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Development of prebiotic galactooligosaccharide enriched buttermilkand evaluation of its storage stability

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Summary. Prebiotic Galacto-oligosacchrides (GOS) is reportedly present in human milk and elicits bacterial growth i.e. beneficial bacteria in human intestines. The aim of the present study was to produce GOS through trans-galactosylation and development of GOS based buttermilk. Trans-galactosylation process was carried out in pasteurized milk (100 °C) to produce prebiotic GOS. Yogurt was produced from GOS containing milk and then after the churning of yogurt buttermilk was produced from trans-glycosylated milk.Results showed that, enzyme concentration between 400-600µL/5ml can produce GOS in 30min and 1hr samples. Chemical analysis and sensory evaluation of plain/control and GOS containing buttermilk showed no remarkable difference. While shelf life study showed that there was no significant difference between the overall quality of buttermilk in glass and pouch packaging. Buttermilk in pouch packaging maintained its stability for 6 days without adding any kind of preservative.

Key words: Buttermilk, galacto-oligosaccharides, prebiotic, transgalactosylation

Introduction

Since 20th century greater emphasis is placed on diet and nutrition due to growing concern regarding the diet related diseases prevalence on global scale (1). The concept of "functional foods" is recently introduced, extensively investigated shortly after, and moved forward to somehow fully grown field. Given the widely accepted fact that functional foods are associated with health benefits (2) so they are prepared with specific, either alone or in combination, with more than one specific bioactive component, usually of plant origin. The advancement made in microbial enzyme technology has further modified functional food sector. Specific microbial enzymes can be used to modify, synthesize or convert foods constituents to more beneficial compounds within foodsthat can attain health benefits or at least decrease the risk of certain diseases (3). With this trend, trans-galactosidase enzyme is reportedly used that can convertmilk lactose to galactose-oligosaccharides (GOS), a well-known and valuable prebiotic (4). This reaction may be beneficial for increasing the milk tolerance for people with lactose intolerance andgrowth of gut microbiota (5).

Galacto-oligosacchrides is not digestible and can be fermented in the small intestine by microbiota (6). Growing body of evidences have shown that GOS is associated with reduction in cholesterol (7), diarrhea, lower intestinal inflammation and increases mineral absorption (8), improve immunity (9), and enhance the growth of beneficial microbiota (6), to name a few. In view of the importance of this dietary fibre, technologically and economically feasible processing methods and product development is necessary. This manuscript reports reliable, easy and cost effective methods for preparation of buttermilk with prebiotic which has comparatively low lactose content.

Materials and methods

D-Glucose (99%) (Merck, Germany), D-Galactose (99%) (Merck, Germany), Lactose (99%) (Mer-

ck, Germany), NaHPO4 (96%) (Merck, Germany), 1-Butanol (99%) (Merck, Germany), Ethanol (99%) (Merck, Germany), 2-Propanol, (99%) (Merck, Germany), H2SO4 (<96%) (Merck, Germany), NaOH (99%) (Merck, Germany), Thymol (90%) (Eyer®), oNPG (99) % (Sigma-aldrich, Germany) and LB broth (MP Biochem, France) were purchased.

Bacterial cell growth and enzyme production

β-galactosidase enzyme was isolated from *Escherichia coli (E.coli)*, which was initially from University of Natural Resources and Life Sciences Vienna Department of Food Science and Technology Division of Food Biotechnology (Vienna, Austria). This strain has gene of *Lactobacillus reuteri* L103 which has ability to produce more active enzymes than taken from normal non recombinant *E.coli*.

Bacterial strains and culture conditions

Escherichia coli containing β-galactosidase gene from *Lactobacillus reuteri*L103 was cultured as the source of the β-galactosidase enzyme. *E.coli* cells were grown on Luria-Bertani broth (LB) containing the appropriate antibiotics (100 μg/mL ampicillin) required for maintaining the plasmids. Bacterial culture was grown at 37 ˚C in incubator for 14-16 hours in 5 mL LB medium. Safety measures such asfiltration of lab through UV light, use of ampicillin and gloves were used to avoid contamination.

