirty six to fifty six percent of protein. Latest studies showed that, an aqueous extract of soybean, soymilk , and its fermented product have vast biological properties and are a good supply of bioactive peptides and can be used as a health enhancing constituent in functional foods. It is an brilliant source of peptides, have antihypertensive, anti-cholesterol, and antioxidant behavior, and appear to avert cancer. Significance in soy isoflavones is based on statistics suggesting potential in preventing prostate, lowering cholesterol levels, and breast cancers, osteoporosis, cardiovascular disease and is also helpful in relieving menopausal symptoms. The peptides dispensed from soy protein in the GI tract significantly increases their nutritious effects. The bioactive peptides formed by soybeans possess varied and sole health benefits.

Benefits of Soy Milk:

- **Improvement in lipid profile** - The foremost significant feature of soy milk is its capability to enhance your blood lipid profile. Soymilk fat is mainly unsaturated fat with zero cholesterol level, unlike dairy milk, which comprises high saturated fat and cholesterol level. The fatty acids in soy mik (mainly monounsaturated and polyunsaturated) can block the transportation of cholesterol into your blood stream. According to studies conducted, habitual intake of soymilk or its product can drastically decrease the LDL and triglycereide level in blood and raise the level of HDL. If one has high cholesterol or family record of coronary heart diseases then soymilk is an ideal drink.

- **Blood vessel integrity is strengthen** – The presence of omega-3 and omega-6 fatty acids along with influential phyto-antioxidants in soymilk can efficiently protect blood vessels from lesions and hemorrhage. These compounds shield the lining cells from free radical attacks and cholesterol deposits by binding themselves to the blood vessel lining. The binding of these nutrients leads to an

increase in the fluidity and elasticity of blood vessels so that they are much more lasting to blood pressure changes.

- **Aid in weight loss** – There is naturally low sugar content in soymilk, unlike regular milk. In comparsion to soymilk which contain only 7 grams of sugar per cup , cow's milk contains around 12 grams of sugar. The cup of complete soymilk has only 80 calories, which is the alike of skim milk. In addition, soymilk contain monounsaturated fatty acid which helps in weight loss by reducing the intestinal absorption of fat. Consumption of soy milk also gives the belief of fullness for longer time as it gives more doses of fibre.

- **Prevention from osteoporosis** - An additional age and hormone related disease is Osteoporosis. Soymilk contain phytoestrogen which avoid the damage of bone mass and aids in gather speed of absorption of calcium by body. For the utmost advantage, one should be certain to purchase the soy milk that contain extra calcium and vitamin D.

- **Pregnancy –** Women hoping to get pregnant are excepted to have same benefits as other women do so intake of soy products for the period of pregnancy can be encouraged. Only reliable dietary bases of vitamin D is Fortified milk and fortified soymilk. All other dairy products contain slight or no vitamin D. Soymilk can be alternative for women who are susceptible to the sun or for those who don't like being outdoors to receive enough vitamin D. Women who don't like normal milk , Soymilk can may also be an substitute.

- **Cardiac -** Serum concentrations of cholesterol, LDL and trigylcerides have been found to decrease by the consumption of soymilk rather than using animal protein thus benefiting indiviulas that have high cholesterol. Although numerous theories exist but the exact method of mechanism is not recognised . One of the theory proposed that absorption of cholesterol is impaired or changed. Other theory postulate that phytoestrogens bind to estrogen receptors and create alike effects including lowering LDL and increasing HDL, vasomotor tone changes, and arterial wall function.

- **Obesity and Diabetes –** According to newest studies, soy protein contributes to the control of hyperglycemia and lowering body weight, hyperlipidemia, and hyperinsulinemia. These characteristics may be valuable to both nondiabetic and diabetic persons in the managing of obesity and blood sugar.

- **Helps in Prevention form Cancer -** Genistein, which is one of the phytochemicals found in soy, can decrease the threat of cancer. Development of cancer is hindered by stopping tumors from creating blood vessels that is essential for nourishment for their growth. One portion a day i.e. one cup of soymilk is efficient for cancer avoidance .

- **Menopausal Symptoms -** Phytoestrogens acts like synthetic estrogen that guards women from bone loss and keep up a healthy heart this therefore determines its value. Bone and calcium balance in postmenopausal women is also influenced by soy protein derived from soymilk and its product. Women who were not getting hormone replacement therapy for them outcomes were markable. In the case of young, healthy women who were still menstruating results were not same .

- **Due to lack of galactose in soymilk, it can easily replace breast milk for children with galactosemia** - A little percentage of children are born with a situation known as galactosemia, a condition in which digestion of any type of animal milk is prevented, including breast milk for those group of individuals soy is a safe choice.

- **Safe for people with lactose intolerance, or milk allergy** - Milk and dairy products consists of milk sugar called lactose, which is made from two sugar components, glucose and galactose, it is necessary for the body to split lactose into its two sugar components which can then be absorbed into the bloodstream. If these two sugars reside connected, uncomfortable symptoms like bloating, gas, loose stools and stomach pain can result which results into lactose

intolerance. Soymilk does not consists of lactose as it is derived from the soybean plant.

Probiotics are nutritional supplement containing potentially valuable live microorganism (bacteria or yeasts), which when managed in ample amounts, provide a confer health advantages to the host. They are directed in diverse quantities that allows for different colon colonization. These products assist in bracing health encouraging flora and suppressing the pathologic colonization and disease spread. Probiotics diverge in their capability to ferment the bioactive fibres. To produce fermented milk with hypotensive activity, many industrially utilized dairy starter cultures which are proteolytic in nature to some extent are used. e.g .*Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus thermophilus*. The proteolytic system of lactic acid bacteria (LAB) is very well characterised.

