Evaluation of prebiotic activities of resistant starch from potatoes

Dissertation submitted in partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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UNDER THE GUIDANCE OF

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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, SOLAN

MAY 2018

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CERTIFICATE

This is to certify that the work reported in the B.TECH thesis entitled **"Evaluation of Prebiotic activities of resistant starch from potatoes"** submitted by **Ankita Chakravarty and Moksh Tandon** at **Jaypee University of Information Technology, Waknaghat, India** is a bonafide record of their original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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DATE-…….

DECLARATION

We do here by declare that the work reported in the B.TECH thesis entitled **"Evaluation of prebiotic activities of resistant starch from potatoes"** submitted at **Jaypee University of Information Technology, Waknaghat, India** is an authentic record of our work carried out under the supervision of **Dr. Gunjan Goel.** We have not submitted this work elsewhere for any other degree or diploma.

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ACKNOWLEDGEMENTS

We would take this opportunity to express our sincere gratitude towards our mentor, **Dr. Gunjan Goel, Associate Professor,** Department of Biotechnology and Bioinformatics, **Jaypee University of Information Technology, Waknaghat, India** for his persistent support and incomparable guidance, and most of all for his unmatched patience, attention and care. His efforts put in us far outweigh any of our efforts put in this project and for making corrections as and when required. We are truly grateful to him.

We would also like to express our sincere gratitude to **Dr. Pinky Raigond, division of crop physiology, biochemistry and postharvest technology, central potato research institute, Shimla** for her guidance and support.

We would like to give special thanks to **Dr. Sudhir Kumar,** Professor and Head, Department of Biotechnology and Bioinformatics for providing facilities which enabled us to complete this project work successfully.

We are grateful to **Dr. Samir Dev Gupta**, Director (Admin), **Brig Maj Gen Rakesh Bassi** (Retd.) director (Academic affairs). We would also take this opportunity to thank Ms. **Kavita Sharma (**PhD), **Mr. Sampan Attri** (PhD), **Mr. Deepak Sharma** (PhD) and **Mr. Baleshwar** for their continuous assistance during the practical work.

Finally we are thankful to our family, friends for continuous encouragement throughout the process of project. The accomplishment would have not been possible without them.

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LIST OF ABBREVIATIONS AND SYMBOLS

RS	Resistant starch
CPRI, Shimla	Central Potato Research institute
SEM	Scanning electron microscopy
℃	Degree Celsius
SCFA	Short chain fatty acid
GIT	Gastro intestinal tract
GRAS	Generally regarded as safe
ABTS	2,2'- Azino-bis(3-ethylbenzothiazoline-6-sulphoni acid)
DNS	3,5- Dinitrosaliscylic acid
ACE	Angiotensin converting enzyme
CFU	Colony forming unit
MRS	De Man, Ragosa and Sharpe medium

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ABSTRACT

Resistant starch is one of the varieties of starch which is reported for the prebiotic effects for probiotic cultures. This starch is not available to human digestible enzymes but is processed and fermented in colon which results in the manufacturing of short chain fatty acids as beneficial end products for gut micro biota. Recent scenario among customers, researchers and manufacturers of health products aim to make foods that tend to offer extra heath benefits with essential nutrition. Now a day's resistant starch from potato is gaining attention as a functional food ingredient due to its unique physiochemical properties. However, the function of resistant starch in Indian potato varieties has not been evaluated yet.

Combining resistant starch with probiotic lactic acid cultures proliferates their growth efficiency and helps to enhance their metabolic system. Hence, in our project we used five lactic acid cultures in amalgamation with resistant starch and screened all the prebiotic, probiotic and finally synbiotic characteristics of the substrates.

CHAPTER-1

INTRODUCTION

Recent scenario among customers, researchers and manufacturers of health products aim to make foods that tend to offer extra heath benefits with essential nutrition. Recently resistant starch (RS) from potato is gaining attention as a functional food ingredient due to its unique functional and physiochemical properties. However, the role of RS in Indian potato varieties has not been evaluated yet.

Resistant starch is gaining much attention now a days as it has a unique property of escaping digestion in our upper GIT and directly goes to our colon where it gets fermented by colonic bacteria and hence results in increasing their number promoting many health benefits. Resistant starch is present naturally in food products but is also supplemented to foods by adding up of extracted or manufactured types of resistant starch. Enzymatic treatment of RS from Kufri Bahar potato variety was carried out in CPRI, Shimla.

Probiotics were discovered by Elie Metchnikoff. These are the live bacteria or yeasts that are considered good for our health. These bacteria are present in our gut and prevent us from onset of different diseases such as diabetes, cancer. They promote our gut health and strengthen our immune system. Although there can be some side effects of probiotics but taken in fare amounts can pose many benefits on our digestive system.

Prebiotics refer to the non digestible food ingredients that are known to augment the growth and number of microorganisms that are there in our gut. These prebiotics are fermented in our large intestine and forms SCFA that are responsible for energy production. These SCFAs are utilized by probiotics to increase their growth. Commonly used prebiotics in labs are inulin and oligofurctosaccharides.

CHAPTER-2

REVIEW OF LITERATURE

Milk is a white liquid which is produced by the mammary glands of mammals. There are ranges of health benefits related with milk as it contains vitamins, minerals. Cow milk is a source of potassium, which helps in improving vasodilatations and reduces blood pressure. It also contains a high amount of saturated fat and cholesterol, which have been linked to an engorged risk of heart disease**.** It may also surround small amounts of vitamin B2, or riboflavin, vitamin B12, B6. Magnesium may also be present.

The main purpose of milk fermentation by lactic acid bacteria is in order to enhance its shelf life and also enhance its nutritional content.

Lactobacillus strains are the most commonly and dominantly used starter culture in fermentation of milk. Also in many products made from cow milk, there are naturally occurring bioactive peptides present. They have been regarded as 'Generally regarded as safe organisms (GRAS)'.

2.1- Probiotics-

Probiotics are the living microorganisms that have various profits to the host when ingested in sufficient amount. The strains often used as probiotics comprise *Lactic acid bacteria* and *Bifidobacteria.*

According to K Gogineni et al. (2013), probiotics mechanism of action includes:

- Enrichment of epithelial barriers
- Improved adherence to intestinal mucosa
- Affiliated blockage of pathogen union
- Spirited elimination of pathogenic microorganisms
- Production of anti microbial substances and intonation of immune system.

Probiotics have been known to pose many benefits on our gut as the beneficial bacteria present there can protect us from various diseases and improves out health. Probiotic are mainly available in fermented dairy products as when ingested orally they can be deteriorated by acidic condition in stomach.

2.2 - Prebiotics-

According to the research of Joanne Slavin et al. (2013) prebiotics were initially defined as "nondigestable food ingredients that beneficially affect the host by selectively stimulating the growth and /or activity of one or a limited number of bacteria in the colon, thus improving host health". *Lactobacillus* and *Bifidobacteria* are major targets of prebiotics.

There are some set criteria for a substance to be regarded as a prebiotic that is non digestibility and property to escape digestion in stomach, must be selectively fermented by gut micro flora and stimulate the growth of beneficial bacteria. These plant derived products are classified under oligosaccharides. Fructans and galactans are the two categories of these oligosaccharides sources that can fuel the growth of beneficial bacteria. Different prebiotics are used to encourage the growth of bifidobacterium and lactobacillus. Studies have also found that prebiotics, not only stimulates the development of beneficial gut bacteria, but can also slow down the growth of harmful and pathogenic microorganisms in the gut[24].

Inulin and galacto-oligosaccharides are the commonly used prebiotics in food labs.

2.3 - Synbiotic-

Aggregation of prebiotics and probiotics is referred to as synbiotic and is of area of extensive research in current world. A synbiotic product constructively affects the host in improving the endurance and spurt of live microbial nutritional supplements in the gastrointestinal tract by selectively motivating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria.

