Formulation of peptides rich fermented dairy beverage with multiple targets against diabetes using a proteolytic *Lacticaseibacillus rhamnosus* NCDC 24

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Abstract

Globally, more than one in 10 adults is now living with diabetes. There is a growing list of countries where one in five or even more adults have diabetes. Milk derived peptides have been reported to exert antidiabetic properties. The current study was designed to develop a fermented dairy beverage rich in peptides with Dipeptidyl peptidase-IV and α-glucosidase inhibitory and antioxidant properties using a single proteolytic culture Lacticaseibacillus rhamnosus NCDC 24, which could normalize the blood glucose levels in diabetic individuals. Six beverage formulations were prepared using combinations of skim milk and milk proteins and fermented with L. rhamnosus NCDC 24. Formulations were analysed for physicochemical, microbiological, sensorial and biofunctional properties.

Nutrient mix supplementation was found to positively impact the growth and proteolytic activity of the culture and Formula 3 was observed to display maximum peptide content and bioactivities after 48 h of fermentation. However, all the beverages were sensorially acceptable only up to 24 h of fermentation beyond which hedonic score fell below 7.0 due to low pH and high acidity at extended fermentations. Further, effect of inoculum concentrations on bioactivities of formula 3 was investigated. At 1x10^7 CFU/mL inoculum level, beverage exhibited elevated peptide content (0.745±0.06 mg/mL Leucine equivalent), DPP-IV (44.1±2%) and α-glucosidase inhibitory (36.68±2.4%) and ABTS (685.9±15.8 µM/L Trolox equivalent) and DPPH (82.6± 4.3%) scavenging activities. Findings foster the potential application of multifunctional peptides rich fermented dairy beverage developed using single strain as a dietary treatment.

Keywords: α-glucosidase, Antioxidant activity, Bioactive peptides, Diabetes, Dipeptidyl Peptidase-IV, *Lacticaseibacillus rhamnosus* NCDC 24.

Introduction

Diabetes mellitus is currently one of the major health issues that have escalated to alarming levels. International Diabetes Federation confirms diabetes to be one of the fastest growing global health problems of the 21st century as more than half a billion (540 million) people are living with diabetes worldwide and it is expected to snowball to 1.3 billion by 2050.[16] Amongst the three common forms of diabetes, type 2 (T2D) is the most frequently happening form and accounts for around 90% of diabetes worldwide. If not properly treated, it may lead to life threatening complications like cardiovascular disorders, renal failure, nerve damage and blindness.[25]

Many classes of glucose-lowering agents are commercially available to regulate T2D but most effective among them are dipeptidyl peptidase-IV (DPP-IV) and α-glucosidase enzyme inhibitors[10]. DPP-IV (EC 3.4.14.5), a ubiquitous enzyme, diminishes the insulin response by quickly degrading the incretin hormones namely glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) which are known to stimulate glucose-induced insulin secretion from pancreas.[23] Inhibition of DPP-IV can help maintaining insulinotropic effect of the incretins. α-glucosidase (EC 3.2.1.20) is tethered to small intestinal brush border, primarily involved in the hydrolysis of complex saccharides to liberate monosaccharides[20].

Moreover, oxidative stress has been suggested to be a common pathway for the pathogenesis of complications in diabetes.[9] Hence focus on antioxidants could help to minimize the development and progression of complications. Recently, milk protein derived bioactive peptides have been discovered to inhibit DPP-IV[30] and α-glucosidase[19] *in vitro* and they are well known for their antioxidant capacity.[32] Bioactive peptides are inactive within the sequence of a parent protein but can be released by gastrointestinal digestion, enzymatic proteolysis and fermentation.[21] Exploring enzymatic hydrolysis, a large amount of research was established for the production of DPP-IV. α-glucosidase inhibitory peptides are not economical for large scale industrial production and application.[48]

Conversely, generation of peptides endowed with specific bioactivities in milk products through fermentation by potent proteolytic lactic acid bacteria (LAB) as starter culture represents a cost-effective approach. The resultant peptides rich functional fermented dairy products may act as natural equivalents for the management of diabetes. Currently,

peptides rich dairy products such as Calpis and Evolus, BioPURE-GMP, Capolac and PRODIET F200/Lactium are available commercially with antihypertensive, anticariogenic, mineral-binding and stress relieving properties respectively[21], but none with antidiabetic properties; hence an attempt was made in the current study.

The present study was contemplated with the objective of formulation of an antidiabetic fermented dairy beverage enriched with α-glucosidase and DPP-IV inhibitory activity and antioxidative peptides. The study also attempted to highlight the changes among fermented beverage formulations with respect to some physicochemical, microbiological, biochemical and sensory characteristics along with biofunctional properties. A proteolytic strain *L. rhamnosus* NCDC 24 was selected on the basis of previous studies[34] carried out in our lab focusing on different bioactivities. In addition to the selection of right strain, proteolytic processes also require optimized and controlled growth factors and environment for maximal production rates of biotechnologically important compounds. Hence, nutrients mix including glucose, free amino acids, minerals and vitamins in addition to buffering agents were utilized during formulation of beverage.

Material and Methods

Bacterial strains and chemicals used: *L. rhamnosus* NCDC 24 was procured from National Collection of Dairy Cultures (NCDC), Karnal, India. Caseinate and whey protein isolate (WPI) were procured from MAHAN®, ACE international, New Delhi. A-glucosidase, DPP-IV from Porcine Kidney, 2, 2- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS], DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox were procured from Sigma-Aldrich, USA.

Formulation of protein matrix and optimizing incubation time for fermented beverage: Protein matrix was formulated by mixing skim milk, caseinate and WPI in different ratios (Table 1) to obtain soft and flowable curd with maximum bioactivities for the development of antidiabetic beverage. Fermentation with proteolytic culture *L. rhamnosus* NCDC 24 was performed according to the protocol mentioned as flowchart in fig. 1. A volume of 25 ml homogeneous sample from each beverage was aseptically drawn every 24 h during 72 h of incubation. Viable cell count, pH, acidity, viscosity and peptide content were determined. Samples were also analyzed for bio-functional and sensory attributes.