Prebiotics are non-digestible substances, which offer promising physiological effect on the host by selectively promoting the positive growth or activity of a limited number of indigenous bacteria in the colon that develop host health. Key targets for the prebiotic approach are bifidobacteria and lactobacilli, as these intestinal bacteria show numerous helpful effects upon humans. A food product which contain both probiotics and prebiotics is named as synbiotic which result in a rise in the probiotic count and the lessening of pathogen microorganisms in the gut. The sensory profile, physicochemical and rheological features of probiotic-fermented products is improved by manifestation of prebiotic.

Drawbacks linked with the fermented milk, mainly the rising frequency of the lack of tolerance to lactose and the level of cholesterol, made it necessary to discover some other non-dairy sources as appropriate substrate for the probiotics. Soy is an brilliant raw material for the increase of probiotic non-dairy functional foods to beat the

restrictions linked with dairy products. On the basis of these drawbacks our project was designed with two objectives as follows:

a) Preparation of soymilk and its analysis along with evaluation of acidification rate of lactic cultures
b) Effect of prebiotic on growth of selected probiotic culture and characterization of synbiotic product

2.Literature Review

There are over 400 recognized bacterial species in the biomass that produce powerful metabolic action and are of great significance for human health. This leads to devastation of helpful bacteria accept the ones which are resistant, pathogenic. This ecology gets interrupted when visible to toxics in the form of polluted water or food as well as use of antibiotics then this ecology gets disrupted [1,2]. Awareness of complex correlation between food and health has challenged researches to propose functional food that not only provide basic nutrition but also has health benefit properties [3].The utilization of foods and beverages containing these health benefiting probiotic microorganisms is a growing worldwide trend [4].

2.1 Probiotics are living microorganisms which, when ingested in ample amount, helps in providing different health benefits to the host [5]. Probiotic bacteria should be able to resist low pH and stay alive in gastric pH, they should be able to stand in the intestinal tract that contains bile salts in specific concentrations, they should be able to cling on to the intestinal mucosal cells, should possess long shelf life, should substitute and restore the intestinal microflora and they should provide clinically proven benefits.

2.2 Prebiotic is a non-digestible substance, such as FOS and inulin, used as safe component supplements to fermented milk [6] that offers helpful physiological effect on the host by selectively improving the complimentary growth or activity of a narrow number of indigenous bacteria [7]. Addition of prebiotics also increase the sensory outline, physicochemical and rheological quality of probiotic-fermented products [8]. According to recommendations the capability of probiotic bacteria to ferment oligosaccharides can be an important characteristic because microflora that becomes conventional in the colon is majorly influenced by the ease of use of carbohydrates that escape adsorption and metabolism in the small intestine [9, 10, 11]. Some carbohydrates, for instance oligosaccharides, are fermented only by particular strains of bifidobacteria and lactobacilli, then diets comprising so-called "prebiotic" substrates could go for those strains of probiotic bacteria. A food product that contains both probiotics and prebiotics is termed as symbiotic [12] which consequently increases the probiotic count and it also lessens undesired microorganisms in the gut [13].The capability of microorganisms to utilize prebiotics

is strain- and substrate-specific. Because of that, one of the essentials for good synbiotic formulation is a proper choice of pro- and pre mixture. Various food products have been developed that contains probiotics, mainly of dairy origins because consumers are usually associated with fermented dairy products and take in health benefit imparted by probiotic cultures incorporated in the product [14].

There are few drawbacks linked with fermented milk that includes the rising occurrence of the lactose intoleranc, the level of cholesterol, high frequency of hypertension, which is one of the predominant risk factors of coronary heart disease, indicates an urgent need for prevention and choice of therapy which make necessary to look atsome other non-dairy sources as appropriate substrate for the probiotics [15].Soy is a brilliant raw material and a substitute of milk for the growth of probiotic in non-dairy functional foods to overcome the limitations related with dairy products. The benefits of soy have attracted the attention in recent times and abundant soy productshave been evaluated as likely probiotic vehicles. Soybean based foods are of great interest because of the confirmation that use of soy proteins 25 gram per day can lower the risk of cardiovascular disease, an sign approved by the U.S Food and Drug Administration in 1999.

2.3 Soy and Soy-based products is an outstanding raw material for the improvement of probiotic non-dairy functional foods to overcome the drawbacks related to dairy products. Soybeans provides high-quality proteins and carbohydrates but are devoid of lactose and cholesterol. It is an abundant resource of nutrition for milk allergy patients, or those who are lactose-intolerant [16]. Soy bean protein, due to its undigested pepsin fraction may have an effect on the fecal excreta of bile acids and also influences the cholesterol metabolism [17].

Soy has an significant impact on the host's well-being as it is an vital source of many other nutrients including dietary fiber, proteins, oligosaccharides, vitamins and trace minerals. Due to presence of oligosaccharides, soy exhibits its significant biological and functional role to meet the prebiotic standards. Soy bean, high quality protein could serve as a complete source of protein for vegetarians [18]. Soybean has 20% oil contents, 40% protein, 15% saccharides, 15% dietary fiber and 10% others [19].