The health benefits of using synbiotics includes when consumed by humans: Amplified levels of *lactobacilli* and *bifidobacteria* and impartial gut micro biota, enhancement of liver function in cirrhotic patients, improvement of immune modulating ability and prevention of bacterial translocation etc. (Zhang et al. 2010).

Now a day's cow milk is gaining much attention because of its physical and chemical properties. Although milk contain lactose in huge amounts people convert the milk into curd that contains less amount of lactose and hence, can be consumed even by lactose intolerant individuals.

In our project we made curd as a fermented symbiotic product from cow's milk inoculating the milk with *Lactobacillus paracasei (CD4)* as a probiotic and Resistant starch as a prebiotic [33].

2.4 - Mechanism of action of probiotics-

Probiotics influence many mechanisms of epithelial barrier by lessening apoptosis of intestinal cells or amplified mucin production. *Lactobacillus rhamnosus GG* was able to avoid cytokine-induced apoptosis in intestinal epithelial cell models by restricting tumor necrosis factor (TNF). Probiotics induces host cells to fabricate peptides that hamper with pathogens, and avert epithelial invasion.

According to the researcher Floch, M. (2011), these bacteria's fight with invading pathogens for binding sites to epithelial cells. *S.boulardi* (another type of probiotic organism)secrets a high temperature liable factor which has shown to be in charge for decreased bacterial adherence. *Lactobacillus casei* has shown to widen total and pathogen specific secretary IgA levels upon disease in mice by stimulating B cells class switching to IgA.

There are many factors that may influence the probiotic mechanism of action from food processing to ingestion. But many methods like microencapsulation can augment the survival and stability of these microorganisms in foods. Researchers are still working on the foods rich probiotic microbes to increase their functionality when they pass on through our GIT.

2.5 - Mechanism of action of prebiotics-

Prebiotics can have straight adverse effects on the configuration of the colonic mucosa (Poldbeltsev et al., 2006). No exact effects are as such reported for this mechanism but it is reported that short chain fatty acids are to blame for the change of configuration of mucosa. Prebiotics also arouse the development and metabolic activities of some *bifidobacteria* and *lactobacilli*, which thus increases creation of the luminal bacterial enzyme b-glycosidase, which hydrolyses the glycosidic bond of isoflavone conjugates. The different mechanisms of prebiotic actions are shown in the figure below.

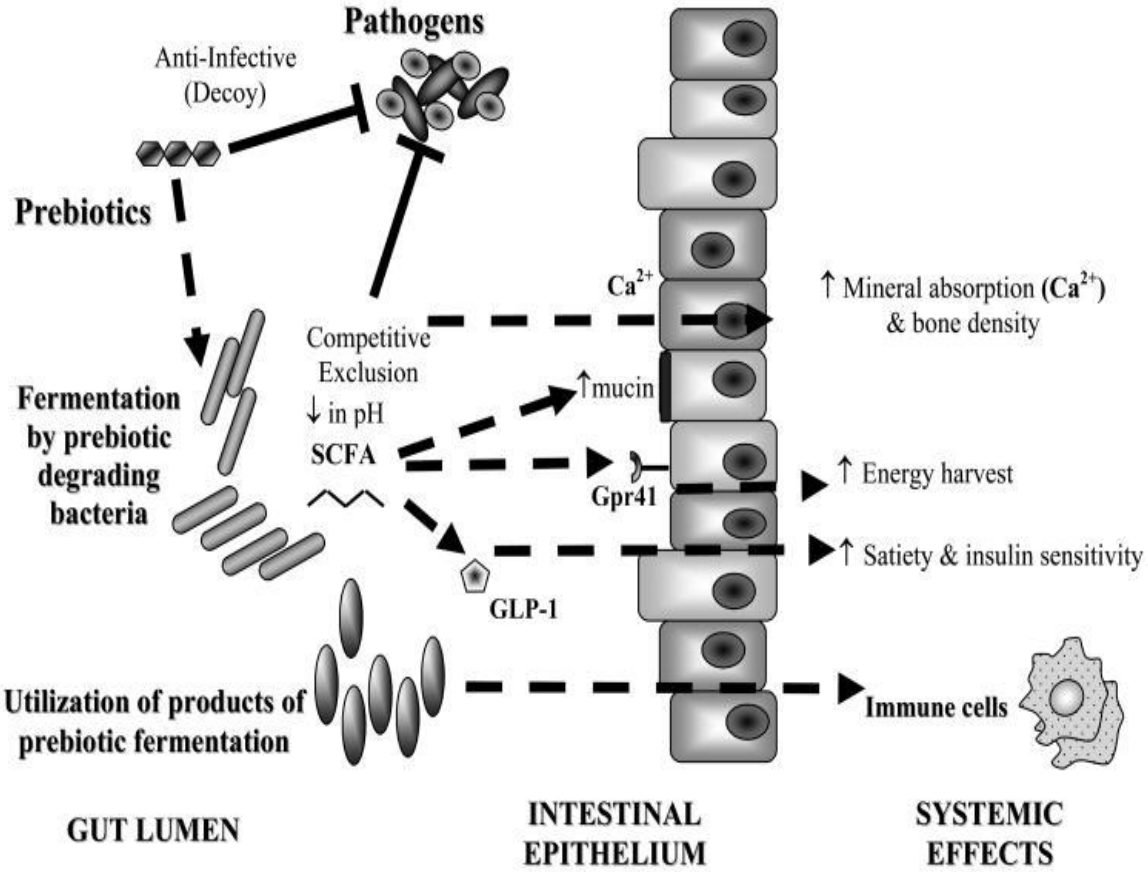


Figure 2.1 - Different mechanism of prebiotic activity (Saulnier, Det al., Mechanisms of probiotic and prebiotic: considerations for enhanced functional foods, 2013)

2.6 – Resistant starch-

Scientists, researchers and consumers have been working to develop foods with additional health benefits with basic requirements such as foods having low glycemic index. Glycemic index of food affects the sugar levels in blood and cause deadly health problems like diabetes and obesity. Glycemic index of a food is inversely related to its starch content. Starch, the most profuse storage polysaccharide in plants and is the chief constituent of diet. Most of the starch is taken up in gelatinized form, which can be readily digested. Reduced digestibility of RS is influenced by a lot of internal and external factors such as behavior and nature of food, botanical origin of starch, food processing and physiology .properties of starch depending upon the arrangement of two different molecules in the granule ie., amylose and amylopectins. Resistant starch (RS) was first termed by Englyst et. al. (1982) and described as a small fraction of starch that is resistant to hydrolysis by exhaustive enzymes- alpha-amylase and pullulanase treatment, *in vitro*. The content of RS in food is extremely influenced by food preparation manner and processing techniques.The concept of bio-availability of resistant starch and its uses as dietary fibre and prebiotic component has evoked new interests in new generation. Resistant starch is a prebiotic non-digestible fibre compound that is not accessible to human digestive enzymes but is fermented in colon, producing short-chain fatty acids [37]. RS can be used as prebiotic composition to promote the growth of beneficial probiotic micro biota in human intestine. Resistant starch has been extracted and optimized using three different methods namely, Autoclaving-cooling cycles (without enzymatic treatment), treatment with single enzyme (α amylase) and treatment with two enzymes (α amylase and amyloglucosidase). Type of resistant starch used must be based on their properties. There are five types of resistant starch that can be utilized in foods to improve out gut micro biota.

Table 2.1 – Types of resistant starch (Diane F. Birt et.al Resistant Starch: Promise for Improving Human Health, 2013)

TYPE	DESCRIPTION	EXAMPLE
RSI	Physically inaccessible starch	Coarsely ground or whole-kernel grains
RSII	Granular starch with the B- or C-polymorph	High-amylose maize starch, raw potato, raw banana starch
RSIII	Retrograded starch	Cooked and cooled starchy foods
RSIV	Chemically modified starches	Cross-linked starch and octenyl succinate starch
RSV	Amylose-lipid complex	Stearic acid-complexed high-amylose starch

Physical or chemical treatments can alter the levels and properties of resistant starch in our foods. Hence, before using resistant starch in our project different tests were performed to evaluate its properties such as swelling power, SEM.