Total protein content of each formulation was measured before and after fermentation by Kjeldahl IDF block digestion method 981.10 of the AOAC International and Bradford[6] method respectively and the protein utilization by the culture during fermentation was calculated.

Preparation of water soluble extracts (WSE): Samples from each beverage were mixed thoroughly and centrifuged (HERMLE, Germany) for 30 min at 10,000 rpm.[42] Supernatants as WSE were collected, filtered using 0.45µm membrane (Millex® - HV, MERK Ireland), freeze dried (Labconco, USA) and stored at -20 °C until further analysis.

Estimation of peptides content: Peptides generation in fermented formulations was determined by O-phthaldialdehyde (OPA) method.[11] Briefly, OPA reagent was prepared by mixing 25 mL of 0.1 M disodium tetraborate, 2.5 mL of 20% sodium dodecyl sulfate, 1 mL methanol containing 40 mg of OPA, 0.1 mL of β-mercaptoethanol and final volume was made up to 50 mL with deionized water. A volume of 150 µL WSE was mixed with 3 mL of OPA solution and absorbance was measured at 340 nm using the double beam UV-VIS spectrophotometer (UV 1800, Shimadzu, Japan) after 2 min. Concentration of peptide was calculated from standard curve of L-Leucine.

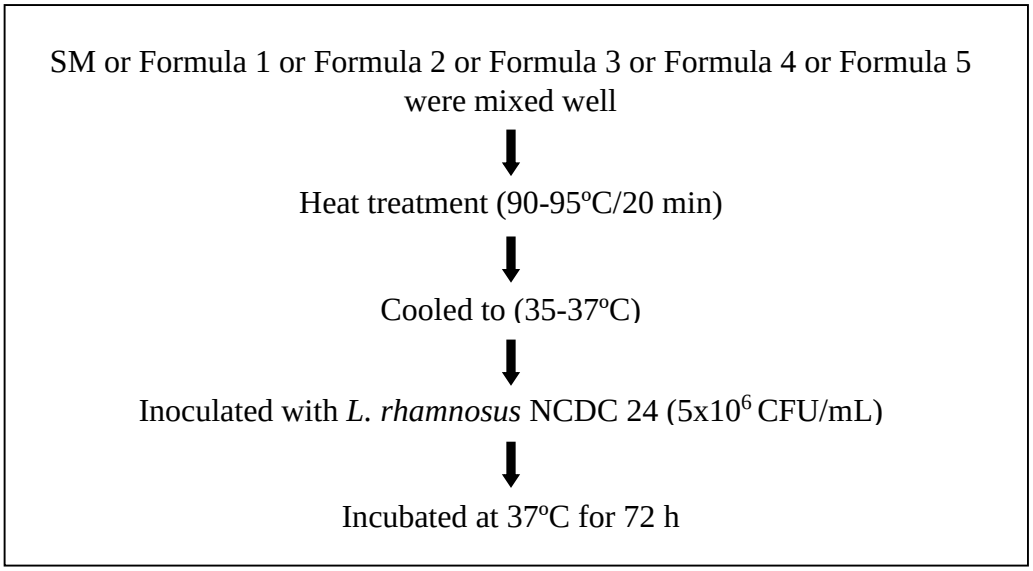

Fig. 1: Flow diagram for the preparation of fermented beverage formulations

Table 1
Milk and milk protein formulations for beverage preparation

S.N.	Formulation Code	Description
1	SM	Skim Milk
2	Formula 1	Skim milk + 1% WPI
3	Formula 2	80% Skim milk + 2% WPI
4	Formula 3	50% Skim milk + 50% Reconstituted milk protein mix [Caseinate (2%) & WPI (1%)] + 0.5% glucose + 0.1% nutrient mix
5	Formula 4	Reconstituted milk protein mix [Caseinate (2%) & WPI (1%)] + 1% glucose + 0.2% nutrient mix
6	Formula 5	25% Skim milk + 75% Reconstituted milk protein mix [Caseinate (2%) & WPI (1%)]+ 0.75% glucose + 0.15% nutrient mix

DPP-IV inhibition assay: DPP-IV inhibitory assay was performed as described by Lacroix and Li-Chan.[22] Lyophilized WSE was dispersed in 100 mM Tris buffer (pH 8.0). Essentially, 25 µL of samples was added to 25 µL of 1.59 mM Gly-Pro-p-nitroanilide, the substrate and mixtures were incubated at 37°C for 10 min. Reaction was started by adding 50 µL of DPP-IV (0.01 U/mL). After 60 min, reaction was stopped by adding 100 µL of 1 M sodium acetate buffer (pH 4.0). The optical density (OD) of the resulting solution was read at 400 nm in the microplate reader. Percentage inhibition was calculated using following equation:

DPP-IV inhibition (%) = [(A-B) – (C-D)]/ (A-B) × 100

where A = OD_{400} of substrate + enzyme at 60 min, B = OD_{400} of substrate + enzyme at 10 min, C = OD_{400} of substrate + enzyme + WSE at 60 min and D = OD_{400} of substrate + enzyme + WSE at 10 min.