Soy based products decrease menopausal symptoms, and also avoid, lessens and cure diseases such as cancer, atherosclerosis and osteoporosis [20]. Soy milk contains phytoestrogen that establishes shielding effect against age-related bone loss and other chronic diseases. It is rich in iso-flavonoids and is associated with lowering risk of many diseases, such as prostate and breast cancers, cardiovascular diseases and osteoporosis [21]. In addition, iso-flavonoids are anti-cancerous and has also proved its hormone varying, estrogenic and anti-estrogenic properties. saturated fat content is low in soybean so it could be helpful in reduction of coronary diseases [18].

Fermented foods are element of the human diet and can be used to increase the shelf life and for better digestibility, nutritional value and safety [22-25]. Fermentation of food by lactic acid bacteria is a procedure where desirable microbes and their enzymes can be used to change fermentable sugars present in the food into lactic acid and other fractional products.

2.4 Properties of LAB

The LAB are Gram–positive cocci or rods with low-GC. They can tolerate acidic conditions in and are usually non-spore forming bacteria which are related by their familiar metabolic and physiological characteristics. They can grow both in the existence and lack of oxygen. Lactic acid is the major metabolic end product of products generated LAB as a result of carbohydrate fermentation. LAB generate proteinaceous, bacteriocins that becomes an additional barrier for spoilage and pathogenic microorganisms.

The fall in pH, which is helpful in the conservation of food is due to lactic acid which is formed byLAB. Growth of many other food spoilage microorganisms is inhibited by lowered pH. Shelf life of food (e.g., yogurt, pickles, cheese) is prolonged by low pH environment by inhibiting contamination by microbes that are ordinary in the kitchen or able to grow at refrigeration temperatures [26,27]. Another inhibitory aspect for potential food pathogens is the reduced oxygen level through lactic acid fermentation. Antibacterial compounds also known as bacteriocins are created by

LAB. Bacteriocins act by creating holes through the membrane that surrounds the bacteria. Therefore, this action of bacteriocins makes it lethal to the bacteria. Lactic acid and other metabolic activity products add to the organoleptic and textural profile of a food item. The industrial significance of the LAB is more manifested by their GRAS status because of their ubiquitous occurrence in fermented foods and their input to the intestinal microflora of human mucosal surfaces.

2.5 LAB as probiotic

LAB contains an important factor that has some valuable role in the GI tract. Therefore, a better co-relation between the intestinal bacterial populations contributes in the development of new approaches for the treatment and/or prevention of several diseases [28]. Probiotics are live microbes that are used as food supplements which helps host by balancing intestinal microbial population[29-31]. Probiotic LAB belong to the genera *Lactobacillus, Bifidobacterium, Enterococcus, Lactococcus, Streptococcus, and Leuconostoc* [32, 33]. Probiotics are consumed in the products like yoghurt, fermented milks, or other fermented foods in which they are supplemented [30]. Traditionally, to restore gut health these products possessing living organisms are being used. [34]. Since these useful bacteria have a positive impact on human health, therefore, it is added as probiotics in variety of food items [34, 35].

Probiotic bacteria belongs to genus Lactobacillus that are naturally present in fermented foods and pathogens invading the gastrointestinal tract that are responsible for causing diseases can be prohibited by sustaining proper intestinal flora by proper intake of probiotics or fermented foods. Probiotics have a great potential in providing better nutrition, optimizing gut ecology, improving the immune system, soothing intestinal disorders and promoting overall health because of their capability to compete with pathogens for adhesion sites, to modulate the host's immune response or to antagonize pathogens [35,36].

Bifidobacterium species are stringently anaerobic, they invade in the large intestine and approximately, 30 species have been recognized that reside in the large intestine.

These microbes are used as supplements in dairy products such as yoghurt and baby formula milk or their growth can also be encouraged in the large intestines by prebiotics through diet. They help in the breakdown of lactose, production of lactate ions from lactic acid and vitamin synthesis [33]. Generally, probiotic bacteria do not colonize the human intestinal tract permanently, but some strains can rapidly colonize and adapt the native microbiota [34]. Use of LAB should be promoted for high-qualityhealth because they are highly helpful organisms.

Brief plan of work

1) Preparation and analysis of soymilk
    - pH
    - Total solids, moisture and ash content
    - Protein estimation
    - Phenolic content
    - Fat content
    - Antioxidant activity by ABTS method
    - Reducing sugar by DNS

2) Maintenance and activity rating of lactic cultures.

3) Fermentation of soymilk.

4) Effect of prebiotic on growth of selected probiotic.

5) Synbiotic product and its characterization.

3.Materials and Methods

3.1 Chemicals:

Sodium bi-carbonate, Sodium Potassium tartarate, Copper sulphate, Folin & Ciocalteu's phenol reagent, Bovine serum albumin, ABTS, ethanol, methanol (HPLC grade), phenolphthalein indicator, sodium carbonate, tannic acid, inulin(chicory), FOS , sodium hydroxide, sodium chloride.

3.2 Procurement of Milk & Cultures:

The pure lactic cultures were obtained from the lab, JUIT. Strains used were as follows :

TABLE 1: Different LAB Strains

Sr no.	Sample	Strain
1	4	*Lactobacillus paracasei* (CD4)
2	7	*Lactobacillus gastricus* (BT M7)
3	9	*Brevibacillus aydinogluensis* (BT M9)
4	13	*Brevibacillus thermoruber* (CD13)
5	14	*Enterococcus* sp. (GTM14)
6	29	*Brevibacillus thermoruber* (HM29)
7	34	*Brevibacillus thermoruber* (HM34)
8	75	*Lactobacillus* sp.
9	78	*Lactobacillus* sp.
10	84	*Lactobacillus* sp.
11	90	*Lactobacillus* sp.
12	94	*Lactobacillus* sp.
13	98	*Lactobacillus* sp.
14	100	*Lactobacillus* sp.
15	C1	*Weissella confuse* (CD1)
16	LRGG	*L. rhamnosus GG* (LRGG)

3.3 Preparation of soymilk:

Soymilk was produced by soaking dried soybeans and grinded in blender(as in *Fig 1* and *Fig 2*). 100g of soybeans was taken which yields to 1000ml of soymilk. The milk was then autoclaved at 121°C and stored at 4°C. It was further analyzed for estimation of moisture content, ash content, pH, fat and protein content [37].