After the bacterial growth, 0.8 mlof the grown culture (culture A) was transferred into 40 mLLB medium and was grown again at 37 ˚C for 16 hrs. Then,culture A (2mL) was transferred into 2000 mLLB medium and was grown again at 37 ˚C until optical density of this media was between 0.3-0.6, then isopropyl-β-D-thiogalactoside (IPTG) was added (final conc. in media 0.1 mM), after adding IPTG, bacteria was grown at 25 ˚C for 16 hours. These three steps were carried out again and again until the requirednumbers of cells were collected.

Collection and cell breakdown

Cells (90 ml) were collected by centrifugation (Micro 200R, Japan) (5000rpm, 15min, 4˚ C) when lag phase of microbial growth was reached. The cells obtained were washed twice in 50mM sodium phosphate buffer pH 6.5 and suspended in same buffer and stored at 4˚ C. Supernatant was drained while cellswere breakdown with sonicator (Ultrasonic homogenizer) for 2 min at 13000 rpm and cell debris were removed by ultra-centrifugation (Centrifuge 5810R, Japan) (12000rpm, 20min, 4˚ C) to obtain the unpolished cell extract/ crude enzyme (35 mL).

Enzyme assays

β-Galactosidase (β-gal) activity was determined using O-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrates (Nguyen et al. 2006). 20 μL crude enzyme sampleswere added to 480 μL of 22 mM ONPG in buffer (50 mM sodium phosphate buffer, pH 6.5). After 10 minutes, reaction was stopped by adding 750 μL Na_2CO_3 (0.4 M). The release of O-nitrophenol was assessed by measuring the absorbance at 420 nm.

Transgalactosylation experiment on buttermilk

Buttermilk15 mLwas taken in 3 separated falcon tubes. Reaction was started by adding enzyme β-galactosidase 50μL, 200 μL and 400 μL. Reaction were carried out for 4 hrs at 37˚ C and agitation (230 rpm) was applied by putting in shaker. Samples were withdrawn after 0hr, 30 mins, 1hr, 1.5hr, 2hr, 3.5hr, and 4hrs. The enzyme was deactivated by dipping in boiling water for 3 minutes.Similarly, from each time point 400 μL samples were drawn each time dipped in boiling water. The samples were then mixed with 180 μL, centrifuged again. After centrifugation, enzyme was settled at the bottom of falcon tubes while supernatant was kept in hot oven at 70º C for about one hour. After the oven treatment, 20 uL supernatant was diluted with180uLdistilled water and was used for TLC analysis.

Thin Layer Chromatography (TLC)

Almost 1.0 μL from each prepared solution, as mentioned above, along with standards (glucose, galactose, lactose) was taken and applied on silica gel plate, dried the plates for 20 minutes and placed in running buffer used in TLC was prepared by water, n-propanol, ethanol, and n-butanol in the ratio of 2:3:3:2 respectively. Silica gel plates were put in the chromatographic tank till the buffer reached the 2/3rd of the plate at the upper end. TLC was run for 45 minutes. After that, the plates were taken out of the chromatographic tank

and dried in the hot air oven at 100°C. Staining buffer (0.6 g thymol+5ml H_2SO_4(95%) + 95ml ethanol) was sprayed on the dried plates. Sugar stains were visible after 110 °C heating in hot air oven which shows the formation of glucose,galactose and GOS.

Analysis of buttermilk

Buttermilk was analyzed for the following parameters like fat, protein, Lactose, Ash and pH was determined according to the methods of AOAC (10).

Standards and buffers used for TLC

The standards used for thin layer chromatography were of following concentration; 20g/lGalactose, 20g/l Lactose, 20g/l Glucose. 0.02g of each standard samples were taken anddissolved in 1ml of distill H_2O. TLC staining buffer composition was: 0.6 g thymol+5ml H_2SO_4(95%) + 95ml ethanol.