Determination of α-glucosidase enzyme inhibitory activity: α- glucosidase enzyme inhibitory activity was assayed according to the modified chromogenic method reported by Ramchandran and Shah.[36] A volume of 50 µL α-glucosidase solution (0.2 U/mL) was preincubated at 37°C for 10 min with 25 µL of the WSE. Sample was replaced with potassium phosphate buffer in the control. Reaction was initiated by addition of 25 µL of pNPG (0.2 mM in 0.1 M potassium phosphate buffer, pH 6.8) and carried out for 30 min at 37°C. Then reaction was terminated by addition of 100 µL of Na_2CO_3 solution (0.1 M, pH 9.8). Inhibition of α-glucosidase was determined by measuring the OD of the p-nitrophenol released from pNPG at 405 nm using microplate reader. Percentage α-glucosidase inhibitory activity was calculated using the following equation:

α- glucosidase inhibition (%) = [1- ($OD_{(sample)}$)/($OD_{(control)}$)] x 100

Determination of ABTS free radical scavenging activity: ABTS free radical scavenging ability was assessed using the method given by Ramesh et al.[37] A volume of 20 µL WSE was reacted with 180 µL working ABTS solution in dark, prepared by mixing ABTS with potassium persulphate. After 10 min, decrease in absorbance was read at 734 nm using microplate reader. Trolox was used as a reference standard and the ABTS scavenging activity was expressed as µM/litre of trolox equivalent antioxidant capacity (TEAC).

Determination of DPPH free radical scavenging activity: DPPH scavenging ability was determined as previously described.[18] A total of 100 µL of 0.3 mM DPPH methanolic solution was mixed with 100 µL of WSE and allowed to react at room temperature for 30 min. Absorbance was measured at 517 nm and percentage scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = \left[1 - \frac{[\text{Abs (sample)} - \text{Abs (blank)}]}{\text{Abs (control)}}\right] x\ 100$$

Methanol plus WSE solution was used as a blank, while DPPH solution plus 1 mM Trolox was used as a control.

Sensory evaluation: All the beverage formulations were fermented for 24 h and refrigerated immediately. Then coagulum was broken down using hand blender and roasted. Powdered cumin seeds as natural flavour @ 0.1% and salt @ 0.05% were added and mixed well to prepare beverage. 50 ml of beverage in PET bottles was served to expert panel comprising of 10 judges for sensory characteristics evaluation. Each judge rated the fermented beverage on a 9-point hedonic scale under five liking attributes i.e. colour and appearance, flavor, consistency, mouthfeel and overall acceptability.

Effect of inoculum concentration on techno-functional properties of fermented beverage: The beverage that displayed highest DPP-IV and α- glucosidase inhibitory and antioxidant activities, was further selected and studied for the effect of inoculum levels $5x10^6$, $1x10^7$ and $5x10^7$ CFU/mL of *L. rhamnosus* NCDC 24 on pH, acidity, cell viability, peptide content and biological activities of fermented beverage.

Statistical analysis: All the trials were performed thrice and data were evaluated by Analysis of Variance (ANOVA)

wherever appropriate using GraphPad Prism 5.0 statistical tool package followed by Tukey's test. Level of significance was set at $P < 0.05$. Data are displayed as mean ± standard error of mean (SEM).

Results and Discussion

Viable *L. rhamnosus* NCDC 24 counts: Viability of *L. rhamnosus* NCDC 24 was determined by plating appropriate dilutions in MRS agar. As shown in table 2, all the formulations showed significant increase in viable cell log counts upto 24 h of incubation. Significantly higher bacterial population was observed in formulations having supplement mix i.e. formula 4, 5 and 3 with 9.14, 8.89 and 8.81 log CFU/mL cells, compared to non supplemented formulations i.e. formula 1 and 2 and fermented skim milk with 8.43, 8.38 and 8.31 log CFU/mL cells respectively ($p<0.05$). Results are in agreement with the investigation by Liu and Tsao[26] where addition of yeast extract was evidenced to enhance the counts of LAB by providing several growth promoting compounds such as vitamins, amino acids and other growth factors.

Oliveira et al[31] found that supplementation of casein hydrolysate stimulates the growth of *S. thermophilus* and *Lactobacillus* in yogurts. Further at 48 and 72 h of fermentation, slight decrease in cell viability was observed in all fermented products and the decline in growth may be attributed to the acid injury of cells as a result of increased acidification during fermentation.[46] The viability of more than 8.2 log CFU/mL cells in supplemented formulations even after 72 h of incubation may be possibly due to the presence of high peptide content which can be presumably utilized by *L. rhamnosus* NCDC 24 for their survival. Pastar et al[33] also reported that peptides and amino acids released by proteolytic *L. rhamnosus* BGT10 meet the nutrient requirement and contribute to the minimal decline in cell population during extended fermentations.

pH and acidity of beverage formulations: The pattern of changes in pH and titratable acidity of the beverage formulations during fermentation is presented in table 2. A decrease in the pH accompanied by an increase in acidity was observed in all formulations and values were ranged from 4.51±0.53 to 4.87±0.45 and 0.71±0.06 to 0.84±0.07% LA respectively after 24 h of incubation. The trend was persistent until end of fermentation.

The initial pH values were observed to be influenced by the composition of the formulations. Though the formulations with nutrients mix have higher initial pH, great fall (Formula 4, 5 and 3 with pH 4.51±0.53, 4.55±0.64 and 4.58±0.39 respectively) was seen at all intervals of fermentation compared to non-supplement formulations (Formula 2 and 1 and SM with 4.73±0.52, 4.81±0.47 and 4.87±0.45 respectively). The palpable reason may be higher growth and metabolic activities of *L. rhamnosus* NCDC 24 in supplemented formulations during fermentation.[12] In case of acidity, formula 4 has showed significantly high % LA ($p<0.05$) and we presume that the conversion of glucose present in formula 4 into lactic acid was more faster compared to the lactose in other products.