2.7– Mechanism of action of resistant starch -

Resistant starch has a unique property of escaping digestion in our upper GIT and passes to the large intestine where it gets fermented by the action of the micro flora present there. Eating food with plentiful fiber has long been supposed to defend against colorectal cancer. More recently, resistant starch has accredited attention for possible prevention of colon cancer and inflammatory bowel diseases. Many hypotheses have been anticipated for the potential mechanism by which colon carcinogenesis may be tainted by resistant starch.

The most common hypothesis center includes:

- Modification of the water-holding ability of the fecal stream

- Amendment of the micro biota
- Escalating SCFA production.

These bacteria present in out intestine produce short chain fatty acids (acetate, propionate and butyrate). According to Z. Zhou et al. (2013) once the Resistant starch is fermented these SCFA are responsible for providing the energy to the bacteria present in our gut and hence, increases their number. Butyrate, the main energy source for colonocytes, is actively transported into cells by a Na^+-dependent co transporter. This cell membrane transporter plays a role as a tumor suppressor gene and is silenced by hypermethylation in human aberrant crypt foci (a precancerous lesion) and colorectal cancer. Butyrate has been regarded as most significant SCFA as in cell culture, butyrate has antitumorigenic properties, like dropping cell proliferation and inducing apoptosis of colorectal tumor cell lines.

Consumption of resistant starch has been known to be having many potential benefits on human health. Products with low glycemic index can perk up and control of obesity and diabetes and subsequently reduce the risk of cardiovascular disease. All types of RS are not beneficial to the cholesterol level in blood. The production of SCFA by bacterial fermentation of RS in the large intestine is dogged by the composition and properties of RS. Slow digestibility of RS leads to the slow release of glucose. RS has physiological profit of soluble fibers and a positive contact with the colonic health by escalating the crypt cell production rate or declining colonic epithelial atrophy in contrast with no-fiber diets. Nutritional value of food is expressed by a tendency to reduce the calorific value of meals, especially in developed countries. RS has gained superior interest because it is a natural food component which is neutral to the organism and adds little calorific value to food. Dietary fibers, including RS, promote beneficial physiological effects, including laxation, blood cholesterol attenuation and blood glucose attenuation [37].

CHAPTER-3

MATERIALS AND METHOD

3.1 – Chemicals-

Phenolphthalein, N/10 NaOH, 95% ethanol, gallic acid/tannic acid, FC reagent, sodium carbonate, ABTS, potassium per sulfate, 80 %ethanol, absolute ethanol, DNS reagent (DNS, crystalline phenol, sodium sulphite, 1% NaOH, glucose, ethyl acetate, ACE solution, HHL substrate, HCL,MRS media (De Man, Rogosa and Sharpe agar),HCL-KCL buffer, Tris-maleate buffer, pepsin solution, alpha amylase, amyloglucosidase, sodium acetate buffer.

- Milk sample from the local breed of cow (Waknaghat) was collected and autoclaved to remove the indigenous bacterial population of milk so that effectively of our probiotic strains can be checked.

- Protocol of our project is divided into four phases in which various tests for characterization of milk are performed - a) raw milk b) fermented milk (probiotic) c) Isolation of resistant starch and its characterization d) synbiotic product and its storage study.

3.2 - Tests performed for raw milk are as follows-

3.2.1. Total solid substrate (TSS)-

One or two drops of milk were put on to the refractometer with the help of dropper and the reading was observed.

3.2.2. pH and Acidity –

pH was observed through pH meter with a sample amount of 10 ml.

Acidity was checked through titrating with N/10 NaOH and using phenolphthalein as an indicator. Sample was kept the same.

3.2.3. ABTS assay for antioxidant activity –

a) ABTS stock was prepared by mixing (0.096 g) 25 ml ABTS and (0.0165 g) 25 ml potassium per sulfate.

b) The solution was mixed with 80 % ethanol and was left for incubation overnight.

c) With the help of spectrophotometer, the OD of the solution was set to 1 or less than 1 taking it as standard.

d) In a 96 well plate, 2.9 ml ABTS was mixed with 100 µl of sample that was taken in triplicates. Also a blank was taken as control.

e) Absorbance was noted at 734 nm and scavenging effect was calculate using the formula-

$$[1- (absorbance_{sample} / absorbance_{control})] * 100 \quad \text{eq. (1)}$$

3.2.4. Fat estimation by UV spectrophotometry-

a) 30 µl and 60 µl of milk sample were mixed in duplicates respectively with 3 ml of absolute ethanol at -20°C.

b) All the vials were stored at -20°C for one hour.

c) The vials were then centrifuged at 13,000 rpm for 15 minutes and then were permitted to reach the room temperature.

d) Absorbance was noted at 208 nm using absolute ethanol as blank.

3.2.5. Moisture content –

a) An empty petri plate was weighed and after addition of 20 ml of milk sample the plate was again weighed and was kept for drying in a hot air oven.

b) After 24 hours, sample was cooled and allowed to reach room temperature and moisture content was measured using the formula-

$$\textbf{Moisture content (\%)} = \{w_1 - w_0\}\{w_2 - w_0\}/\{w_1 - w_0\} * 100 \quad \textbf{eq. (2)}$$

Where, w_o = weight of empty moisture

w_1 = weight of moisture dish and sample

w_2 = weight of cooled sample.

$$\{w_1 - w_0\}\{w_2 - w_0\} = \textbf{Weight Loss} \quad \textbf{eq. (3)}$$

$$\{w_1 - w_0\} = \textbf{Weight of the sample} \quad \textbf{eq. (4)}$$

3.2.6. DNS assay –

a) DNS reagent (1 g DNS + 0.2 g crystalline phenol +0.05 g sodium sulphite in 100 ml of 1% NaOH) and glucose stock solution (1 mg/ml) were prepared.

b) Different concentrations of glucose were prepared ranging from 0.1 to 1 with corresponding addition of distilled water to it.

c) Also different dilutions of sample were prepared {1:10, 1:100, and 1:1000}.

d) 3 ml of DNS reagent was added to each test tube and were covered.

e) Then, the tubes were kept in pre heated water bath at 90°C till the color changes.

f) To all the test tubes 5 ml of water was added.

g) 200 µl from each test tube was taken into 96 well plate and the absorbance was noted at 540 nm.

h) Standard graph was plotted between glucose concentration and absorbance to obtain the unknown concentration.

3.2.7. Total phenolic content (TPC)-

According to the procedure followed by Cecilia Velazquez Vazquez et. al (2014)

To calculate total phenolic content 100 ml of milk sample was transferred to round bottom flasks and was kept at -80°C for freezing. Then the freezed milk was lyophilized for 24 hours and approximate 50 g of lyophilized powder was obtained.

a) Weighed 3 g of the powder and 20 ml of 95% ethanol was added to it in a flask.

b) Mixed the solution by keeping it on magnetic stirrer with hot plate at 30 °C for 1 hour at 300 rpm.

c) Centrifuged at 5°C for 15 minutes at 7800 rpm.

d) The extract obtained was decanted and quantification by FC method was done.

e) Gallic acid standard/ tannic acid (0.5 mg/ml) was made.

f) Different concentrations of gallic acid were prepared and certain amount of water was added.

g) 12 µl of sample, 50 µl of water, 13 µl of FC reagent, 125 µl of sodium carbonate was mixed with distilled water and vortexed the solution.

h) The solution was kept for 1.5 hours of incubation and after that absorbance was noted at 750 nm.

i) Standard graph was plotted between concentration and absorbance.