Quantification of Galacto-oligosaccharides

Quantification of Galacto-oligosaccharides was done by measuring lactose, D-galactose and D-glucose through Megazyme kits (Megazyme International, Ireland) and detailed as:

D-glucose Assay conditions

About 3.0 ml of GOPOD reagent was added to 0.1 ml of buttermilk solution and incubated at 40°C to 50°C for 20 minutes. Absorbance was read against reagent blank at 510 nm wavelength in order to get values of ΔAsampleand ΔAD-glucose standard.While for the lactose and D-galactose, absorbance was carried out at 340 nm, and water was taken as blank.Total galacto-oligosaccharides produced were calculated by following formula:

GOS (g/L) =A – B+C+D

Where;A= Initial lactose concentration (g/L); B= Lactose concentration after transgalactosylation (g/L); C=Galactose concentration after transgalactosylation (g/L); D=Glucose concentration after transgalactosylation (g/L)

Preparation of buttermilk

The raw milk was inoculted with 2.5% of mixed culture of *Lactobacillus balgaricus* and *Streptococcus thermophillus* and filled into clean glass containers (250 ml volume) and incubated at 45° C for 3-4 hours. After the curd formation it was cooled to 4 °C (11).
Yogurt was then taken in a butter churner and was agitated vigorously until the whey was separated from the fat. Butter/ fat was removed and remaining liquid (which was buttermilk) was collected for further analysis.

Production of prebiotics GOS

Pasturization of 10 litre of raw milk was done at 85°C for 15 seconds. The β-galactosidase enzyme (35 ml) was added in milk at that temperature optimized at initial trials. Then 100 µL of this milk was used to check the enzyme activity and immediately 400 µL of ONPG (chromogenic substrate o-nitrophenyl-β-D-galactopyranoside) was used for enzyme deactivation at 95 °C for 5 minutes and then cooled to 45 °C temperature.

Chemical Analysis

Once the buttermilk containing prebiotic galacto-oligosaccharides was produced, it was analyzed for the quantity of protein, fat and SNF, pH, glucose, galactose and lactose (Table 4).

Organoleptic acceptability

Prebiotic galacto-oligosaccharide containing buttermilk was presented for sensory evaluation by a semi-trained panel of 10 judges (12). For this purpose judges were contacted through personnel contacts, emails and fliers and consent forms were signed. The judges were given a performa, they marked it according to their individual acceptability. Evaluation was carried out by the panelists using 15-cm unconstructed line for parameters of color, flavor, taste, consistency and overall acceptability on a sensory evaluation.

Shelf life study of buttermilk containing GOS

Pouch and glass packaging of prebiotic GOS buttermilk was done to check the shelf life and stability of the new product. Glass bottles were used for glass packaging. Bottles were sterilized before packaging. Shelf life of the new products was checked by sensory evaluation which was carried out for 6 days. A panel of 10 judges (each day) participated in the evaluation and marked the given performa according to their acceptability of the product. The results of collected data

were analyzed through paired sample T test and two way ANOVA analyses by using SPSS version 16.0.

Results

Chemical analysis of Trans-galactosylated buttermilk

Results showed that enzyme concentration between 400-600μL/5mL can produce GOS in 30min and 1hr samples. By increasing time all lactose present in milk was converted into galactose and glucose by breaking all β-linkages present in lactose. Results showed no significant difference (p>0.05) in the composition of plain and prebiotic buttermilk, suggesting safety of the prebiotic for human consumption (Table 1).

Sensory evaluation of plain and prebiotic buttermilk

Sensory attributes of plain and GOS containing butter milk is presented in Table 2 and Figure 1. According to the results, significant difference was found (P≤0.05) in flavor, taste and consistency of plain and prebiotic GOS containing buttermilk. However, overall acceptability remained similar (P=0.08) for both type of milk. Moreover, GOS buttermilk was slightly sweet as compared to plain buttermilk, but sensory panelist scored preferences was found to be not significantly different (P = 0.335).