100g of soybean

↓

Soaked in clean water for overnight

(Fig 1)

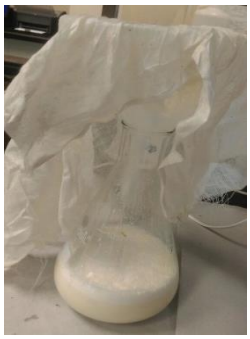
Rinse in water (10 times the weight of soybean) and ground into paste

↓

(Fig 2)

↓

Slurry sieved through muslin cloth



(Fig 3)

↓

Autoclaved the soy milk and analyzed it

(Fig 4)

3.3 Analysis of soymilk:

3.3.1 pH: -

pH of fermented soymilk was estimated with the help of pH meter [37].

3.3.2 Determination of Moisture Content in Soymilk: -

In an empty petri-desh ,around 20ml of the soymilk sample was weighed and was then dried in a hot air oven. After 24 hrs sample was cooled down in a desiccator and weight was measured [37].

Moisture content % = (W1−W0) – (W2−W0)/ (W1−W0) × 100

Where:

W0 = Weight of empty moisture dish

W1 = Weight of moisture dish and sample

W2 = Weight of dessicated sample

(W1 – W0) – (W2 – W0) = Weight loss

(W1 – W0) = Weight of sample

3.3.3 Determination of Ash Content in Soymilk:

In empty clean crucible 2 ml of milk sample was taken and placed in a muffle furnace at 500°C - 600°C. After 3 hrs, the crucible was removed from the muffle furnace and cooled in a desiccator, and ash weight was noted down [37].

Ash content % = (W2−W0)/ (W1−W0) × 100

Where:

W0 = Weight of empty crucible

W1 = Weight of sample and crucible

W2 = Weight of ash and crucible.

3.3.4 Simple UV Spectrometric Method for Measurement of Milk Fat Content:

- 30 µl of soymilk sample was added to 3 ml of absolute ethanol at −20°C.
- All vials were plugged and stored for 1 hr at 20°C.
- Samples were centrifuged at 13,000 rpm for 15 min and allowed to reach room temperature.
- Sample absorbance was taken at 208 nm [38].

3.3.5 Lowry Method of Protein Estimation in Soymilk :

Reagent preparation :

- Lowry A – 2% sodium carbonate was dissolved in 0.1 N sodium hydroxide.
- Lowry B – 1% copper sulphate was dissolved in 100 ml of distilled water.
- Lowry C- 2% sodium potassium tartarate.
- Lowry solution- 49 ml A + 0.5 ml B + 0.5 ml C.

Folin solution- 2N : distilled water (1:1)

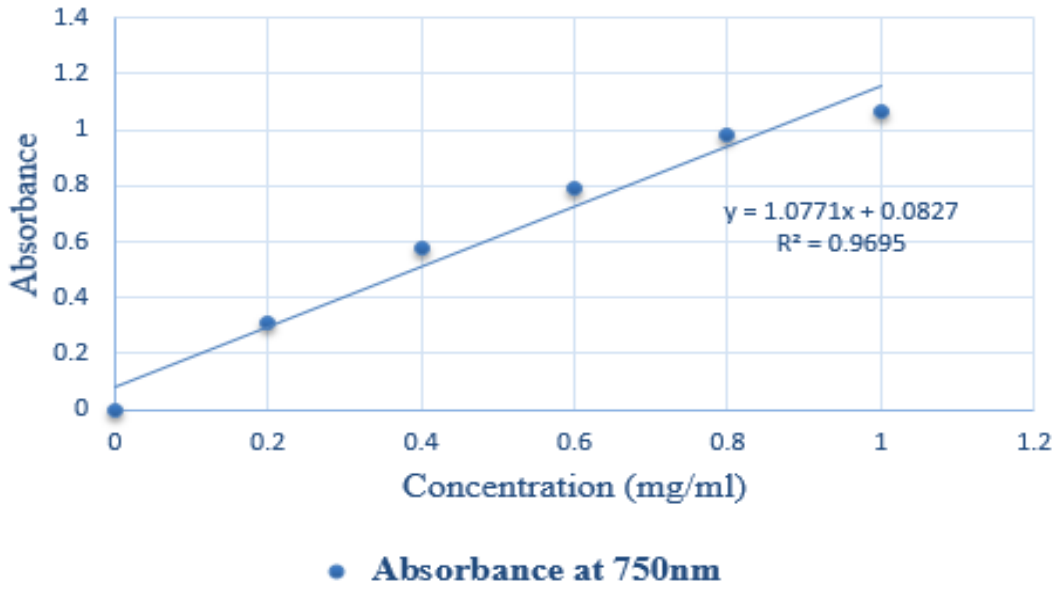
Standard – BSA(1mg/ml)

Procedure :