3.2.8. ACE activity -

According to the procedure followed by Zhang et al. (2009)

a) In a falcon a pinch of sample was taken and mixed with 200µl of Phosphate Buffer Saline Ph 7.4.

b) From the dissolved sample 50µl was taken in an eppendorf into which equal amount of 1 unit of ACE solution was added.

c) To the above solution 50µl of substrate (HHL) solution was added.

d) All the vials were kept at 37°C for 30 minutes incubation.

e) After incubation 150µl of 1M/L HCL was added in order to terminate the reaction.

f) 1ml of Ethyl Acetate was added to above solution for the extraction of Hippuric acid

g) Centrifuged the sample solution at 8000rpm for 10 minutes at 4°C.

h) Organic phase from centrifuged sample was transferred to a fresh eppendorfs.

i) Then the sample was kept in an oven at 100°C for evaporation.

j) 3ml of distilled water was added after evaporation to the residue.

k) Absorbance was measured at 228nm with using distilled water as blank.

Inhibition Equation –

ACE inhibitory activity = [(X-Y)/(X-Z)] **eq. (5)**

Where, X = Absorbance of enzyme + substrate

Y = Absorbance of enzyme + substrate + sample

Z = Absorbance of substrate only

3.3 - Test performed for fermented milk-

- **Procurement of cultures-**

The Lactic cultures used in the project were obtained from –

1. *Lactobacillus rhamnosus GG* (Standard strain)
2. *Lactobacillus paracasei* (CD4 from curd)
3. *Lactobacillus gastricus* (BTM7 from butter milk)
4. *Brevibacillus aydynoglunsis* (BTM9 from butter milk)
5. *Lactobacillus fermentum* (K75 from fermented wheat flour dough)

Also Skim milk from commercial market was used for the uniform revival of lactic culture.

- **Lactic culture maintenance-**

Above mentioned 5 lactic cultures were maintained individually in MRS broth by inoculation of each strain into it and were kept at 37°C for overnight to observe the growth in a shaking incubator.

- **Revival of the lactic cultures-**

1. Five test tubes containing 5ml of skim milk were autoclaved.

2. After the tubes were cooled 2ml of the cultures from MRS broth were inoculated individually and kept for growth.
3. After incubation setting of the curd was checked in each of the tubes.

- **Fermentation of cow milk-**

70ml of milk was taken in 5 different flasks and inoculated the flask with 3ml of respective cultures from skim milk tubes and were incubated at 37°C overnight.

Fermentation was observed the next day.

- **Analysis of fermented cow milk-**

1ml of sample of each strain was taken in eppendorfs and was used to count CFU/ml and the rest were set for lyophilization to obtain powered fermented cow milk.

3.3.1. pH and acidity-

10ml of fermented cow milk were taken in different beaker and preceded with checking of ph and acidity for 2 consecutive hours.

3.3.2. Counting of CFU/ml-

- Prepared 400ml of MRS broth (9g Agar and 21.65g MRS) for 18 plates to check the CFU/ml.
- After cooling of the autoclaved MRS broth 18 plates were prepared and were left to solidify.
- Serial dilution of each strain were done till 8^{th} dilution and spreading of 6^{th}, 7^{th} and 8^{th} dilution was done.
- The plates were kept at 37°C for growth.
- After 48hours colonies were counted and CFU/ml was calculated using the formula.

CFU/ml = number of colonies formed * dilution factor/ volume of culture plate **eq. (6)**

3.3.3. Total Solid Substrate (TSS)-

For each of the 5 strains same procedure was followed as above with the help of refractometer.

3.3.4. Total phenolic content (TPC)-

The procedure followed for extraction of phenolic content and quantification by FC method was same as done for raw cow milk and further standard graph was plotted between concentration and absorbance.

3.3.5. ACE activity-

The procedure followed was same as followed for the raw cow milk. The ACE inhibitory activity was calculated using the same formula as above (eq.5).

3.3.6. Antioxidant activity by ABTS method-

The extract obtained after centrifugation of the lyophilized sample with the addition of 95% ethanol was used for the procedure of ABTS. Rest procedure is same as followed for the raw milk.

Scavenging effect of the 5 strains was calculated as per the above mentioned formula (eq. 1).

3.4 - Isolation of Resistant starch from Kufri Bahar potato variety-

1. Autoclaving-cooling cycles (without enzymatic treatment)-

- Potato starch of variety Kufri Bahar was gelatinized (starch to water, 1:5) at 90°C.
- 100 g of gelatinized starch was autoclaved (in pressure cooker) for half an hour.

- Autoclaved starch was stored at 4ºC (in refrigerator) overnight. Autoclaving and cooling cycles were repeated twice. In total three autoclaving and cooling cycles were given to increase resistant content.
- Afterwards autoclaved-cooled starch was lyophilized.

2. Isolation of RS with single enzyme (alpha - amylase)-

- 100g of gelatinized starch (starch to water, 1:5) was mixed with 1L if HCL-KCL buffer (pH 1.5) and 20 ml of pepsin solution (1gm pepsin/ 10ml KCl-HCl buffer).
- Sample was incubated at 40 ºC for 1hr in water bath with continuous shaking at 1000rpm.
- After cooling down to room temperature, 900ml of tris – maleate buffer (pH 6.9) and 100ml of α amylase (4g/100ml tris –maleate buffer) was added.
- Sample was incubated at 37 ºC for 16 hrs with continuous shaking. Sample was centrifuged and residue was retained.
- Residue was washed multiple times distilled with water to remove sugars and centrifuged.
- After centrifugation, residue was lyophilized to obtain dried starch.

3. Isolation of RS with two enzymes(alpha amylase and amyloglucosidase)-

- 180g of gelatinized starch (starch to water, 1:5) was mixed with 1L if HCL-KCL buffer (pH 1.5) and 20 ml of pepsin solution (1gm pepsin/ 10ml KCL-KCL buffer). Sample was incubated at 40 ºC for 1hr in water bath with continuous shaking at 1000rpm.
- After cooling down to room temperature, 900ml of tris – maleate buffer (pH 6.9) and 100ml of α amylase (4g/100ml tris –maleate buffer) was added.
- Sample was incubated at 37 ºC for 16 hrs with continuous shaking. Sample was centrifuged and residue was retained.
- Residue was washed multiple times with distilled water and centrifuged to remove sugars.
- 100ml of 0.4M sodium acetate (pH 4.75) and 100ml of amyloglucosidase was added to the residue and vortexed.

- Sample was incubated at 60 ºC for 45 min in water bath and centrifuged. Residue was washed with distilled water multiple times and lyophilized.

3.5 – Characterization of resistant starch content-

Physio-chemical analysis was done by performing the following tests-

a) Swelling power-

- 0.5g of the resistant starch (one enzyme treated S1, two enzyme treated S2 and autoclaved gelatinized S3) was taken and dissolved in distilled water and the mixture was vortexed.
- The mixtures were heated at 60°C and 90°C for 30 minutes in a boiling water bath respectively.
- The suspensions were cooled and were subjected to centrifugation at 5,000 RPM for 15 minutes.
- The supernatant was discarded and the weight if the residue was noted respectively.

Swelling power = weight of sediment/ weight of dry sample **eq. (7)**

b) Scanning electron microscopy (SEM) –

Samples were sent for SEM analysis.

3.6 - Prebiotic activity of resistant starch –

- All the five probiotic cultures were screened for checking their utilization with resistant starch and proliferating.
- Initially, 11% reconstituted skim milk powder was autoclaved with supplementation of 0.001% and 0.25% of alpha amylase treated resistant starch.

- After the prepared solutions were cooled, tubes were inoculated with cultures at the rate of 1% (i.e. each culture had two tubes of 0.001% and 0.25% of RS).
- The tubes were kept at 37°C overnight incubation.
- After incubation, fermentation was examined for viable cell counting of all the cultures and their concentrations were done for the fifth and sixth dilution, pH and titratable acidity.
- A control tube without addition of resistant starch was also incubated in parallel.