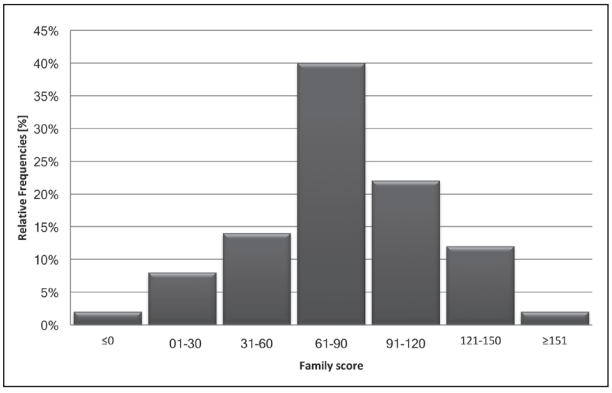

Figure 1. Average Score of Sensory Attributes of plain and prebiotic buttermilk

Shelf Life Study

Buttermilk containing prebiotic galacto-oligosaccharides was pouch packed and glass packed by sterilizing glass bottles and pouring buttermilk in it. Buttermilk was stored at refrigeration temperature for 6 days. Sensory evaluation was carried out for 6 consecutive days to check the shelf life of prebiotic GOS containing buttermilk in both types of packaging.

Table 1. Chemical analysis of buttermilk

Analysis	Plain Buttermilk	GOS containing Buttermilk	Independent samples t- statistic	P Value
Protein	3.233±0.015	3.133±0.100	1.061	0.184
Fat	0.6±0.057	0.533±0.057	1.000	0.561
Ash	0.633±0.073	0.666±0.016	-.267	1.000
Lactose	3.906±0.057	2.763±0.057	3.583	0.073
pH	4.533±0.048	4.433±0.176	2.121	1.000

Note: All differences are statistically non-significant (p>0.05)

Table 2. Sensory analysis of prebiotic buttermilk with plain buttermilk

Parameters	Plain Buttermilk	Prebiotic Buttermilk	Independent samples t statistic	P-value
Colour	8.6±0.665	10.3±0.866	-1.169	0.185
Flavour	8.3±0.568	9.2±1.111	-0.826	0.334
Taste	8.7±0.264	9.8±0.896	-1.281	0.484
Consistency	7.6±1.106	8.5±0.472	-0.988	0.591
Overall Acceptability	7.2±1.457	10.4±0.568	-2.476	0.932

Note: All differences are statistically non-significant (p>0.05)

Table 3. Shelf life of sensory attributes of prebiotic buttermilk in different packages

OAA	Consistency	Taste	Flavour	Colour*	Parameters		
7.22±0.55	7.61±0.32	8.73±0.33	8.39±0.31	8.69±0.45	Day 1	Glass	Packaging
10.46±0.20	8.50±0.42	9.82±0.11	8.39±0.31	10.31±0.50		Pouch	
9.66±0.42	8.53±0.45	8.76±0.53	10.6±0.05	8.6±0.44	Day 2	Glass	Packaging
10.88±0.05	9.34±0.23	9.86±0.09	11.15±0.89	8.97±0.18		Pouch	
7.68±0.1	9.40±0.33	9.11±0.03	8.19±0.19	8.5±0.45	Day 3	Glass	Packaging
9.39±0.33	10.76±0.15	10.06±0.05	9.67±0.43	10.02±0.02		Pouch	
7.68±0.1	9.40±0.33	9.11±0.03	8.67±0.44	8.53±0.45	Day 4	Glass	Packaging
9.39±0.33	10.76±0.15	10.06±0.05	9.53±0.24	9.17±0.08		Pouch	
8.95±0.10	9.62±0.44	9.52±0.43	9.92±0.08	9.68±0.32	Day 5	Glass	Packaging
10.33±0.1	9.24±0.17	9.92±0.08	10.37±0.46	10.3±0.50		Pouch	
7.68±0.1	9.40±0.33	9.11±0.03	8.67±0.44	8.53±0.45	Day 6	Glass	Packaging
9.39±0.33	10.76±0.15	10.06±0.05	9.53±0.24	9.17±0.08		Pouch	

Note: *$p<0.05$

Discussion

The ancient and effective role of prebiotics in our diet is not clear. Continual researches to clear our concepts of the health welfares of prebiotics through latest in-vitro and in-vivo studies would assist from the time complexity delivered by archaeology. A prebiotic was first known as a 'food ingredient that is not digestible and which constructively affects the host by selectively motivating the growth or activity of one or a restricted number of bacteria in the colon, and in this way improves the host health (13). As the archaeological evidence discloses, prebiotics have long been part of the human food and in amounts in some areas and time that far surpass those currently used by modern populations (14).