Table 2
Cell viability, pH, acidity, peptide content of beverage formulations at different time intervals during fermentation

Parameters		Beverage formulations[1]					
	Time (h)	SM	Formula 1	Formula 2	Formula 3	Formula 4	Formula 5
Viable cell count (log CFU/mL)	0	6.05 ± 0.15^{aA}	6.04 ± 0.17^{aA}	6.07 ± 0.13^{aA}	6.06 ± 0.12^{aA}	6.05 ± 0.04^{aA}	6.07 ± 0.15^{aA}
	24	8.31 ± 0.24^{aB}	8.43 ± 0.21^{bB}	8.38 ± 0.27^{abB}	8.81 ± 0.26^{cB}	9.10 ± 0.31^{dB}	8.89 ± 0.28^{cB}
	48	8.04 ± 0.21^{aC}	8.17 ± 0.32^{bC}	8.09 ± 0.26^{abC}	8.44 ± 0.19^{cC}	8.78 ± 0.27^{eC}	8.63 ± 0.25^{dC}
	72	7.93 ± 0.18^{aC}	7.99 ± 0.26^{aD}	7.91 ± 0.14^{aC}	8.26 ± 0.24^{bD}	8.57 ± 0.24^{dD}	8.39 ± 0.17^{cD}
pH	0	6.63 ± 0.33^{aA}	6.67 ± 0.59^{aA}	6.68 ± 0.61^{aA}	7.09 ± 0.61^{aA}	7.18 ± 0.59^{aA}	7.13 ± 0.71^{aA}
	24	4.87 ± 0.45^{cB}	4.81 ± 0.47^{bcB}	4.73 ± 0.52^{bB}	4.58 ± 0.54^{aB}	4.51 ± 0.51^{aB}	4.55 ± 0.64^{aB}
	48	4.38 ± 0.47^{cC}	4.25 ± 0.64^{bC}	4.29 ± 0.43^{bC}	4.19 ± 0.46^{aC}	4.09 ± 0.53^{aC}	4.13 ± 0.58^{aC}
	72	4.12 ± 0.37^{cD}	3.94 ± 0.71^{bD}	3.98 ± 0.37^{bD}	3.85 ± 0.35^{aD}	3.79 ± 0.41^{aD}	3.91 ± 0.46^{abD}
Acidity (% LA)	0	0.130 ± 0.03^{aA}	0.141 ± 0.04^{aA}	0.152 ± 0.06^{aA}	0.145 ± 0.04^{aA}	0.165 ± 0.05^{aA}	0.153 ± 0.06^{aA}
	24	0.730 ± 0.05^{aB}	0.760 ± 0.07^{aB}	0.740 ± 0.07^{aB}	0.710 ± 0.07^{aB}	0.840 ± 0.06^{bB}	0.770 ± 0.07^{abB}
	48	1.080 ± 0.06^{aC}	1.150 ± 0.08^{bC}	1.170 ± 0.08^{bC}	1.100 ± 0.08^{abC}	1.230 ± 0.09^{cC}	1.098 ± 0.09^{aC}
	72	1.210 ± 0.06^{aD}	1.280 ± 0.07^{abD}	1.260 ± 0.07^{aD}	1.230 ± 0.09^{aD}	1.340 ± 0.07^{bD}	1.310 ± 0.06^{bD}
Peptides content (mg/mL eq. of Leucine)	0	0.089 ± 0.017^{aA}	0.096 ± 0.0^{aA}	0.105 ± 0.02^{aA}	0.102 ± 0.014^{aA}	0.091 ± 0.03^{aA}	0.103 ± 0.02^{aA}
	24	0.542 ± 0.045^{bB}	0.581 ± 0.05^{cB}	0.599 ± 0.07^{cB}	0.644 ± 0.06^{dB}	0.513 ± 0.05^{aB}	0.549 ± 0.06^{bB}
	48	0.63 ± 0.043^{bcC}	0.65 ± 0.06^{cC}	0.66 ± 0.06^{cC}	0.71 ± 0.07^{dC}	0.593 ± 0.07^{aC}	0.625 ± 0.06^{bC}
	72	0.654 ± 0.06^{bcC}	0.67 ± 0.054^{cC}	0.685 ± 0.06^{cdC}	0.73 ± 0.06^{dC}	0.640 ± 0.08^{aC}	0.66 ± 0.05^{bC}

[A-D] Mean values in the same column with different superscript upper case letters are significantly different at $P < 0.05$.
[a-d] Mean values in the same row with different superscript lower case letters are significantly different at $P < 0.05$.
[1]Values are expressed as mean ± standard error (n=3)

Acidity of all the products was increased with incubation time and significantly greater at 72 h of fermentation. Observations were in accordance with studies of Shu et al[41] and Solieri et al[43] where inverse relationship between pH and acidity was observed during lactic fermentation.

Peptide content of fermented milk formulations: Peptides generation in different beverage formulations was evaluated in terms of free NH_3 groups liberated on hydrolysis of protein during fermentation. As shown in table 2, the concentration of peptides augmented significantly during fermentation from 0 to 48 h for all formulations tested (P<0.05). Though there was a further increase in peptide content at 72 h, the difference was non-significant (P>0.05) when compared to 48 h. Formula 3 was found to have significantly high peptide content among all formulations with 0.73±0.06 mg Leu/mL whereas formula 4 showed lowest peptide content (0.652±0.07 mg Leu/mL) after 72 h of fermentation.

Milk proteins are broken down as a result of cleavage of peptide bonds by proteinases and peptidases of LAB consequently increasing the levels of free amino groups in fermented milks.[8] Results were consistent with the findings of Panchal et al[32] wherein linear increase in peptide content was observed with incubation time during milk fermentation by proteolytic *L. helveticus, L. plantarum* and *L. fermentum*. Though the formula 1, 2 and SM have significantly greater protein content than formula 3, the relatively higher peptide content in formula 3 may be the result of high growth and proteolytic activity of *L. rhamnosus* NCDC 24. Rubak et al[38] screened four different *Lactobacillus* species such as *L. fermentum* S206, *L. rhamnosus* R2, *L. kefiri* JK17 and *L. delbrueckii* BD7 for proteolytic activity in milk and found *L. rhamnosus* R2 to be the most proteolytic with highest peptide content in fermented milk. Strong positive correlations between cell population, acidity, protein utilization and peptide content in fermented formula 3 demonstrate that nutrient mix supplementation improves the growth and proteolytic capability of *L. rhamnosus* NCDC 24 in milk.