3.7. Selection of starter culture for synbiotic product-

Gelatinized amylase treated resistant starch from Kufri Bahar variety has stimulatory effect on growth of probiotic cultures however this effect is strain specific indicating that all the probiotic cultures do not have the enzymatic machinery to ferment and utilize resistant starch. Therefore, CD4 *Lactobacillus paracasei* was used for further study.

3.8 – Preparation of synbiotic product-

- Cow milk was autoclaved with 100 ml each in reagent bottle with 0.01% resistant starch in four bottles and four bottles as control (i.e. without resistant starch).
- After cooling, 2ml of CD4 *Lactobacillus paracasei* CD4 was inoculated in all the bottles and kept at 37°C for overnight incubation.
- The storage study was preceded by examining for the viable cell counting, viscosity and pH.
- The study was done on zeroth day, seventh day and so on and the samples were stored at 4°C and left undisturbed for rest of the days.

CHAPTER-4

RESULTS AND DISCUSSION

4.1- Analysis of raw cow milk-

- **pH**: 6.25
- **Total Solid Substrate (TSS)**: 10.5°brix
- **Acidity:** 2.1
- **Fat Estimation:** 4.5%
- **Antioxidant activity-** Negligible

Physio-chemical properties of the cow milk were up to the standard values and the antioxidant content in the sample was negligible. ABTS is a commonly used to measure of antioxidant property. It is basically used in food and agriculture industry. In this method ABTS cation radical is formed by the loss of an electron by nitrogen atom of ABTS which absorbs light at 734nm. During this reaction the formed radical cation is blue in color due to the addition of sodium persulphate. Antioxidant activity is therefore, inversely proportional to the noted absorbance.

- **Moisture Content-**

Moisture Content = 16.74%

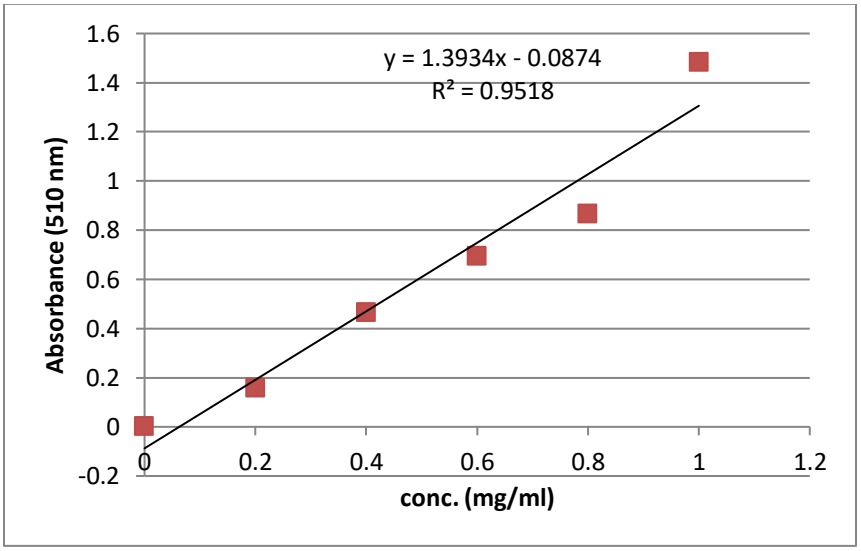
Weight loss = 354.9g

Weight of the sample = 21.2

- **DNS Method for estimation of reducing sugar-**

Table 4.1 - Absorbance of standard and samples at 510 nm

SAMPLE	ABSORBANCE (510 nm)
0	0
0.2	0.156
0.4	0.464
0.6	0.691
0.8	0.865
1	1.480
Blank	0.83
Sample (1:10)	3.690



Graph 4.1-Graph b/w OD and Concentration of Glucose.

As form our results obtained-

Reducing sugar concentration in sample 1 = 1.55µg/ml

Reducing sugar concentration in sample 2 and 3 were Negligible

For the estimation of the concentration of the reducing sugar in a sample DNS method is used**.** 3,5- dinitrosaliscyclic acid in alkaline condition when reacts to a reducing sugar (glucose) the resultant complex formed is orange in color which is known as 3-amino,5- nitrosaliscyic acid. The intensity of the color represents the index of the reducing sugar. This reaction is carried out under alkaline conditions so as to reduce the interference by oxygen in the process of glucose oxidation. Sulphite in sodium sulphite therefore absorbs the dissolved oxygen. The sugar concentration in our sample was observed in the dilution of 1:10.

- **ACE Activity-**

Angiotensin-converting enzyme or ACE is a fundamental constituent of the renin-angiotensin system (RAS), which tends to control blood pressure by amending the volume of fluids in the body. It converts the hormone angiotensin I to the active vasoconstrictor angiotensin II. Hence, ace increases BP by constricting the blood vessels. ACE is a zinc metalloenzyme. The zinc ion is vital for its activity, because it directly takes part in the catalysis of the peptide hydrolysis. The ace inhibitory activity in our sample was 1.16.

- **Total Phenolic Content-**

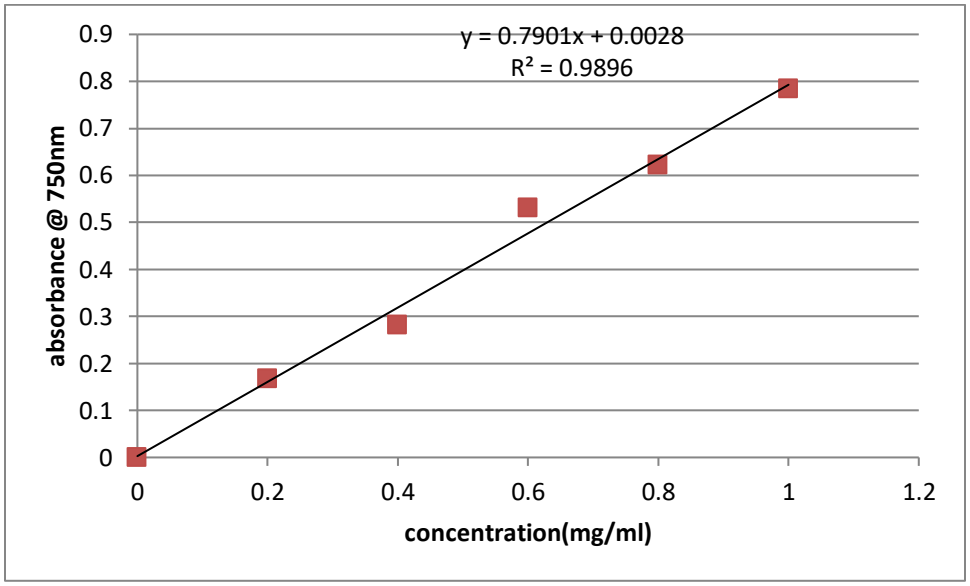
As from our results Phenolic content in our sample = 0.07mg TAE/ml

Table 4.2 – Absorbance of standards and samples at 734 nm

STANDARD	ABSORBANCE (734 nm)
0.2	0.168
0.4	0.282
0.6	0.531

0.8	0.622
1	0.784

SAMPLE	ABSORBANCE (750nm)
Blank	0.129
Sample (1:10)	0.06



Graph 4.2- Represents the relation between Concentration and absorbance

During the reaction of phenolics with FC reagent it is known that the molybdate ion of sodium molybdate gets reduced and as a result becomes phenolate anion which reduces FC reagent. The reaction results the formation of a green-blue complex which is independent of the structure of phenolics. However, this method of phenolic content is non-specific. It is just an oxidation reduction reaction. The default value of phenolics in cow milk reported is 0.7± 0.6. To increase the phenolic concentration of raw cow milk the sample was freeze dried and was heated in order to increase the phenolic concentration.