The production of galacto-oligosaccharide from lactose, by utilizing β-galactosidase enzyme, has been widely examined over the last 50 years because of the functional assets of GOS as prebiotics. The importance in GOS synthesis has increased since its addition in Japanese legislation concerning foods for stated health use (15).

Buttermilk, whey or lassi is a popular drink especially in Punjab regions of Pakistan and India. Buttermilk and lassi has many health benefits. Lassi is a mixture of yogurt, water and many water soluble vitamins. Lassi can be salty or sweet and has more or less the consistency of a smoothie. It provides calcium, vitamin B12, zinc and proteins. Vitamin B12 is very important for converting blood glucose into energy (16). While buttermilk is the liquid remaining after the removal of the butter fat (17), the nutritional value of buttermilk is similar to that of skim milk. It is an excellent source of potassium, vitamin B12, calcium and riboflavin.it is also a good source of phosphorus and contains zinc, magnesium, niacin, thiamin, folic acid and vitamin B6. Low in fat, buttermilk is rich in lactic acid and nitrogen (18).

Buttermilk is also recommended to the individuals suffering from lactose intolerance (19). Lactose intolerance is caused by insufficient production of the lactose enzyme, which is necessary for the breakdown and absorption of lactose, the carbohydrate found only in dairy products. Undigested lactose leads to bloating, nausea, abdominal pain and excessive gas. When dairy products are created by a fermentation process, they contain significantly less lactose. This is especially true for yogurts, aged cheese (e.g cheddar, swiss, Colby) and buttermilk (20).

Use of GOS as food ingredient have been found beneficial because GOS has wide options of usage in food products. Constancy of GOS towards salivary break down and oral microbiota makes GOS less cariogenic carbohydrate alternative in confectionary and chewing gums. GOS have low glycemic index and low calorie index because they are not digested by gastric juice and pancreatic enzymes while passing through

the small intestine. Its glycemic index is almost 50% less than that of sucrose, which makes GOS preferable for diabetic patients and a low calorie food. They are very much soluble and have good moisture holding ability. Their properties are similar to sucrose in such a way that they can improve the mouth feel, texture of food with pleasant taste (21).

In the present study, milk was obtained from UVAS Ravi campus and was transgalactosylated. Yogurt was produced from this milk by adding culture and after the churning of butter from yogurt, butter milk was collected and stored at refrigeration temp for further analysis. 5ml of milk samples were taken for enzyme analysis 0ul, 100ul, 200ul, 300ul and 800ul respectively samples was collected at 30min and 1hr and so on. Enzyme was denatured by putting in hot water and samples were analyzed on TLC with lactose and Yakut Oligomate as standard.

Result showed that. transgalactosylation occurred at 30 mins and at 1 hour. Clear bands could be seen with glucose, galactose and lactose standards. It showed that enzyme can produce GOS (galacto oligosaccharides) in 30min and 1hr samples. By increasing time all lactose present in milk was converted into galactose and glucose by breaking all linkages present in milk lactose. In this study, GOS were produced in milk by transgalactosylation process at optimized conditions.

Buttermilk was prepared with good organoleptic attributes. Organoleptic attributes of the buttermilk containing prebiotic GOS were checked and it was concluded that there was no significant difference between plain buttermilk and prebiotic galacto-oligosaccharide enriched buttermilk. Parameters like color, flavor, taste, consistency and overall acceptance were checked. Chemical analysis were done for plain and GOS containing buttermilk. Chemical analysis showed no significant difference between the composition of both buttermilk, which shows that galacto-oligosaccharides don't change the composition of buttermilk.

Mean values for chemical analysis of buttermilk were: protein (3.133±0.1) while for fat, ash and lactose values were (0.533±0.06), (0.666±0.03) and (3.906±1.1) respectively.