DPP-IV inhibitory activity: DPP-IV inhibitory activity of beverage formulations determined during fermentation is depicted in fig. 2. Inhibition was significantly increased in all formulations up to 48 h of incubation; however, eventually significant decrease was observed except in formula 3, 4 and 5, possibly due to extended hydrolysis of DPP-IV inhibitory peptides into inactive or less active peptides.[41] Among the beverages, formula 3 has showed highest inhibitory activity at all the time intervals of fermentation followed by formula 5 and formula 4. This may be due to increased DPP-IV inhibitory peptides with increased peptide content as this order correlates with the peptide content of formulations. Moreover, it has been reported that generation of high number of proline (at 2nd position from N-terminal end) containing peptides may contribute to the maximum DPP-IV inhibitory activity.[29] β-casein which is rich in proline derived peptides are prone to exert great DPP-IV inhibitory activity.[22] A study by Fan et al[13] reported that β-casein is more susceptible to the degradation by proteases of *Lactobacillus* and causes to produce many beneficial peptides. Similar investigation was carried out by Ni et al[28] to prepare yogurt with DPP-IV inhibitory activity and the identified peptides in the study were mostly β-casein derived. Further, lowest DPP-IV inhibitory activity was found in the fermented SM having no significant difference with formula 1 and 2 which may be due to the interference of non active high molecular whey derived peptides obtained after proteolysis.[45]

α- glucosidase inhibitory activity: α- glucosidase inhibitory activity of *L. rhamnosus* NCDC 24 fermented beverage formulations is depicted in fig. 3. After 24 h of fermentation, activity in all fermented formulations raised significantly, maximum recorded in the formula 3 with 28.48±2.61% flowed by formula 2 and formula 5. Bioactivity of all formulations was further improved after 48 h of fermentation. Formula 3 and formula 2 with 34.14±2.12 and 32.95±2.56% respectively have exerted greater activity. Significant fall of activity appeared at 72 h except in formula 1 for which non-significant differences were found between 48 and 72 h (p>0.05).

Amongst all, fermented SM displayed lower α- glucosidase inhibition after 24 h of fermentation and found non-significant changes during extended fermentation. The mechanism behind α-glucosidase inhibitory activity of peptides is unknown; however, Bharatham et al[5] suggested that non-saccharides may block the enzyme's active site via hydrophobic interactions. First such investigation has been carried out by Ramchandran and Shah[36] revealing the release of α-glucosidase inhibitory peptides from skim milk by different LAB. Ayyash et al[4] compared the generation of α-glucosidase inhibitory peptides from fermented bovine and camel milk by *L. acidophilus* and reported non-significant differences for both the milks with 29 and 26% inhibition respectively. In another recent study, α-glucosidase inhibitory activity of 13.74% and 11.14% was reported by *L. fermentum* M7 and *L. fermentum* M2 in bovine milk on 24 h of fermentation.[19]

ABTS free radical scavenging activity: Development of ABTS radical scavenging activity during fermentation of milk protein formulations was determined. As shown in fig. 4, ABTS radical scavenging activity increased significantly with incubation time up to 48 h (P<0.05) whereas non significant differences were found between 48 and 72 h of fermentation in all fermented formulations (P>0.05). Similar observations were shown in previous studies where in antioxidant activity was increased with incubation time of fermented milks and highest was found at 48 h with *L. plantarum* 55[1] and *L. fermentum* M4[32] and 111 h with *L. casei* PRA205.[43] Moreover, these time differences were depending on the proteolytic ability of the *Lactobacillus* species.[3]

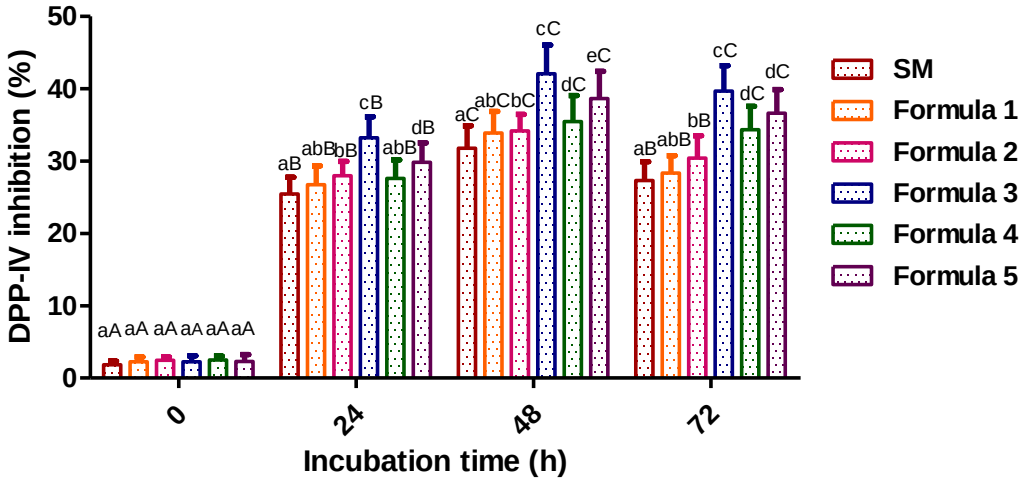

Fig. 2: DPP-IV inhibitory activity of different beverage formulations fermented by *L. rhamnosus* NCDC 24.
Different upper case letters (A-C) above the bars indicate significant differences between time intervals at $P < 0.05$.
Different lower case letters (a-e) above the bars indicate significant differences between beverage formulations at $P < 0.05$.
Values shown are the mean ± standard error (n=3)