- 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard were made.
- Dilutions was done for the sample.
- 5ml of Lowry reagent was added and vortexed and then was kept at 37°C for 15 min.
- 0.5 ml of FC reagent (1N) was added to each test tube and incubated for 30 min in dark and absorbance was taken at 750 nm.
- Standards curve was plotted as shown in *Fig 5.*

TABLE 2:Protein Estimation of Soymilk Sample by Lowry's Method

S. No	Concentrationof BSA stock solution(mg/ml)	Volume of BSA solution (µl)	Volume of water(µl)	Total volume (ml)	Volume of lowry's reagent (ml)		Volume of FC reagent(ml)		Absorbance at 750nm
1	Blank	0	1000	1	5	Incubation for 10 min at room temperature	0.5	Incubation for 30min at 30°C	0
2	0.2	200	800	1	5		0.5		0.3125
3	0.4	400	600	1	5		0.5		0.5775
4	0.6	600	400	1	5		0.5		0.794
5	0.8	800	200	1	5		0.5		0.9785
6	1.0	1000	0	1	5		0.5		1.065
7	Sample (1/100)	1000	0	1	5		0.5		0.23



Graph 1 : Standard Curve of Protein Estimation by Lowry's Method

3.4.6 ABTS Method to Estimate Antioxidant Activity of Samples:

The scavenging activity was estimated by ABTS method [39]. ABTS (7mM in water) was prepared by mixing stock solution with potassium per sulphate (2.45mM) in an equal quantities and left to stand for 12-16 h at room temperature in dark until reaching a stable oxidative state.

With 80% ethanol , ABTS solution was diluted to an absorbance of 0.80 ± 0.05 at 734 nm.

- 100µL of sample was mixed with 2.9 ml of ABTS solution and the mixture was allowed to stand at room temperature for 30 min in dark condition.
- The absorbance was determined at 734 nm.

Scavenging effect (%) =

[1 - (Absorbance of sample / Absorbance of control)] x 100

- Absorbance of sample was observed as 0.506 at 734nm.

3.4.7 Estimation of Reducing Sugar by DNS Method:

Preparation of DNS reagent:

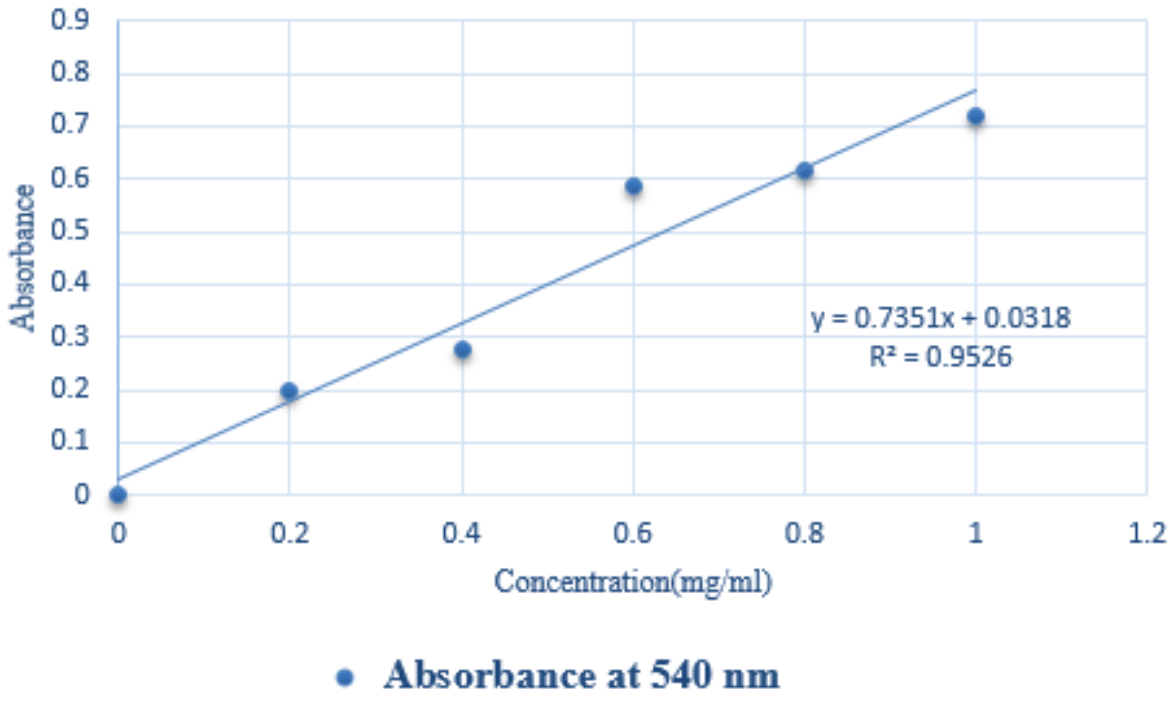
- 1 g DNS, 200 mg crystalline phenol and 50 mg sodium sulphite was dissolved in 100 mL 1% NaOH by stirring.
- Stored at 4°C.Since the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.

standard : glucose 1mg/ml

- DNS was prepared just before mixing stock solution to 3m1 of DNS in test-tube.
- Test-tubes were plugged and placed in boiling water bath for 5 min and cooled down.
- OD was taken at 540nm, step was repeated for samples provided and standards were plotted to estimate the concentration of the unknown samples.