In a study conducted in USA on Chemical Composition, Probiotic Survivability and Shelf Life of Symbiotic Buttermilk, buttermilk was made using a commercial mesophilic starter CHN22 and the probiotics. The control buttermilk was made using CHN22 and the symbiotic buttermilk were evaluated for chemical composition, probiotics survivability, mold, yeast and coliform counts. Changes in pH, titratable acidity and proteolysis were also checked during storage at 4°C for 12 weeks. The chemical composition of the control and symbiotic buttermilk were: protein 3.29±0.05 and 3.30±0.02%; fat 3.28±0.04 and 3.26±0.06%; carbohydrate 4.55±0.05 and 5.16±0.06%; total solids 11.81±0.05 and 12.42±0.03%; ash 0.69±0.03 and 0.70±0.01%, respectively. There were significant differences in pH and titratable acidity between the control and symbiotic buttermilk ($p<0.05$). There was no major difference in proteolysis between the two samples. Results indicated that the symbiotic buttermilk might be considered as a functional food (23).

In another study in Spain on physicochemical properties of buttermilk with protein, fat, ash and lactose (24.82±0.02), (30.11±0.07), (5.22±0.05) and (39.35±0.03) respectively (these values shows the values of total solids), there was an overall difference in the chemical composition of buttermilk, which might be due to differences in the original quality of milk and processing conditions. In this study all visible fat was removed from milk and after the churning of buttermilk fat was reduced to 0.5 % which makes it ideal drink for the patients of diabetes and cardiovascular disease and for those who are on weight reduction program (22).

Results of paired t-test of the present study showed that there was no significant difference in flavor, taste and consistency of plain and prebiotic galactooligosaccharide containing buttermilk as $P>0.05$ but there was a significant difference in overall acceptability of galactooligosaccharide containing buttermilk as it was slightly sweet $P=0.08$. Flavor of plain buttermilk was intense than GOS containing buttermilk but it was not significantly different P value = 0.335 ns.

Buttermilk containing prebiotic galacto-oligosaccharides was pouch packed in University of Veterinary and Animal Sciences, Ravi Campus and glass packed by sterilizing glass bottles and pouring buttermilk in it. Buttermilk was stored at refrigeration temperature for 6 days. Sensory evaluation was carried out for 6 consecutive days to check the shelf life of prebiotic

galactooligosaccharide containing buttermilk in both types of packaging. Results showed that there was significant difference in colour of both packaging P<0.05. Results showed that there was no significant difference in taste in both packaging P>0.05.

Consistency of buttermilk was also checked in both packaging for 6 days. Results showed that there was no significant difference between the consistency of buttermilk in both packaging as p>0.01. Although, solids began to accumulate at the bottom of the bottles in glass packaging and required to shake the bottle every time before consumption.

Results of overall acceptability of buttermilk in both packaging showed that there was no significant difference between the overall quality of buttermilk in both packaging (P>0.05).Buttermilk in pouch and glass packaging maintained its stability for 6 days without adding any preservative at refrigeration temperature.

Conclusion and suggestions

The main aim of this project was to produce galacto-oligosaccharides enriched buttermilk to make it a value added product of high nutritional value and check its shelf life and stability. Buttermilk is an industrial waste which is discarded after the production of butter and cream. Buttermilk provides us protein, minerals, vitamins and is low in fat but with additional functional ingredient GOS will increase its worth. From the present study it can be concluded that the GOS enriched buttermilk can be produced. GOS stability under various pH and temperature conditions increases the options of its unification with other food products. Transgalactosylated buttermilk comparison with plain buttermilk did not show any significant difference in flavor, taste, consistency and overall acceptability of prebiotic buttermilk but there was difference in the colour of both (i.e plain and prebiotic) buttermilks due to the different packaging. Two types of packaging i.e Glass and Pouch packaging was done to check the shelf life and stability of the new product. Results showed that buttermilk in pouch packaging maintained its stability for about 6 days without adding any preservative. Also the functional product prepared was of good bifidogenic effect according to the results. It is recommended that GOS enriched buttermilk should be introduced in the market after proper packaging and safety evaluations. The stability of GOS enriched buttermilk should be checked for more than 6 days and effect of other types of packaging e.g tetra pack should be checked on prebiotic GOS enriched buttermilk. Efforts should be made to create awareness among the general population about the role of prebiotics in human health.

Declaration of interest

Authors declare that there is no conflict of interest.

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