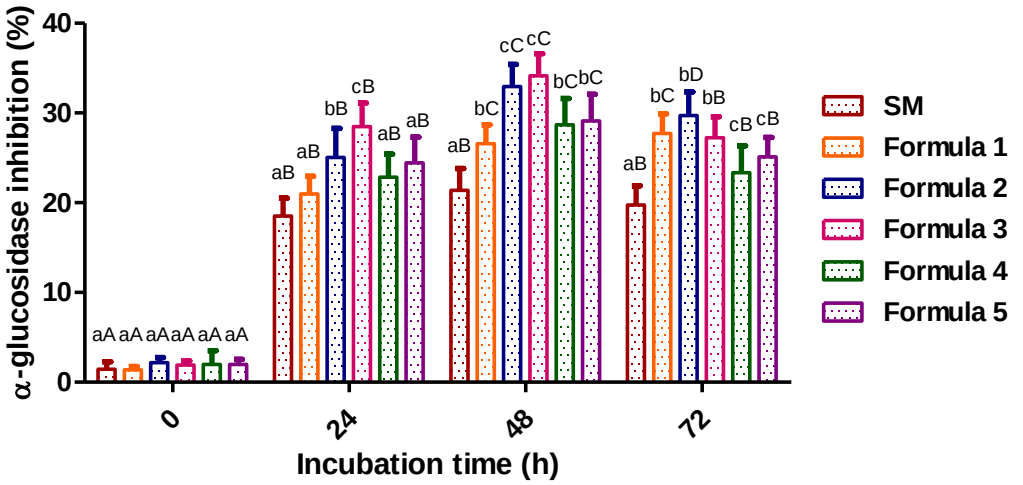

Fig. 3: *α*-glucosidase inhibitory activity of different beverage formulations fermented by *L. rhamnosus* NCDC 24.
Different upper case letters (A-D) above the bars indicate significant differences between time intervals at $P < 0.05$.
Different lower case letters (a-d) above the bars indicate significant differences between beverage formulations at $P < 0.05$.
Values shown are the mean ± standard error (n=3).

Maximum ABTS scavenging activity was found at 48 h of fermentation in formulations and the activity ranged from 568.17 to 648.97 µM/L Trolox equivalent with highest in fermented formula 3 and lowest in skim milk respectively. Higher peptide content in formula 3 due to the application of nutrient mix may be the reason for greater antioxidant activity among other formulations.[40] Peptide released in fermented milks has potential to neutralize ROS, either by direct reduction via electron transfer or by radical quenching via H-atom transfer leading to more stable species.[36] Similar results were demonstrated by Ramesh et al[37] and Aguilar-Toala et al[1] wherein ABTS scavenging activity of 490 and 270 µM/L Trolox eq. has been reported in fermented skim milks by *L. rhamnosus* NCDC 24 and *L. plantarum* 55 respectively.

DPPH free radical scavenging activity: DPPH free radical scavenging activity of fermented formulations is shown in fig. 5. There was significant increase in activity from 0 to 24 h and was found to be decreased later at 48 and 72 h of fermentation in all formulations. It may be due to the degradation of DPPH scavenging peptides into inactive peptide fragments or free amino acids.[41] Length of the peptides is presumably conceded to play an important role in scavenging DPPH radical, as it has been shown in a study that length of 23 DPPH scavenging peptides from fermented milk ranged between 9 to 18 amino acids.[44] DPPH scavenging activity of formulations after 24 h of fermentation ranged between 58.69±3.24 in SM and 74.35±5.12% in formula 3. The high peptide content due to elevated proteolytic activity may be responsible for greater

antioxidant activity in formula 3. Our results are in agreement with previous findings where degree of milk protein proteolysis during fermentation was highly correlated with DPPH scavenging activity of fermented milks.[39] Shu et al[40] demonstrated that addition of different growth promoting nutrients such as glucose, casein peptone and calcium lactate increases protease expression in *L. casei* L61 which further leads to improved protein hydrolysis during fermentation of milk that generates more bioactive peptides.

Hence, it may be the reason for high antioxidant activity in formula 3. The high DPPH scavenging activity in formula 2 after Formula 3 may be attributed to the presence of more whey protein derived peptides as explained by Unal and Akalin.[46] Moreover, it was also reported that addition of 1-2% whey protein hydrolysates to skim milk increases its antioxidant activity from 21 to 88%.[28] Farvin et al[14] suggested that the milk derived peptides act as electron donors that are able to react with free radicals to convert them into stable products.

Sensory evaluation: Although, all the fermented formulations displayed significantly high peptide content and greater bioactivities after 48 h of fermentation, they were sensorially unacceptable due to low pH, high acidity and some off flavours.

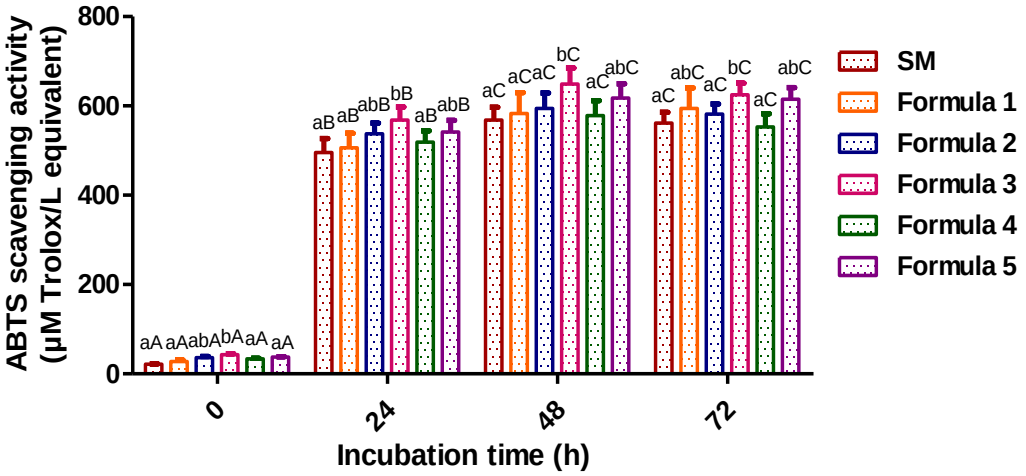

Fig. 4: ABTS scavenging activity of different beverage formulations fermented by *L. rhamnosus* NCDC 24.
Different upper case letters (A-D) above the bars indicate significant differences between time intervals at $P < 0.05$.
Different lower case letters (a-e) above the bars indicate significant differences between beverage formulations at $P < 0.05$.
Values shown are the mean ± standard error (n=3).