TABLE 3: Estimation of Reducing Sugars in Soymilk by DNS Method

Sr no.	**Sugar concentration (mg/ml)**	**Volume of water (ml)**	**Volume of DNS reagent (ml)**		**Volume of water (ml)**	**OD at 520 nm**
1	0.0	1.0	3	Incubation at 60°C for10 min	5	0.00
2	0.2	0.8	3		5	0.20
3	0.4	0.6	3		5	0.27
4	0.6	0.4	3		5	0.58
5	0.8	0.2	3		5	0.61
6	1.0	0.0	3		5	0.71
7	Sample(1/100)	-	3		5	0.25



Graph 2: Standard Curve of Reducing Sugars by DNS Method

3.4.8 Phenolic Content by FC Method

Preparation of 15% sodium carbonate solution:

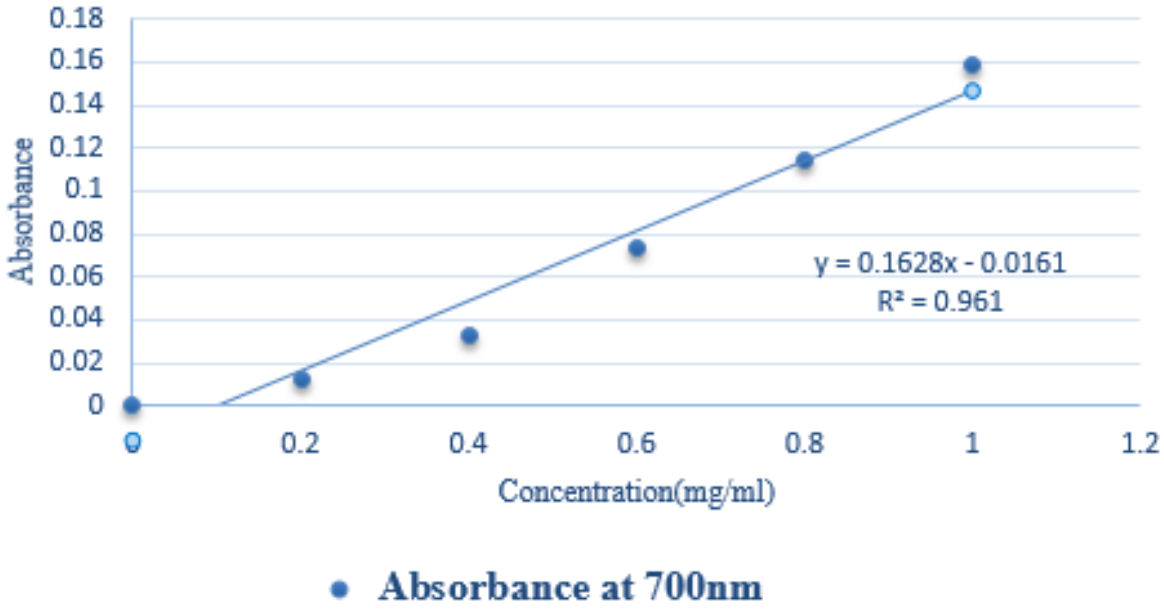
- 3g of sodium carbonate was dissolved in 20ml of distilled water by simply stirring.

Dilution were prepared of tannic acid solution and final volume was raised up to 200µl with distilled water.

- To 200µl of sample, 8.3ml of water was added.
- 0.5ml FC reagent was added and sample was vortexed.
- Added 15%Na_2CO_3 to each sample and vortexed.
- Incubated for 30min in dark and O.D was measured [37].

TABLE 4:Estimation of Phenolic Content in soymilk sample by FC reagent Method

S.No	**Concentration of stock solution (ml)**	**Volume of stock solution (µl)**	**Volume of water(µl)**	**Total volume (ml)**	**Volume of distilled water(ml)**	**Volume of FC reagent (ml)**		**Volume of 15% Na_2CO_3 (ml)**		**OD at 700nm**
1	Blank	-	1000	1	8.3	0.5	Vortex the sample	1	Vortex the sample and incubate for 30 min in dark	0.0000
2	0.2	200	800	1	8.3	0.5		1		0.0125
3	0.4	400	600	1	8.3	0.5		1		0.0325
4	0.6	600	400	1	8.3	0.5		1		0.0735
5	0.8	800	200	1	8.3	0.5		1		0.1145
6	1.0	1000	-	1	8.3	0.5		1		0.1585
7	Sample	1000	-	1	8.3	0.5		1		1.0505



Graph 3: Standard Curve of Phenolic Content by FC Reagent Method

3.5 Maintenance of lactic culture:

The lactic cultures were revived by inoculating each culture individually in MRS broth (de Man, Rogosa and Sharpe) and were incubated at 37°C for 24 hrs.

3.6 Acidification rate of lactic cultures:

- Filled the burette with N/10 NaOH solution.
- Mixed the milk sample thoroughly.
- 10 ml milk was transferred in conical flask.
- Equal quantity of distilled water was added in the flask.
- 3 to 4 drops of phenolphthalein indicator was then added and solution was stired.

- Rapidly titrated the contents with N/10 NaOH solution by continuous addition of alkali drop by drop and shaking till the content color changes to pink colour which remains constant for 10 to 15 seconds.
-Lowest point of meniscus was observed for the reading of alkali in the burette.

Calculation:

$$\% \text{ Lactic acid} = \frac{\text{No of ml. of 0.1 N NaOH solutions required for neutralization} \times 0.009}{\text{Sample weight}} \times 100$$

(Weight of sample = Volume of milk x specific gravity)

3.7 Preparation of Fermented milk:

- 5ml of skimmed milk was added in test tubes and sterilized at 121°C.
- The test tubes containing skimmed milk were inoculated with 200µl of different lactic cultures.
- After the incubation of 24hrs at 37°C , 200ml of soymilk bottles were inoculated with 200µl of different fermented skimmed milk.
- Incubated at 37°C for 24hrs.