Figure 4.1 – lyophilized milk

4.2 Analysis of fermented milk-

Table 4.3 – Analysis of fermented milk with probiotic cultures

Lactic culture used	pH	Acidity	TSS (°Brix)	TPC (µg/ml)	Antioxidant activity (%)	ACE inhibitory	Viable count log CFU/ml
***Lactobacillus paracasei* CD4**	4.64	2.8	6	0.25	negligible	4.91	7.60
***Lactobacillus gastricus* BTM7**	4.65	2.7	10	0.89	negligible	1.5	8.36
***Brevibacillus Aydinogluensis* BTM9**	4.44	2.5	10	0.24	29	3.58	8.25
***Lactobacillus rhamnosus GG* LRGG**	3.80	5.3	8	0.34	negligible	0.083	8.49
***Lactobacillus fermentum* K75**	4.83	3.2	8.8	0.75	2	6.67	8.34

Raw milk	6.25	2.1	13	0.31	negligible	0.67	0

The general trend of obtaining an effective strain is reduction of pH which leads to increase in acidity with time. But as data from our results no such trend was observed and hence, ph and acidity of each strain for the 0^{th} hour was taken into account and further tests were done considering all the 5 strains.

Raw milk batch used for the analysis of the fermented milk was different from the batch used for the analysis of raw cow milk. The TSS content of all the lactic acid cultures used in our project ranges from 6 to 10 °brix.

All the further experiments were done by lyophilizing the milk. Antioxidant activity was observed only in BTM9 and K75. Others were negligible in spite of exposing the milk to fermentation.

Total phenolic content was carried out which ranged from 0.24 to 0.89 µg/ml.

Similarly, Ace inhibitory activity in fermented milk ranged from 0.083 to 6.67 with control having ace inhibitory activity of 0.67

Figure 4.2 - Five fermented milk flasks

- **CFU/ml -**

Figure 4.3 – MRS tubes of lactic acid cultures

Figure 4.4 – Skim milk tubes

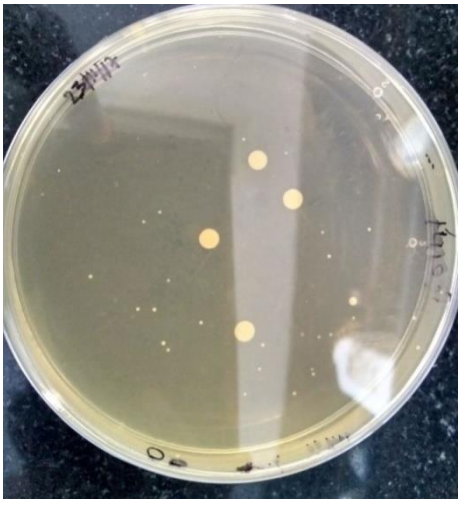


Figure 4.5 – Colonies of LRGG on MRS plate

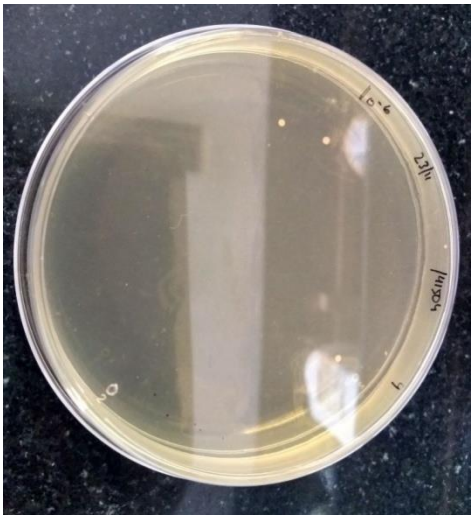


Figure 4.6 - Colonies of CD4 on MRS plate

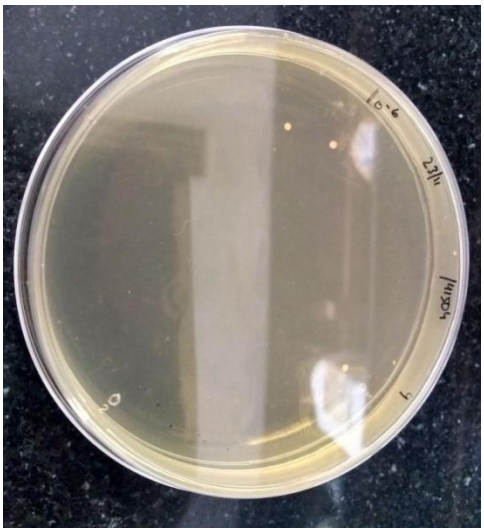


Figure 4.7 - Colonies of BTM7 on MRS plate

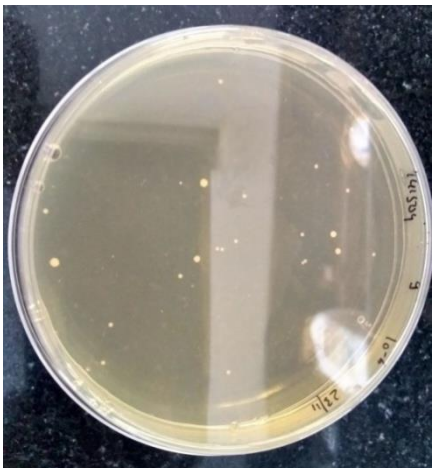


Figure 4.8 -Colonies of BTM9 on MRS plate

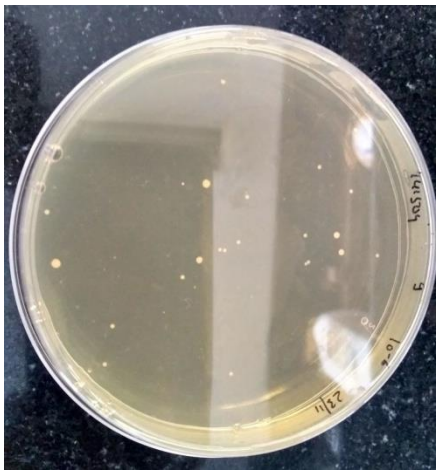


Figure 4.9 - Colonies of K75 on MRS plate

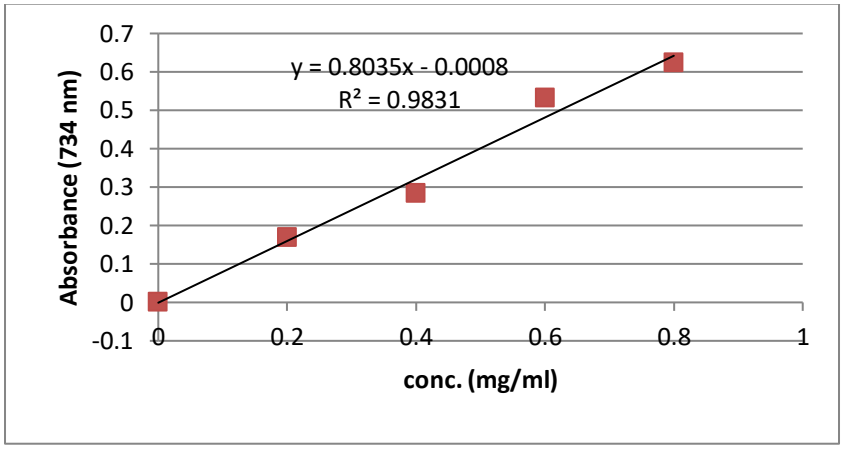
Calculating the CFU/ml gives the viable bacterial cell population. For convenience the results are given as CFU/ml for liquids and CFU/g for solids.

- **Total phenolic content-**

Table 4.4 – Absorbance of standard and samples at 734 nm

STANDARD (TANNIC ACID)	ABSORBANCE (734nm)
0	0
0.2	0.168
0.4	0.282
0.6	0.531
0.8	0.622
1	0.784

SAMPLE	ABSORBANCE @750NM
Blank	0.129
Sample (1:10)	0.06



Graph 4.3- Relation between conc. and OD at 734 nm.