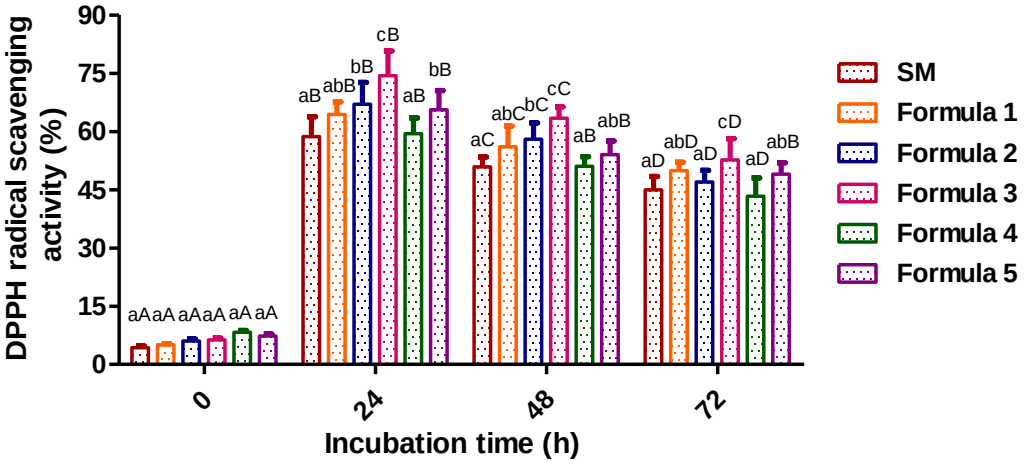

Fig. 5: DPPH free radical scavenging activity of different beverage formulations fermented by *L. rhamnosus* NCDC 24.
Different upper case letters (A-D) above the bars indicate significant differences between time intervals at $P < 0.05$.
Different lower case letters (a-e) above the bars indicate significant differences between beverage formulations at $P < 0.05$.
Values shown are the mean ± standard error (n=3)

Hence, formulations fermented for 24 h served for sensory evaluation. Toned milk fermented using mixed dahi culture NCDC 167 was used as control. All beverage formulations were acceptable and found non-significant differences in overall acceptability except for formula 4 when compared to the control. Complete usage of reconstituted dried non fat milk powders in formulation may be explained for relatively low sensory score for formula 4.

Among the formulations, highest overall acceptability score was obtained for Formula 1 (7.9±0.41) followed by formula 2 (7.85±0.43), formula 3 (7.82±0.23), SM (7.81±0.26), formula 5 (7.5±0.34) and formula 4 (7.2±0.27). Greater consumer acceptability for formula 1 and 2 could be attributed to the good flavour and mouthfeel contributed by WPI.[17] Low sedimentation in formula 1 and 2 may be because of the water-holding, thickening and gelling properties of whey proteins as high viscosity was observed in these beverages.[15] Formula 3 with highest peptide content and bioactivities has obtained overall acceptability score of 7.82±0.23, which implies that it was very much liked and the scores of other sensory attributes were also above acceptable limit i.e. 7.5.

The proteolytic potentials of *Lactobacillus* cultures not only produce bioactive peptides, but also affect the taste and texture properties of the products. Studies revealed that high proteolytic strains display less acidifying activity during fermentation and storage.[2] Hence, the formulation formula 3 exhibiting maximum DPP-IV and α- glucosidase inhibitory and antioxidant activities along with high consumer acceptability, was selected for further studying the effect on bioactivities during fermentation over different inoculum concentrations of *L. rhamnosus* NCDC 24.

Effect of inoculum concentration on cell viability, pH and acidity of Formula 3: The differences in cell viability, pH and acidity of beverage fermented at different inoculum level are summarized in table 3. It can be seen that the viable cell counts in fermented beverage increased from 8.81 log CFU/mL at $5x10^6$ CFU/mL inoculum level to 9.33 and 9.57 log CFU/mL at $1x10^7$ and $5x10^7$ CFU/mL inoculum levels respectively. The pH of the beverage decreased from 4.62±0.06 at $5x10^6$ CFU/mL to 4.54±0.05 and 4.35±0.04 at $1x10^7$ and $5x10^7$ CFU/mL inoculum concentration whereas acidity increased from 0.71±0.01 at $5x10^6$ CFU/mL to 0.75±0.01 and 0.89±0.02 at $1x10^7$ and $5x10^7$ CFU/mL inoculum level respectively. There were no significant differences in pH and acidity of fermented beverage at inoculum concentrations of $5x10^6$ and $1x10^7$ CFU/mL but they significantly varied at $5x10^7$ CFU/mL.

Shu et al[41] also reported similar trend for pH and acidity in fermented goat milk at increased inoculum concentrations. Moreover, they described that it may be due to high initial cells that grew faster and produced more lactic acid and other organic acids, peptides and amino acids which led to increased titratable acidity and decrease in the pH.