3.8 Effect of prebiotic on growth of selected probiotic:

- Cultures were revived in MRS broth(as in *Fig 5*).
- Cultures were inoculated in skimmed milk (as in *Fig 6).*
- Different concentrations of prebiotics inulin and fructoligosacchride (0%, 0.5%,1%,1.5%) were added to soymilk along with different probiotic cultures(4, 7, 9, 13, C1 and LRGG) shown in *Fig 7.*
- All samples were incubated at 37°C.
- Serial dilutions of the samples were made in saline water (0.85 % w/v NaCl).

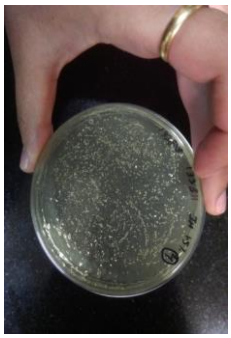
- Aliquots of 50 µl were taken from each bottle and was spread onto MRS agar plates.
- Incubated for 48 hrs at 37°C for determination of cell count as shown in *Fig 8* and *Fig 9*[40].

(Fig 5)

(Fig 6)

(Fig 7)

(Fig 9)

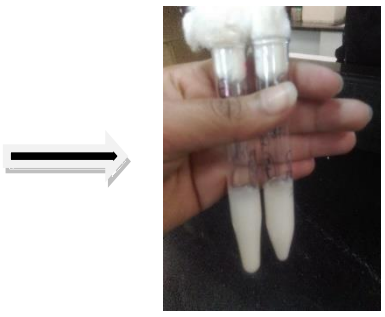


(Fig 8)

3.9 Synbiotic product and its characterization:

- Cultures were revived in MRS broth (as in *Fig 10*).
- Cultures were inoculated in skimmed milk (as in *Fig 11).*
- Different concentrations of prebiotics inulin (0%, 0.5%,1%,1.5%) were added to soymilk to ferment along with different probiotic lactic strain 7 shown in *Fig 12* .
- All samples were incubated at 37°C.
- Titratable Acidity and pH of synbiotic product (fermented soymilk) was calculated.
- Samples were taken for HPLC analysis.

(Fig 10)



(Fig 11)

(Fig 12)

4.Results and Discussion

4.1 Analysis of milk :

To test the quality of milk its analysis is done. It is reported that pH of processed soymilk is usually 7.30 which is neutral while the pH of lab processesed soymilk was resulted to be 6.68 which is near to the neutral condition.

The amount of water contained in the substance is its moisture or water content. Soymilk contains a good quantity of moisture content in it which was 93.9%.

Mineral content in the substance is refered to its ash content, and is determined by burning a given amount of material under given conditions and measuring the residue.

The average ash percentage in soymilk sample was found to be 2.35%.

The average value of whole soluble solids was calculated by refractometer and was found to be as 6°brix.

The average value of fat in soymilk was found to be 3%.

The average value of protein content was fond to be 0.059 mg/ml.

Phenols, a class of chemical componds which is sometimes called phenolics, consist of a hydroxyl group (—OH) which is bonded directly to an aromatic hydrocarbon group. Based on the number of phenols units present in a molecule, Phenolic compounds are classified as simple phenols or poly phenols. Examples of naturally going on phenols are tyrosine, thymol and gallic acid. The average worth of phenolic content in soymilk was 0.511 mg/ml of tannic acid equivalent.

An antioxidant prevents or delay some types of cell injure caused by free radicals formed in our body by preventing and inhibiting oxidation of these molecules. The antioxidant strength of plant extract or food product has been measured by ABTS assay. In this assay, by adding sodium persulfate ABTS is transformed to its radical

cation. This cation absorbs light at 734nm and is blue in color.The average value of antioxidant was calculated to be 49.12.

In order to estimate reducing sugar or occurence of free carbonyl group which are reducing sugars DNS (Di nitrosalicylic acid) method is used. It oxidizes aldehyde functional group. The average assessment was calculated as 0.304 mg/ml (1/100).

TABLE 5: Analysis of Soymilk

pH	6.68
Ash content	2.35%
Moisture content	93.9%
TSS	6° Brix
Phenolic content	0.511 mg/ml of tannic acid equivalent
Fat content	3%
Protein content	0.059 mg/ml
Antioxidant activity by ABTS	49.12
Reducing sugar by DNS	0.304 mg/ml (1/100)

4.2 Acidification rate of lactic cultures:

It was analysed that among all the lactic culture combinations,cultures (4, 7 , 9 , 13, C1 and LRGG) were utmost appropriate probiotic starter cultures for fermention of soy milk. These starter cultures showed good acidification rate by decreasing the fermentation time.

pH of different fermented soymilk sample was determined with the help of pH meter.

The lactic cultures were evaluated for acidity using Horral Elliker's test. The pH of the fermented milk dropped from 6.8 to 6.19 with increase in acidity from 0.2 to 0.4 after incubation for 4 hrs.