4.3 – Extraction of resistant starch-

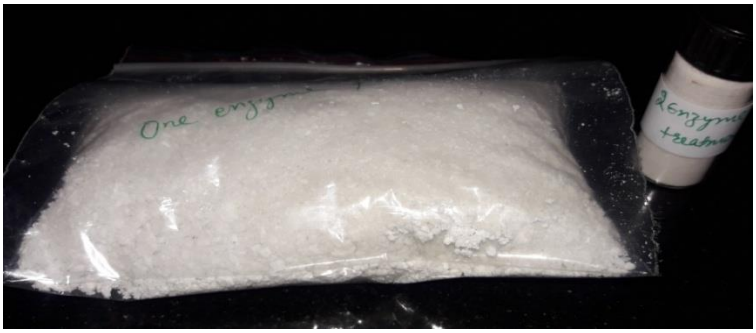


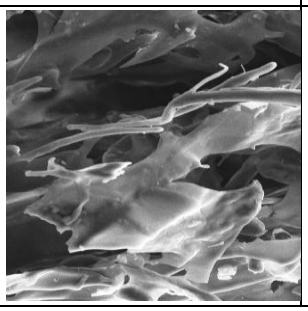
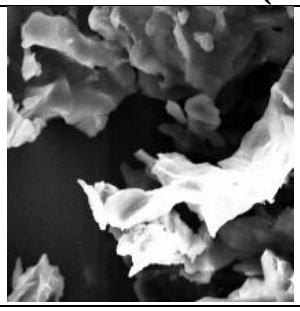
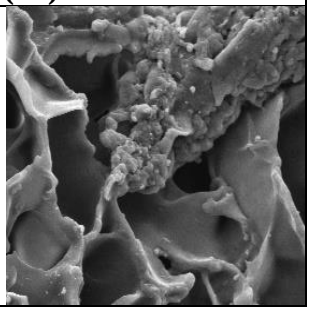
Figure 4.10 - Resistant starch extracted in CPRI Shimla in lyophilized form

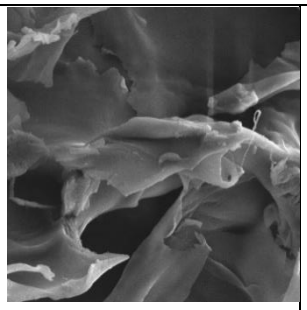
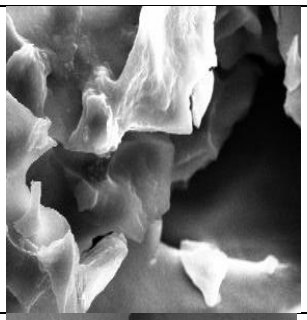
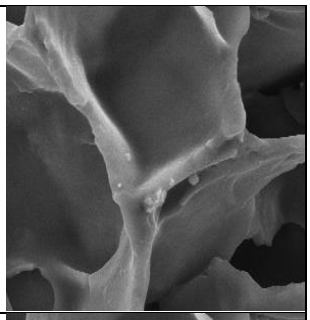
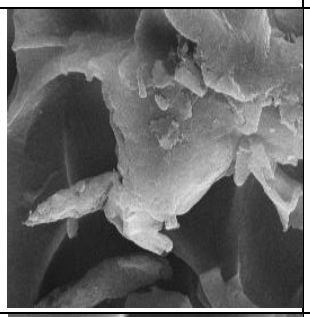
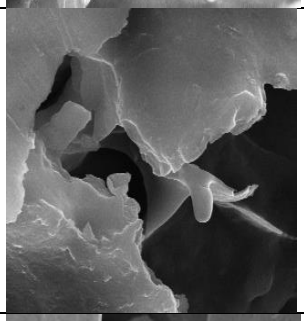
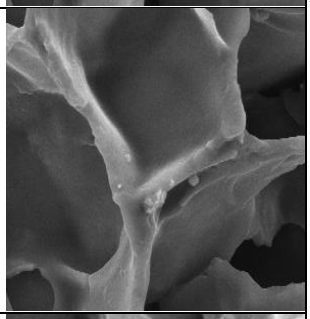
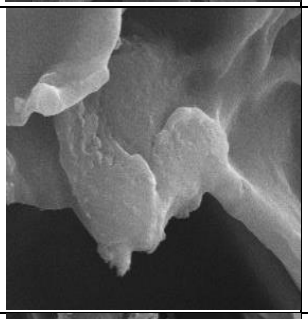
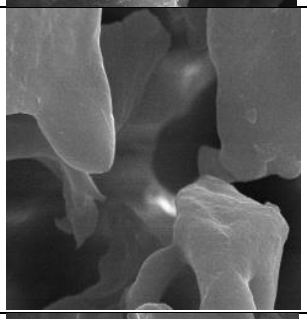
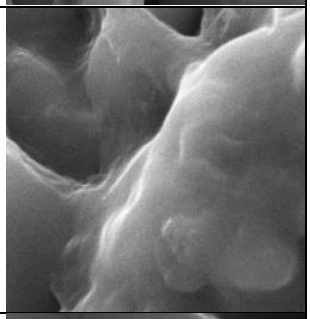
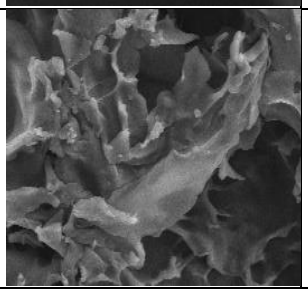
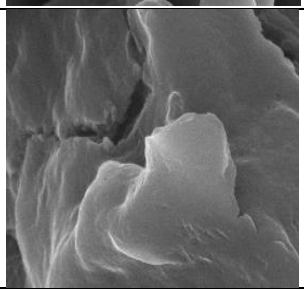
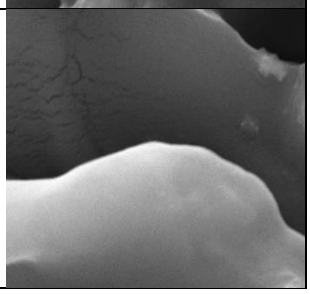
4.4 - Characterization of resistant starch-

- **SEM –**

a) SEM analysis at different magnifications of three different RS samples:

Table 4.5 – SEM analysis of RS at different magnification

MAGNIFICATION	ALPHA-AMYLASE TREATED RS (S1)	ALPHA -AMYLASE + AMYLOGLUCOSIDASE TREATED RS (S2)	AUTOCLAVED GELATINIZED RS (S3)
500X			

1.00 KX			
2.50 KX			
5.00 KX			
10.00 KX			

SEM micrographs of S1 and S2 showed irregular, dense and loosely packed structures whereas S3 showed smooth and amorphous structures as it was only to gelatinization effect and temperature. Smooth structure of RS exposes to more microbial activity. More amorphous type of structure leads to the inhibition of enzyme treatment activity.

b) Oxygen and carbon content of three different samples of resistant starch:

Table 4.6 – Oxygen and carbon content of three different samples of RS

	ONE ENZYME TREATED (S1)	**TWO ENZYME TREATED (S2)**	**AUTOCLAVED GELATINIZED (S3)**
Carbon content	52.53	52.55	48.33
Oxygen content	47.38	47.44	51.66

- **Swelling power** –

Table 4.7 – Swelling power of three samples of RS at two different temperatures

TEMPERATURE (°C)	**ONE ENZYME TREATED (S1) %**	**TWO ENZYME TREATED (S2) %**	**AUTOCLAVED GELATINIZED (S3) %**
60	0.07	0.02	0.03
90	0.09	0.05	0.04

The swelling power of one enzyme treated resistant starch increased by 2% from 60°C to 90°C**.** Whereas the swelling power of two enzymes treated RS increased by 3%. The normal autoclaved gelatinized RS had an increasing swelling property of 1% with respect to two different temperatures. Thus, this showed that with increase in temperature, swelling power of RS enhances which is a desirable characteristic of RS.