Effect of inoculum concentration on peptide content: As presented in table 3, significant increase in peptide content in formula 3 fermented at $1x10^7$ and $5x10^7$ CFU/mL inoculum levels was observed when compared at $5x10^6$ CFU/mL ($p<0.05$) whereas non significant differences were found at $1x10^7$ and $5x10^7$ CFU/mL initial counts of *L. rhamnosus* NCDC 24 ($P>0.05$). It may be due to the higher demand for peptides and free amino acids for the survival and growth of *L. rhamnosus* NCDC 24 at high initial cells.[36] Our results are in agreement with the observations of previous investigation where in diminution of free amino group content was reported at higher inoculum level (> $5x10^7$ CFU/mL) of *L. plantarum* LP69 in fermented milks.[41]

Authors demonstrated that the nutrients in milk could not meet the growth requirement of *L. plantarum* LP69 at high inoculum level, which led cells' faster utilization of peptides and amino acids released during fermentation in the product. Moreover, higher inoculum level may contribute to excessive acid production which negatively impacts the cell's proteolytic ability.[7]

Effect of inoculum concentration on DPP-IV and α-glucosidase inhibitory activities: DPP-IV and α-glucosidase inhibitions in formula 3 were significantly augmented at $1x10^7$ CFU/mL inoculum level compared to the activities at $5x10^6$ CFU/mL ($p<0.05$) (Table 3). Further, at $5x10^7$ CFU/mL DPP-IV inhibition was slightly decreased but non-significant when compared to the inhibition at $1x10^7$ CFU/mL inoculum level. In case of α- glucosidase inhibitory activity, it was found to be diminished significantly at $5x10^7$ CFU/mL inoculum and there was no significant difference between the activities at $5x10^6$ and $5x10^7$ CFU/mL. It might be due to the degradation of α- glucosidase inhibitory peptides at higher inoculum level as there is more demand for peptides and free amino acids for growth.[36]

It was confirmed in further study by Shu et al[41] wherein decreased bioactivities were observed in fermented milks at > $5x10^7$ CFU/mL inoculum levels of *L. plantarum* LP69.

Chen et al[7] and Li et al[24] observed that an inoculum concentration of $1x10^6$ CFU/mL exerted significantly greater ACE-inhibitory activity when compared to higher initial cells of *L. helveticus* IMAU80872 and *L. casei* IMAU20411 respectively and they suggested that lower initial cells might have offered less competitive environment in terms of nutrients and metabolites for growth and generation of bioactive peptides. These differences in optimum inoculum levels of various *Lactobacillus* species for production of different bioactive peptides may be due to the differences in their proteolytic abilities.

Effect of inoculum concentration on ABTS and DPPH free radical scavenging abilities: As shown in table 3, beverage fermented using $1x10^7$ CFU/mL inoculum has displayed maximum scavenging activities against both the free radicals.

Table 3
Effect of inoculum concentrations on cell viability, pH, acidity, peptide content and bio-functional activities of beverage (Formula 3)

Parameters[1]	**Inoculum conc. of *L. rhamnosus* NCDC 24 (Log CFU/mL)**		
	$5x10^6$	**$1x10^7$**	**$5x10^7$**
Viable cell (Log CFU/mL)	8.81 ± 0.09^a	9.30 ± 0.08^b	9.57 ± 0.07^c
pH	4.62 ± 0.04^a	4.54 ± 0.054^a	4.35 ± 0.06^b
Acidity (%LA)	0.71 ± 0.04^a	0.75 ± 0.035^a	0.89 ± 0.025^b
Peptide content (mg/mL eq. of Leucine)	0.644 ± 0.06^a	0.745 ± 0.064^b	0.755 ± 0.05^b
Bio-functionalities			
DPP-IV inhibitory activity (%)	33.24 ± 1.8^a	44.09 ± 2.1^b	41.67 ± 1.51^b
α- glucosidase inhibitory activity (%)	28.96 ± 2.03^a	36.68 ± 2.40^b	29.75 ± 2.84^a
ABTS Scavenging activity (µM Trolox eq.)	568.58 ± 18.9^a	685.97 ± 15.8^b	634.69 ± 16.6^c
DPPH scavenging activity (%)	74.39 ± 3.6^a	82.61 ± 4.3^a	63.38 ± 2.9^b

[a-c] Mean values in the same row with different superscript letters are significantly different at $P < 0.05$.
Values expressed are mean ± standard error (n=3)

Further at $5x10^7$ CFU/mL, activity against DPPH was observed to be reduced significantly whereas scavenging activity against ABTS had no significant difference. The altering scavenging abilities against ABTS and DPPH free radicals may be attributed to peptide length, sequence and position of amino acids in the peptides released at varying inoculum levels[44] and the mechanisms of action for the two free radical assays used.[4]

Findings reveal that the peptides responsible for DPPH scavenging activity might be degraded into inactive peptide fragments or amino acids at higher inoculum levels[41] but surprisingly those degraded peptides may still be active against ABTS free radical resulting non significant changes in the activity. Nongonierma and FitzGerald[29] reported that amino acids possess very low DPPH scavenging ability compared to the peptides. Consistent observations were reported in fermented milks at higher inoculum concentrations of *L. helveticus* IMAU80872 and R0389 by Chen et al[7] and Li et al.[24]

Conclusion

Different beverage formulations were fermented using *L. rhamnosus* NCDC 24. Extended fermentations upto 48 h have augmented peptide generation and biofunctional activities in formulations but the beverages were unacceptable for consumption due to high acidity and presence of off flavours. Nutreints supplementation in formula 3 greatly impacted the growth and proteolytic ability of the culture and facilitated maximum protein utiliation thereby high peptide content and bioactivities. There was no distinct difference between beverage formula 3 fermented for 24 h and control in terms of over all accebtability during sensory evaluation.

Increase in inoculum concentrations upto $1x10^7$ cells further improved the bioactivities of formula 3. Therefore, findings of the present study suggest and endorse the potential use of *L. rhamnosus* NCDC 24 as single culture for the development of a dairy beverage with multifunctional peptides through fermentation and the beverage may be applied as a potent antidiabetic therapeutic drink as the generated peptides have multiple targets against diabetes. Further research is warranted to investigate the antidiabetic efficacy of the beverage *in vivo*. Identification and validation of the biologically active peptides generated by the culture would also be beneficial.

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