TABLE 6: pH OF Different LAB cultures

Incubation time (minutes)	**4**	**7**	**LRGG**	**34**	**90**	**29**	**84**	**9**	**13**	**C1**	**14**	**75**	**100**	**78**	**98**	**94**
0	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80
30	6.90	6.83	6.82	6.68	6.77	6.60	6.65	6.63	6.72	6.72	6.80	6.80	6.80	6.80	6.80	6.80
60	6.93	6.84	6.84	6.64	6.64	6.50	6.69	6.58	6.64	6.65	6.80	6.80	6.80	6.80	6.80	6.80
90	6.89	6.85	6.93	6.64	6.64	6.50	6.67	6.54	6.61	6.63	6.80	6.80	6.80	6.80	6.80	6.80
120	6.80	6.78	6.84	6.64	6.63	6.50	6.73	6.50	6.58	6.58	6.80	6.80	6.80	6.80	6.80	6.80
150	6.97	6.96	6.92	6.66	6.63	6.50	6.72	6.44	6.50	6.51	6.80	6.80	6.80	6.80	6.80	6.80
180	6.78	6.61	6.60	6.66	6.63	6.50	6.70	6.40	6.43	6.40	6.80	6.80	6.80	6.80	6.80	6.80
210	6.75	6.40	6.47	6.66	6.62	6.50	6.68	6.29	6.29	6.24	6.80	6.80	6.80	6.80	6.80	6.80
240	6.16	6.19	6.26	6.65	6.60	6.50	6.65	6.20	6.26	6.19	6.80	6.80	6.80	6.80	6.80	6.80

TABLE 7:Titratable Acidity (%) OF Different LAB Cultures

Incubation Time (minutes)	**4**	**7**	**LRGG**	**34**	**90**	**29**	**84**	**13**	**9**	**C1**	**14**	**75**	**100**	**98**	**78**	**94**
0	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
30	0.21	0.21	0.21	0.256	0.12	0.11	0.13	0.15	0.19	0.14	0.22	0.22	0.22	0.22	0.22	0.22
60	0.22	0.22	0.22	0.14	0.13	0.15	0.14	0.15	0.21	0.14	0.22	0.22	0.22	0.22	0.22	0.22
90	0.17	0.20	0.20	0.13	0.13	0.10	0.15	0.15	0.15	0.16	0.22	0.221	0.22	0.22	0.22	0.22
120	0.15	0.15	0.18	0.15	0.09	0.13	0.4	0.14	0.19	0.18	0.22	0.22	0.22	0.22	0.22	0.22
150	0.19	0.22	0.14	0.15	0.10	0.09	0.12	0.13	0.20	0.12	0.22	0.22	0.22	0.22	0.22	0.22
180	0.18	0.15	0.22	0.18	0.13	0.12	0.12	0.15	0.14	0.15	0.22	0.22	0.22	0.22	0.22	0.22
210	0.22	0.26	0.22	0.19	0.15	0.16	0.11	0.40	0.40	0.40	0.22	0.22	0.22	0.22	0.22	0.22
240	0.2	0.4	0.28	0.20	0.20	0.23	0.20	0.40	0.40	0.40	0.22	0.22	0.22	0.22	0.22	0.22

4.3 Effect of prebiotic on selected probiotic:

It was analysed that prebiotics FOS and inulin showed different results on the metabolic activity of probiotic cultures in soymilk. Viability of lactic culture (4 , 7, 9, 13, C1 and LRGG) was enhanced by both prebiotics. It was observed that 1.5% concentration of both FOS and inulin showed equal change in metabolic activity.

a) Fructoligosacchride as prebiotic:

TABLE 8: **Log_{10} CFU/ ml value of Different LAB Cultures with FOS**

Concentration(%)	4	7	LRGG	9	13	C1
0	6.46	6.60	6.54	7.41	7.57	6.85
0.5	7.19	7.51	5.80	6.00	7.53	6.93
1	7.04	6.60	6.60	7.23	7.30	6.90
1.5	7.23	8.34	6.27	6.77	7.49	7.30

b) Inulin as prebiotic:

TABLE 9: **Log_{10} CFU/ml value of Different LAB Cultures with Inulin**

Concentration(%)	4	7	LRGG	9	13	C1
0	6.07	6.69	7.46	6.39	7.23	6.84
0.5	7.17	7.43	5.30	6.69	7.87	7.89
1	7.50	7.80	7.40	6.90	7.49	7.49
1.5	7.86	Uncountable	7.43	6.60	7.74	7.74

4.4 Synbiotic product analysis:

Titration was done to evaluate the acidification rate of culture which was calculated as 0.12.

pH was measured by pH meter which was observed as 4.95.

5.Conclusion

A total of sixteen lactic acid bacterial cultures were evaluated for their acidification rate for further processing of soymilk. Among cultures evaluated, six strains (*Lactobacillus paracasei* (CD4) , *Lactobacillus gastricus* (BT M7), *Brevibacillus aydinogluensis* (BT M9), *Brevibacillus thermoruber* (CD13), *Weissella confuse* (CD1), *L. rhamnosus GG* (LRGG) were selected with higher acidification rate in soymilk. For the maximum proliferation of LAB, two prebiotics (fructooligosaccharide and inulin) were screened at a concentation of (0-1.5%) in soymilk. The effect of prebiotic was evaluated by viable plate assay on MRS agar after 24 h of fermentation of soymilk. Both the prebiotics (FOS and inulin) had similar affect on the LAB count with an increase of log cfu from 6.00 to 7.89 at 1.5%. Based on the results obtained in this study it was concluded that the metabolic activities differ among different LAB in soymilk and FOS and inulin serve as a prebiotic. Further studies are necessary in terms of evaluating the effect of combination of lactic cultures and prebiotic for their beneficial effects from fermented soymilk.

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