4.5 - Prebiotic activity analysis of resistant starch

Table 4.8– Prebiotic analysis of RS with probiotic cultures

PROBIOTIC CULTURES	**CONTROL**	**0.001%**	**0.025%**
***Lactobacillus paracasei* CD4**	7.50	8.36	9.38
***Lactobacillus gastricus* BTM7**	8.07	8.27	8.30
***Brevibacillus Aydinogluensis* BTM9**	6.27	6.47	6.39
***Lactobacillus rhamnosus GG* LRGG**	6.6	6.0	6.30
***Lactobacillus fermentum* K75**	6.0	0	6.77

Viable cell counting at different concentration of resistant starch was calculated in log CFU/ml. The viable count with *CD4* increased by 24% (RS= 0.025%) and increased to 11% (RS=0.001%). No stimulatory increase with *BTM7, BTM9, K75 and LRGG.*

- pH and titrable acidity of the five probiotic cultures were also analyzed in order to check their ability to utilize resistant starch and proliferate.

Table 4.9 – pH and titrable acidity of RS with cultures respectively

PROBIOTIC CULTURES	**CONTROL**	**0.001%**	**0.025%**
***Lactobacillus paracasei* CD4**	5.12	4.98	4.74
***Lactobacillus gastricus* BTM7**	5.16	5.13	5.06
Brevibacillus Aydinogluensis	7.24	7.17	6.97

BTM9			
***Lactobacillus rhamnosus GG* LRGG**	6.05	5.88	5.91
***Lactobacillus fermentum* K75**	6.88	7.17	6.97

PROBIOTIC CULTURES	**CONTROL**	**0.001%**	**0.025%**
***Lactobacillus paracasei* CD4**	5.5	7.5	7.7
***Lactobacillus gastricus* BTM7**	6.3	6	6.2
***Brevibacillus Aydinogluensis* BTM9**	5	5	5.5
***Lactobacillus rhamnosus GG* LRGG**	5.4	6.5	7
***Lactobacillus fermentum* K75**	6	6	4.8

No particular trend from both the tables was observed.

4.6 - Storage study analysis-

- **Viable cell counting –**

Table 4.10 – Log CFU/ml of storage study analysis of the synbiotic product

DAYS	**CONTROL**	**SAMPLE (RS + CULTURE)**
0^{th} Day	5.75	5.95
7^{th} Day	5.97	6.12

More number of colonies was observed in 5^{th} dilution as compared to 6^{th} dilution. This showed that in the process of serial dilution *Lactobacillus paracasei* CD4 utilized more amount of resistant starch to enhance its metabolic rate and hence increased in number.

- **pH and viscosity-**

Table 4.11 – pH and viscosity (Cp) Readings of storage study analysis of synbiotic product respectively

DAYS	CONTROL	PRODUCT
0^{th} Day	3.61	3.63
7^{th} Day	3.58	3.60

DAYS	CONTROL	PRODUCT
0^{th} Day	158.5	163.5
7^{th} Day	1251	1377

CHAPTER-5

SUMMARY AND CONCLUSION

As the diverse cultures were inoculated into the cow milk that have the skill to alter the antioxidant activity of the milk. Practically, for the progress of any type of functional food based on cow milk with various health benefits, different strains of lactic acid bacteria be capable of being used. Angiotensin converting enzyme inhibitory activity showed higher degree of inhibitory activity ranging from 6.67 in L75 culture to as low as 0.083 in LRGG.

Different trends in pH and acidity from the first batch of cow milk to the second batch containing diverse lactic acid cultures showed the random fashion.

Overall five lactic acid cultures were screened for their probiotic properties and based on their pH and titrable acidity, *Lactobacillus paracasei* CD4 showed the best possible trend among all the chosen cultures. Thus we selected this probiotic culture in combination with resistant starch for preparation of synbiotic product and its storage study.

RS was treated with alpha amylase and amyloglucosidase enzyme for further procedure. Characterization of RS was done by SEM and swelling power analysis. Prebiotic analysis of RS was done in 11% reconstituted skim milk and after its various analysis *Lactobacillus paracasei* CD4 and *Lactobacillus gastricus* BTM7 was able to utilize RS for its growth and proliferation with 0.001% (concentration) of RS.

Synbiotic product (curd) was prepared from cow milk containing RS with *Lactobacillus paracasei* CD4 as it showed the best trend with respect to a significant probiotic culture. After analysis of viability cell counting, pH and viscosity was done for storage study which showed the pattern of increasing utilization of RS as a prebiotic to enhance its metabolism.

In conclusion, the isolated lactic acid bacteria's have shown the possibility to enhance the different parameters of the cow milk in combination with resistant starch.

CHAPTER-6

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STIMULATORY EFFECTS OF RESISTANT STARCH FROM KUFRI BAHAR POTATOES ON BENEFICIAL PROBIOTIC LACTIC ACID BACTERIA

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ABSTRACT

Resistant starch is one of the variety of starch which is reported for the prebiotic effects for probiotic cultures. Recently resistant starch from potato is gaining attention as a functional food ingredient due to its unique functional and physiochemical properties. However, the role of RS in Indian potato varieties has not been evaluated yet. The study investigated the stimulatory effect of extracted resistant starch from Kufri Bahar variety of potatoes. RS was extracted using amylase treatment. The RS stimulated the growth of *Lactobacillus paracasei CD4* by 24% when supplemented at a level of 0.025% in skimmed milk based media whereas no stimulatory effect was observed for other lactic strains. The study indicated the prebiotic potential of resistant starch from Indian potato varieties, however the prebiotic effect is strain specific.

Keywords

Prebiotic, Probiotic, Resistant starch, Lactic acid bacteria

OBJECTIVE

Keeping in mind the health benefits of RS and since not much work has been done on the Indian potato varieties; the present work was designed to extract resistant starch from Kufri Bahar potato variety and to check its efficacy in proliferation of probiotic lactic acid bacteria.

MATERIALS AND METHODS

- Resistant Starch was extracted Kufri Bahar variety of potatoes.
- The RS was evaluated for prebiotic effect in skimmed milk based media with *Lactobacillus paracasei* CD4 and *Lactobacillus gastricus* BTM7.

Protocol for extraction and estimation:

Extraction and Estimation of resistant starch

1g of boiled and raw potato

↓

10ml of KCl-HCl buffer (pH 1.5),0.2ml of pepsin to each sample and incubation 37 ° C

↓

Addition of Tris-maleate buffer and 1ml of alpha-amylase

↓

After incubation, the samples were centrifuged

↓

To supernatant add KOH ,HCl and sodium acetate buffer (pH 4.75) , amyloglucosidase was added

↓

The thoroughly mixed sample were centrifuged and supernatant was collected

↓

Both the supernatant were combined to make up the volume 25ml

Prebiotic activity estimation

To estimate the prebiotic effect of RS, skimmed milk tube containing 11% reconstituted skim milk , supplemented with 0.001 and 0.025% RS were prepared

↓

The lactic cultures were added with 1% inoculum and incubated at 37 ° C for 24h. The fermentation was assessed by pH, titratable acidity and viable cell count

RESULTS

The gelatinized RS in Kufri Bahar variety was 0.789% and was used for its prebiotic effect. The final pH of *Lactobacillus paracasei CD4* dropped from 5.12 to 4.7 whereas there were no significant changes with *Lactobacillus gastricus BTM7*. The viable count with CD4 increased by 24% (RS=0.025%) and increase to 11%(RS=0.001%). No stimulatory increase with BTM7.

Fig.1- Tubes containing Alpha-Amylase treated resistant Starch(3.29 and 1.027 g)

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ACKNOWLEDGEMENT

We would like to thank Jaypee University of Information Technology for the technical and infrastructure help.

First International Conference On Futuristic Trends In Network And Communication Technologies
9-10 Feb 2018